10906 J. Agric. Food Chem. 2010, 58, 10906–10910
DOI:10.1021/jf1026907

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Expression of PAT and NPT II Proteins during the Developmental Stages of a Genetically Modified Pepper Developed in Korea

Hyo Jin Kim, Si Myung Lee, Jae Kwang Kim, Tae Hun Ryu, Seok Cheol Suh, and Hyun Suk Cho*

National Academy of Agricultural Science, Rural Development Administration, Suwon, Gyeonggi-do, 441-707, Korea

Estimation of the protein levels introduced in a biotechnology-derived product is conducted as part of an overall safety assessment. An enzyme-linked immunosorbent assay (ELISA) was used to analyze phosphinothricin acetyltransferase (PAT) and neomycin phosphotransferase II (NPT II) protein expression in a genetically modified (GM) pepper plant developed in Korea. PAT and NPT II expression levels, based on both dry weight and fresh weight, were variable among different plant generations and plant sections from isolated genetically modified organism (GMO) fields at four developmental stages. PAT expression was highest in leaves at anthesis (11.44 μg/gdw and 2.17 μg/gfw) and lowest in roots (0.12 μg/gdw and 0.01 μg/gfw). NPT II expression was also highest in leaves at anthesis (17.31 μg/gdw and 3.41 μg/gfw) and lowest in red pepper (0.65 μg/gdw and 0.12 μg/gfw). In pollen, PAT expression was 0.59−0.62 μg/gdw, while NPT II was not detected. Both PAT and NPT II showed a general pattern of decreased expression with progression of the growing season. As expected, PAT and NPT II protein expression was not detectable in control pepper plants.

KEYWORDS: Safety assessment; enzyme-linked immunosorbent assay; phosphinothricin acetyltransferase; neomycin phosphotransferase II; genetically modified pepper

INTRODUCTION

Advances in biotechnology have led to the development of genetically modified (GM) crops for the purpose of increasing food production, with the production and distribution of GM crops increasing rapidly since 1996. At present, the most widely grown GM crops express exogenous genes that confer herbicide tolerance, insect resistance, or both (*1*). Herbicide tolerance, the most widely enhanced trait in transgenic plants, has been achieved in different ways to increase crop weed control. Globally, approximately 75% of GM crops are engineered for increased herbicide tolerance, with most genes used for plant transformation originating from microorganisms or other plants (*2*).

Phosphinothricin acetyltransferase (PAT) is encoded by both *pat* and *bar* genes isolated from *Streptomyces hygroscopicus* and *Streptomyces viridochromogenes* (*3*, *4*) and inactivates the herbicidal compound phosphinothricin (PPT) by acetylation. Herbicide resistance using *bar* and *pat* genes encoding PAT has been produced in several crops, such as soybean, which have received worldwide approval for cultivation and consumption (*2*, *5*, *6*). Neomycin phosphotransferase II (NPT II) is a bacterial enzyme that confers resistance to some aminoglycoside antibiotics through phosphorylation of the 3′-hydroxyl group of the aminoglycoside (*7*, *8*). The gene encoding this enzyme is a common selectable marker used in the production of GM crops as well as cell transformation in bacterial and eukaryotic molecular biology (*9*−*11*).

*Corresponding author. Tel: +83-31-299-1151. Fax: +82-31-299-1122. E-mail: hscho@rda.go.kr.

In Korea, the Rural Development Administration (RDA) has developed GM pepper (*Capsicum annuum* L. Var. 'Subicho') which expresses PAT from the *bar* gene and NPT II as a selectable marker. Expression in T_0 and T_1 plants was confirmed by genomic Southern hybridization, and plants showed herbicide resistance in a 0.3% Basta. Our laboratory isolated homozygotes expressing one copy of integrated gene which showed complete resistance against application of 0.6% Basta, with almost no phenotypic evidence of damage, through subsequent generations. In the present study, T_3 transgenic pepper plants were used for quantitative analysis of PAT and NPT II expression during the stages of plant development. All the experiment were performed from isolated GMO fields, and protein analysis of GM pepper was the first.

According to "The guideline for the Safety Assessment of Genetically Modified Foods" issued by the Korea Food and Drug Administration (KFDA), safety evaluations of GM crops are requested for the purpose of determining whether genetically modified organisms (GMO) are appropriate and safe for consumption (*12*). In accordance with these regulations, food safety assessments are required for GM pepper.

