Table 4
Concordance of digestibility between laboratories, pH 2

Last time detected	0	0.5	2	5	10	20	30	60
Full-length Protein[a] *pH 2*								
Ara h 2	8[b]						1	
BLG								9[b]
BSA	6[b]	2	1					
ConA							6[b]	3
HRP	2	5[b]	2					
Ova						1	3	5[b]
PAT	7[b]	1	1					
RUBISCO	8[b]	1						
STI								9[b]
Sum of labs in agreement[b]	63 (out of 81 possible)							
Percent agreement	77%							
Protein Fragment(s)[a] *pH 2*								
Ara h 2	2			1			1	5[b]
BLG	9[b]							
BSA	2	1	1					5[b]
ConA							1	8[b]
HRP	5[b]			1				3
Ova								9[b]
PAT	7[b]	1						1
RUBISCO	