One important aspect of assessing the safety of GM crops is the characterization of recombinant plants. According to KFDA regulations, information related to characterization of the genetic modification should be provided for any substances expressed in GM plants, including gene product expression levels and specific tissue-specific patterns of expression. In addition, safety assessment data should be provided to demonstrate that the modification has achieved its intended effects and that all expressed traits

 Published on Web 09/28/2010

are expressed and inherited in a stable manner through subsequent generations of GM plants.

Immunoassay technologies with antibodies are ideal for qualitative and quantitative detection of many types of known target proteins in complex matrices. Enzyme-linked immunosorbent assay (ELISA) methods have been widely used because they reduce the need for costly, complicated equipment, decrease analysis times and are suitable for routine analysis of large numbers of samples (*13*−*16*).

This report presents the quantitative ELISA analysis of PAT and NPT II protein expression in GM pepper for the first time in Korea. The purpose of this study was to assess the stability of PAT and NPT II protein expression in replicating generations of GM pepper and to determine the level and pattern of expression in various GM pepper tissues during plant developmental stages. The results are necessary to provide basic data for a food safety assessment of GM pepper.

MATERIALS AND METHODS

Cultivation and Collection of Plant Samples. GM pepper and Subicho (SC), the GM pepper parental plant used as a control, were obtained from the National Academy of Agricultural Science (NAAS) of RDA in Korea. Developing herbicide resistance in the herbicide-susceptible cultivar SC involved modifying SC to express phosphinothricin-acetyltransferase through *Agrobacterium*-mediated transformation.

For the present experiments, plants were grown from seed in a NAAS greenhouse for 4 weeks, and then GM pepper (T_3, T_4, T_5) and SC plants were transplanted to isolated GMO fields Suwon (field 1) and Anseong (field 2) in Korea. The experimental fields were designed as a randomized block in five replicates for each GM pepper end point and two replicates for each SC end point grown on each field. Pepper plant cultivation was carried out in accordance with common local agricultural practices. GM pepper and SC plants were collected at the following four growth stages: seedling (6−8 weeks after planting), anthesis (10−12 weeks after planting, pollen formation), seed maturity (12−16 weeks after planting, green and red fruits formation), and senescence (18−20 weeks after planting). All the leaves, stems, roots, pollen (anthesis), and fruits (mature seeds) were collected from each plot at the two fields. Green pepper and red pepper were randomly sampled in each plot at intervals of four weeks.

Plant materials were transported to the laboratory, where the different sampled tissues were aliquoted for subsequent analysis. For determination of expression levels from multiple generations, healthy leaf tissue was collected from GM pepper (T_3, T_4, T_5) and SC plants which was representative of plants at the seedling stage. For the expressed location and amount of gene products, five plants per GM pepper (T_3), plus two plants from SC, were collected at each of four developmental stages. Fresh leaves were collected from each plant at each site and combined to form the leaf sample. Stems were separated into main stems and reproductive branches. For reproductive structures, the upper and lower stems were not included in the samples. To remove soil particles, the roots were gently washed under running water and then dried on tissue paper. Pollen was collected directly from the field plots, air-dried overnight, and stored frozen at −70 °C. After weighing the samples, individual parts (except pollen) were lyophilized in a freeze drier (Ilshin Lab Co., Ltd., Korea) for 48 h, cut into small pieces (5 mm), mixed well, and then reduced to a fine powder using a mortar and pestle in liquid nitrogen. After reweighing to establish percent dry weight, each powdered sample was mixed thoroughly to ensure homogeneity. Processed samples were stored at −70 °C until preparation for ELISA analysis.

Preparation of Plant Extracts for the ELISA. For PAT protein extraction, 20 mg of each tissue sample (except pollen) was homogenized in 3 mL of phosphate-buffered saline with Tween 20 (PBST buffer) as supplied with the ELISA kit. Each sample was vortexed briefly and then incubated on wet ice for 5 min. Samples were centrifuged at 5000*g* at 4 °C for 5 min, and then the supernatant was used for PAT quantification. For establishing extraction efficiency, after the supernatant was collected, the remaining pellet was extracted in 1 mL of PBST buffer followed by vigorous vortexing. The suspension was kept on ice for 5 min and again centrifuged as described above. The supernatant was recovered and the extraction procedure was again repeated (for a total of three times). All supernatants were transferred into the wells of a microtiter plate, and PAT concentration was determined by ELISA.

For NPT II protein extraction, 20 mg of each powdered lyophilized tissue sample (except pollen) was suspended in 3 mL of PEB (1/10 diluted) buffer supplied with the ELISA kit. Samples were incubated on wet ice for 10 min, vortexed briefly, and then again incubated on wet ice for 10 min. After centrifugation for 10 min at 12000*g* at 4 °C, the supernatant was used for the quantification of NPT II. Extraction efficiency was established as described for the PAT analysis.

ELISA Analysis. Quantitative PAT and NPTII protein expression profiles of GM pepper and SC plants were determined using commercially available ELISA kits (EnviroLogix LibertyLink pat/bar ELISA, EnviroLogix Inc., Portland, ME; Agdia nptII ELISA, Agdia Inc. Elkhart, IN). ELISAs were performed according to the manufacturer's protocols. All samples were incubated in reaction wells for 2 h at room temperature (22 °C), and then the sample absorbance was measured at 450 nm using an ELISA reader (Multiskan EX, Thermo Scientific). All samples were measured in triplicate.

Protein quantification was determined by plotting test sample absorbance values on standard curves generated using purified PAT or NPTII protein standards as supplied with the respective ELISA assay. Results were expressed as μg PAT or NPTII protein per g tissue wet weight with consideration of the dilution factor. The percent dry weight of each sample was then used to convert protein concentrations from gram dry weight (gdw) to gram fresh weight (gfw). For all assessments, corresponding GM pepper and SC plants were grown and analyzed in parallel in order to identify any potential background effects of the plant matrix on the ELISA results.

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

RESULTS

Validation of an ELISA Test Kit for Quantitative Measurements of PAT and NPT II Proteins. To confirm the protein concentration signals produced in the ELISA, PAT and NPT II protein standards were serially diluted and incubated at 22 °C for 2 h. Optical density (OD) showed a linear response to protein concentrations in the range of 0−2 OD units. To estimate the efficiency of PAT and NPT II extraction from different plant tissues, samples of leaves, stems, roots at seedling, pollen at anthesis, and fruits at seed maturity were randomly selected and three sequential extractions of each tissue were performed and measured. The extracted PAT and NPT II protein concentrations from the first extraction ranged from 2.05 to 39.5 ng/mL extract, whereas the second extraction yielded only 0.03−0.94 ng/mL, corresponding to 1−3% of the first extraction (data not shown). Subsequent extractions yielded only trace amounts to 0.1% of the first extraction. As the extraction efficiency of the first extraction was 99% for leaf and fruit and 98% for stem, root, and pollen, sample protein extraction proceeded without consideration for extraction efficiency.

Stability of PAT and NPT II Protein Expression Patterns in Leaf Tissue across Multiple Generations of GM Pepper. PAT and NPT II protein expression levels were evaluated by ELISA over multiple generations of GM pepper. Plant leaf tissues derived from three generations (T_3, T_4, T_5) grown under field conditions were collected at seedling stage from two test fields. PAT and NPT II proteins were present in all generations of GM pepper samples. As expected, PAT and NPT II were not detectable in SC leaves. As shown in **Table 1**, the mean PAT levels measured in leaves from the T_3, T_4 and T_5 generation plants grown in field 1 were 9.95, 10.13, and 10.17 μg/gdw, respectively. The mean PAT levels measured from field 2 were 9.82, 10.06, and 10.10 μg/gdw, respectively, from the T_3, T_4 and T_5 generation plants. There were no significant differences in PAT protein expression between plant generations ($p > 0.05$). Although PAT expression was

Table 1. PAT Protein Levels in Leaf Tissue from Multiple Generations of GM Pepper[a]

	dry weight (μg/g)		fresh weight (μg/g)	
generation	field 1	field 2	field 1	field 2
T_3	9.95 ± 0.02	9.82 ± 0.03	1.89 ± 0.01	1.86 ± 0.04
T_4	10.13 ± 0.08	10.06 ± 0.30	2.03 ± 0.01	1.91 ± 0.02
T_5	10.17 ± 0.04	10.10 ± 0.50	1.83 ± 0.01	2.02 ± 0.06

[a] PAT protein was determined by ELISA. Values are mean ± SD of triplicate measures. ELISA regression curves generated the equation $Y = 0.461x + 0.079$ ($R^2 = 0.998$). There were no significant differences in results from two fields or between plant generations ($p > 0.05$) using Fisher's LSD test.

Table 2. NPT II Protein Levels in Leaf Tissue from Multiple Generations of GM Pepper[a]

	dry weight (μg/g)		fresh weight (μg/g)	
generation	field 1	field 2	field 1	field 2
T_3	12.17 ± 0.46	12.07 ± 0.47	1.95 ± 0.07	1.93 ± 0.08
T_4	12.69 ± 0.19	12.32 ± 0.43	1.90 ± 0.03	1.82 ± 0.06
T_5	12.57 ± 0.96	12.31 ± 0.11	2.12 ± 0.13	2.04 ± 0.02

[a] NPT II protein was determined by ELISA. Values are mean ± SD of triplicate measurements. ELISA regression curves generated the equation $Y = 0.454x + 0.031$ ($R^2 = 0.992$). There were no significant differences in results from two fields or different plant generations ($p > 0.05$) using Fisher's LSD test.

Table 3. PAT Protein Levels (μg/g dry weight) in Various GM Pepper Tissues[a]

		dry weight (μg/g)	
tissue type	stage	field 1	field 2
leaves	seedling	9.95 ± 0.02	9.82 ± 0.03
	anthesis	11.44 ± 0.09	11.26 ± 0.13
	seed maturity	6.96 ± 0.05	6.93 ± 0.09
	senescence	3.65 ± 0.07	3.60 ± 0.04
stems	seedling	1.17 ± 0.03	1.15 ± 0.01
	anthesis	2.11 ± 0.01	2.07 ± 0.04
	seed maturity	1.12 ± 0.01	1.10 ± 0.02
	senescence	0.83 ± 0.02	0.81 ± 0.01
roots	seedling	0.36 ± 0.01	0.35 ± 0.02
	anthesis	0.79 ± 0.02	0.78 ± 0.06
	seed maturity	0.24 ± 0.06	0.22 ± 0.08
	senescence	0.14 ± 0.04	0.12 ± 0.06
pollen	anthesis	0.62 ± 0.01	0.59 ± 0.07
green pepper	seed maturity	11.11 ± 0.03 a	10.05 ± 0.12 b
red pepper	seed maturity	7.40 ± 0.19	7.47 ± 0.13

[a] Values are the average ± SD of triplicate measures. Pollen was analyzed as received and air-dried overnight. Significant difference ($p < 0.05$) between two fields of green pepper is marked using different letters.

slightly higher in field 1 than in field 2, this difference was not statistically significant ($p > 0.05$).

Similar results were observed for NPT II. As presented in **Table 2**, the mean NPT II levels from each generation grown in field 1 were 12.17, 12.69, and 12.57 μg/gdw, respectively. In field 2, the mean NPT II levels were 12.07, 12.32, and 12.31 μg/gdw, respectively. Again, there were no significant differences between plant generations or between regional groups ($p > 0.05$). These data suggest that the consistency of PAT and NPT II levels reflects the inherent stability of transgenic protein expression through multiple generations of GM pepper.

PAT Protein Levels in Various Plant Tissues during Different Growth Stages. PAT protein concentrations were measured in various T_3 plant tissues from two fields at four growth stages: seedling, anthesis, seed maturity, and senescence. PAT protein levels normalized to both dry-weight and fresh-weight are presented in **Tables 3** and **4**, respectively. Results were generally similar between the two fields for each tissue type and each developmental stage, with no significant differences between the two fields ($p > 0.05$) except for the green pepper, where significant field differences were detected ($p < 0.05$).

Table 4. PAT Protein Levels (μg/g fresh weight) in Various GM Pepper Tissues[a]

		fresh weight (μg/g)	
tissue type	stage	field 1	field 2
leaves	seedling	1.89 ± 0.01	1.86 ± 0.04
	anthesis	2.17 ± 0.06	2.14 ± 0.12
	seed maturity	1.32 ± 0.03	1.39 ± 0.07
	senescence	0.69 ± 0.04	0.72 ± 0.02
stems	seedling	0.21 ± 0.01	0.22 ± 0.03
	anthesis	0.40 ± 0.02	0.39 ± 0.05
	seed maturity	0.11 ± 0.01	0.14 ± 0.01
	senescence	0.08 ± 0.00	0.10 ± 0.00
roots	seedling	0.06 ± 0.00	0.07 ± 0.00
	anthesis	0.15 ± 0.02	0.15 ± 0.01
	seed maturity	0.03 ± 0.00	0.03 ± 0.00
	senescence	0.02 ± 0.00	0.01 ± 0.00
pollen	anthesis	0.56 ± 0.01	0.47 ± 0.03
green pepper	seed maturity	1.11 ± 0.01 a	0.95 ± 0.01 b
red pepper	seed maturity	1.33 ± 0.03	1.42 ± 0.02

[a] The percent dry weight of each sample was used to convert protein concentrations from gdw to gfw. Values are the average ± SD of triplicate measures. Pollen was analyzed as received and air-dried overnight. Significant difference ($p < 0.05$) between two fields of green pepper is marked using different letters.

The mean PAT protein expression levels were significantly different among plant tissues ($p < 0.05$) (**Table 3**). The results clearly show that PAT expression levels were the highest in leaves (11.44 μg/gdw and 2.17 μg/gfw) and lowest in roots (0.12 μg/gdw and 0.01 μg/gfw). PAT levels in leaves ranged between 3.60 and 11.44 μg/gdw, and in roots they ranged between 0.12 and 0.79 μg/gdw. PAT levels in stems ranged between 0.81 and 2.11 μg/gdw. At anthesis, PAT expression in pollen (0.59−0.62 μg/gdw) was lower than observed in roots (0.78−0.79 μg/gdw). At seed maturity, green pepper expressed more PAT protein than leaves.

At each developmental stage, the maximum−minimum variability of the PAT levels of each plant tissue was 18−46-fold. Highest PAT variation was observed between roots and green pepper at seed maturity (0.24−11.11 μg/gdw, 46-fold), while the lowest PAT variation was found between leaves and pollen at anthesis (0.62−11.44 μg/gdw, 18-fold). PAT expression in pepper fruits was reduced with the passage of time.

Mean PAT protein expression levels were significantly different among plant growth stages ($p < 0.05$) (**Table 3**). Except for a slight rise in anthesis, PAT expression showed a general and significant decline through senescence. Comparison among the four growth stages revealed that the highest PAT levels were found at anthesis in all tissues. From seedling to senescence, the greatest decline (6.5-fold) in PAT protein expression was observed in the roots (0.78−0.12 μg/gdw). Leaves and stems showed 3.1-fold and 2.6-fold, respectively, declines in PAT expression. Overall differences in PAT expression were higher among tissue samples than among plant growth stages.

NPT II Protein Levels in Various Plant Tissues during Different Growth Stages. NPT II protein concentrations were also measured in various tissues of T_3 plant from two fields at four growth stages. Concentrations were normalized using both dry weight and fresh weight and are presented in **Tables 5** and **6**, respectively. No statistical differences in results were observed between the two test fields ($p > 0.05$), with the exception of roots at anthesis ($p < 0.05$).

Table 5. NPT II Protein Levels (μg/g dry weight) in Various GM Pepper Tissues[a]

		dry weight (μg/g)	
tissue type	stage	field 1	field 2
leaves	seedling	12.17 ± 0.46	12.07 ± 0.47
	anthesis	17.31 ± 0.55	17.03 ± 1.68
	seed maturity	6.36 ± 0.1	6.35 ± 0.10
	senescence	4.90 ± 0.15	4.88 ± 0.52
stems	seedling	9.89 ± 0.27	9.80 ± 0.15
	anthesis	5.37 ± 0.31	5.30 ± 0.21
	seed maturity	1.08 ± 0.03	1.05 ± 0.02
	senescence	1.07 ± 0.01	0.97 ± 0.02
roots	seedling	5.66 ± 0.38	5.76 ± 0.21
	anthesis	2.63 ± 0.06 a	2.89 ± 0.05 b
	seed maturity	0.94 ± 0.08	0.92 ± 0.02
	senescence	<0.01[b]	<0.01
pollen	anthesis	<0.01	<0.01
green pepper	seed maturity	1.38 ± 0.12	1.36 ± 0.13
red pepper	seed maturity	0.66 ± 0.01	0.65 ± 0.01

[a] Values are the mean ± SD of triplicate measurements. Pollen was analyzed as received and air-dried overnight. Significant difference ($p < 0.05$) between roots of anthesis from two fields is marked using different letters. [b] 0.01 μg/g was the detection limit.

Table 6. NPT II Protein Levels (μg/g fresh weight) in Various GM Pepper Tissues[a]

		fresh weight (μg/g)	
tissue type	stage	field 1	field 2
leaves	seedling	1.95 ± 0.07	1.93 ± 0.08
	anthesis	3.41 ± 0.10	3.56 ± 0.04
	seed maturity	1.27 ± 0.07	1.27 ± 0.06
	senescence	1.03 ± 0.03	1.12 ± 0.12
stems	seedling	1.58 ± 0.04	1.57 ± 0.02
	anthesis	0.75 ± 0.04	0.95 ± 0.04
	seed maturity	0.14 ± 0.07	0.14 ± 0.04
	senescence	0.21 ± 0.01	0.21 ± 0.01
roots	seedling	0.57 ± 0.06	0.58 ± 0.05
	anthesis	0.32 ± 0.01 a	0.38 ± 0.01 b
	seed maturity	0.12 ± 0.01	0.11 ± 0.02
	senescence	<0.01[b]	<0.01
pollen	anthesis	<0.01	<0.01
green pepper	seed maturity	0.14 ± 0.03	0.09 ± 0.01
red pepper	seed maturity	0.12 ± 0.01	0.12 ± 0.08

[a] The percent fresh weight of each sample was used to convert protein concentrations from gdw to gfw. Values are the average ± SD of triplicate measures. Pollen was analyzed as received and air-dried overnight. Significant difference ($p < 0.05$) between roots of anthesis from two fields is marked using different letters. [b] 0.01 μg/g was the detection limit.

As was observed for PAT expression, mean NPT II levels were significantly different among plant tissues ($p < 0.05$) (**Table 5**). The results clearly show that NPT II levels were highest in leaves (17.31 μg/gdw and 3.41 μg/gfw) and lowest in red pepper (0.65 μg/gdw and 0.12 μg/gfw). NPT II levels ranged between 4.88 and 17.31 μg/gdw in leaves, 0.97−9.89 μg/gdw in stems, and 0.92−5.76 μg/gdw in roots. Unlike PAT protein, NPT II protein was below the limit of detection in roots during senescence and in pollen. At each stage, the maximum−minimum variability in NPT II levels for each plant tissue was 2.2−9.6-fold. Highest PAT variation was found between leaves and red pepper at seed maturity (0.66−6.36 μg/gdw, 9.6-fold). Lowest NPT II variation was found between leaves and roots at the seedling stage (5.66−12.17 μg/gdw, 2.2-fold). Over time, NPT II expression in pepper fruits was reduced by half.

Mean NPT II protein levels significantly declined through senescence. In addition, mean NPT II expression was significantly different among growth stages for each tissue ($p < 0.05$) (**Table 5**). Comparison of results from the four growth stages revealed that, with the exception of leaves, the highest NPT II levels were found in seedling tissues. For leaves, there was an increase in NPT II expression of 1.4-fold from seedling to anthesis. The 10-fold decline in NPT II expression (9.80−0.97 μg/gdw) was more rapid in stems compared to other tissues. Expression declined in leaves and roots 3.5- and 6.3-fold, respectively. There were no significant differences in NPT II protein expression among plant developmental stages for each plant tissue.

DISCUSSION

Korea has attempted to develop its own GM crops over the past 20 years, with a variety of GM crops being developed by national institutes and university researchers. However, GM crops have not yet been introduced into the commercial market (*1*). While it will take time to approve GM crops for consumption, GM pepper is likely to appear on the commercial market in the near future. Therefore, safety assessment of GM pepper is required.

While there are a few published reports on GM pepper (*1*, *17*, *18*), to date there are no publications presenting the quantitative analysis of proteins expressed in GM pepper samples collected during different plant developmental stages. We investigated the level and site of expression during the life cycle of GM pepper for the first time. In addition, the present study also presents the first detailed report on PAT and NPT II expression patterns in GM pepper grown in two field trials performed in Korea. As shown in **Tables 1** and **2**, the present results confirmed the inherent stability of transgenic protein expression through multiple plant generations. These results suggest that, independent of whether the intended effect of the modification has been achieved, all expressed traits are stable and transgenes are expressed and inherited in a stable manner. Results were also consistent between two test fields, with the exceptions of PAT protein levels in green pepper and NPT II protein levels in roots of anthesis. Therefore environmental factors or gene flow are not likely causes of this variation. According to some reports, the plant source, age of the plant samples, methods of protein extraction and analysis, specific antibody-based reagents used for protein quantification, and protein standards may influence the results of measurements (*19*, *20*). We confirmed that significant differences exist in PAT and NPT II levels among plant developmental stages and tissues. The present results suggest that concentrations of PAT and NPT II proteins decrease gradually over the growing season. Variations in target protein expression during the growing season have been reported by other investigators (*21*−*23*). These variations in protein expression during the growing season clearly indicate the necessity of evaluating specific transgenic plant protein levels during the growing season if detailed expression profiling is required.

Monitoring target protein expression in GM crops is still necessary to provide basic data for the biosafety research (*24*). Because this study was limited to plants grown during one year, results for plants grown over several years are required to validate the correlation of PAT and NPT II levels in different plant tissues and the decline over the growing season. Our study provided the detailed expression pattern of PAT and NPT II in GM pepper in field trials performed in Korea. The main objectives of this study were to assess the stability of transgenic protein expression in replicating generations and to estimate the variability in protein expression between plant tissues, including temporal changes.

The safety assessment of foods derived from GM plants involves methods to identify and detect unintended effects and

procedures to evaluate their biological relevance and potential impact on food safety. In particular, the need for a molecular characterization and assessment of potential unintended effects was identified as a basis for the assessment in all fields (*25*). According to some regulations, detailed information about the level of a protein within different parts of GM crops is one of the important parameters to evaluate the safety (*12*, *25*−*27*). Therefore, our results will provide important information for GM plants exhibiting such unintended traits.

These data are also used both for human and animal food safety assessment and environmental risk assessment, including the potential impact of GM plants on nontarget organisms. Indeed, we conducted a food safety assessment to assess the potential risks to humans and animals from dietary exposure to the PAT and NPT II proteins from the consumption of foods and feeds derived from GM pepper. The amount of pepper-derived food consumed by Koreans that could potentially contain these proteins from GM pepper was estimated using the result of our study and food consumption sheet from the Korea Rural Economic Institute (*28*). As a result, daily intake of PAT and NPT II was 21.17 μg. The daily intake of these is 0.000022% to account for a total protein daily intake of 97.4 g. It indicates that there are no meaningful risks to human health from dietary exposure to either PAT or NPT II from consumption of GM pepper. Future studies will provide an overall safety assessment of GM pepper, including potential food toxins and allergens.

ABBREVIATIONS USED

GM, genetically modified; PAT, phosphinothricin acetyltransferase; PPT, phosphinothricin; NPT II, neomycin phosphotransferase II; RDA, Rural Development Administration; KFDA, Korea Food and Drug Administration; GMO, genetically modified organisms; ELISA, enzyme-linked immunosorbent assay; SC, Subicho; NAAS, National Academy of Agricultural Science; OD, optical density.

LITERATURE CITED

(1) Lee, S. H.; Park, H. J.; Cho, S. M.; Chun, H. K.; Kim, D. H.; Ryu, T. H.; Cho, M. C. Comparison of major nutrients and mineral contents in genetically modified herbicide-tolerant red pepper and its parental cultivars. *Food Sci. Biotechnol.* **2004**, *13*, 830−833.

(2) Xu, W.; Huang, K.; Deng, A.; Luo, Y. Enzyme linked immunosorbent assay for PAT protein detection in genetically modified rape. *Chin. J. Agric. Biotechnol.* **2006**, *3*, 177−181.

(3) Thompson, C. J.; Movva, R. N.; Crameri, R.; Davies, J. E.; Lauwereys, M.; Botterman, J. Characterization of the herbicide-resistance gene *bar* from *Streptomyces hygroscopicus*. *EMBO J.* **1987**, *9*, 2519−2523.

(4) Strauch, E.; Wohlleben, W.; Pühler, A. Cloning of a phosphinothricin *N*-acetyltransferase gene from *Streptomyces viridochromogenes* Tü 494 and its expression in *Streptomyces lividans* and *Escherichia coli*. *Gene* **1988**, *63*, 65−74.

(5) Rathore, K. S.; Chowdhury, V. K.; Hodges, T. K. Use of *bar* as a selectable marker gene and for the production of herbicide-resistant rice plants from protoplasts. *Plant Mol. Biol.* **1993**, *21*, 871−884.

(6) Yamazaki, M.; Son, L.; Hayashi, T.; Morita, N.; Asamizu, T. Transgenic fertile *Scoparia dulcis* L., a folk medicinal plant, conferred with a herbicide-resistant trait using an Ri binary vector. *Plant Cell Rep.* **1996**, *15*, 317−321.

(7) Sanvar, M.; Akhtar, M. Cloning of aminoglycoside phosphotransferase (APH) gene from antibiotic-producing strain of Bacillus circulans into a high-expression vector, pKK223-3. *Biochem. J.* **1990**, *268*, 671−677.

(8) Fuchs, R. L; Heeren, R. A.; Gustafson, M. E.; Rogan, G. J.; Bartnicki, D. E.; Leimgruber, R. M.; Finn, R. F.; Hershman, A.; Berberich, S. A. Purification and characterization of microbially expressed neomycin phosphotransferase II (NPT II) protein and its equivalence to the plant expressed protein. *Nat. Biotechnol.* **1993**, *11*, 1537−1542.

(9) Balbas, P.; Bolivar, F. Design and construction of expression plasmid vectors in *escherichia coli*. *Methods Enzymol.* **1990**, *185*, 14−37.

(10) Southern, P. J.; Berg, P. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. *J. Mol. Appl. Genet.* **1982**, *1*, 327−341.

(11) Fraley, R. T.; Rogers, S. G.; Horsch, R. B.; Eicholtz, D. A.; Flick, J. S.; Fink, C. L.; Hoffman, N. L.; Sanders, P. R. The SEV System: a new disarmed Ti plasmid vector system for plant transformation. *Biotechnology* **1985**, *3*, 629−635.

(12) Korea Food and Drug Administration. *Guidelines for the Safety Assessment Data for Genetically Modified Foods and Food Additives*; KFDA notification No. 2007-60; **2007**.

(13) Grothaus, G. D.; Bandla, M.; Currier, T.; Giroux, R.; Jenkins, G. R.; Lipp, M.; Shan, G.; Stave, J. W.; Pantella, V. Immunoassay as an analytical tool in agricultural biotechnology. *J. AOAC Int.* **2006**, *89*, 913−928.

(14) Asensio, L.; González, I.; Garcá, T.; Martín, R. Determination of food authenticity by enzyme-linked immunosorbent assay (ELISA). *Food Control* **2008**, *19*, 1−8.

(15) Farid, E. A. Detection of genetically modified organisms in foods. *Trends Biotechnol.* **2002**, *5*, 215−223.

(16) Margarit, E.; Reggiardo, M. I.; Vallejos, R. H.; Permingeat, H. R. Detection of BT transgenic maize in foodstuffs. *Food Res. Int.* **2006**, *39*, 250−255.

(17) Park, H. J.; Lee, S. H; Jeong, H. J.; Cho, S. M.; Chun, H. K.; Back, O. H.; Kim, D. H.; Lilleho., H. S. The nutrient composition of the herbicide-tolerant green pepper is equivalent to that of the conventional green pepper. *Nutr. Res.* (*N.Y.*) **2006**, *26*, 546−548.

(18) Song, H. S.; Kim, J. H.; Kim, D. H.; Kim, H. Y. Qualitative and quantitative analysis of genetically modified pepper. *J. Microbiol. Biotechnol.* **2007**, *17*, 335−341.

(19) Mendelsohn, M.; Kough, J.; Vaituzis, Z.; Matthews, K. Are Bt crops safe? *Nat. Biotechnol.* **2003**, *21*, 1003−1009.

(20) Icoz, I.; Stotzky, G. Cry3Bb1 protein from Bacillus thuringiensis in root exudates and biomass of transgenic corn does not persist in soil. *Transgenic Res.* **2007**, *17*, 609−620.

(21) Fearing, P. L.; Brown, D.; Vlachos, D.; Meghji, M.; Privalle, L. Quantitative analysis of Cry1A(b) expression in Bt maize plants, tissues, and silage and stability of expression over successive generations. *Mol. Breed.* **1997**, *3*, 169−197.

(22) Wunn, J.; Kloti, A.; Burkhardt, P. K.; Biswas, G. C. G.; Launis, K.; Iglesias, V. A.; Potrykus, I. Transgenic Indica rice breeding line IR58 expressing a sythetic cryIA(b) gene from *Bacillus thuringiensis* provides effective insect pest control. *Nat. Biotechnol.* **1996**, *14*, 171−176.

(23) Wu, G.; Cui, H.; Ye, G.; Xia, Y.; Sardana, R.; Cheng, X.; Altosaar, I.; Shu, Q. Inheritance and expression of the cry1Ab gene in Bt (Bacillus thuringiensis) transgenic rice. *Theor. Appl. Genet.* **2002**, *104*, 727−734.

(24) Nguyen, H. T.; Jehle, J. A. Expression of Cry3Bb1 in transgenic corn MON88017. *J. Agric. Food Chem.* **2009**, *57*, 9990−9996.

(25) Codex Alimentarius Commission. *Guideline for the Conduct of Food Safety Assessment of Foods Derived from Recombinant-DNA Plants*; Codex Alimentarius Commission, **2003**; CAC/GL 45-2003.

(26) Health Protection Branch. *Guidelines for the Safety Assessment of Novel Foods*; Health Canada: Ottawa, Canada, 1994; Vols. I and II.

(27) European Commission. Regulation (EC) 641/2004 of 6 April 2004 on detailed rules for the implementation of Regulation (EC) 1829/2003. *Off. J. Eur. Union* **2004**, *L102*, 14−25.

(28) Korea Rural Economic Institute. *Food Balance Sheet 2008*; Korea Rural Economic Institute, 2009.

Received for review April 19, 2010. Revised manuscript received September 8, 2010. Accepted September 9, 2010. This research was supported by the National Academy of Agricultural Science (Code 200901FHT020815507), Rural Development Administration, Republic of Korea.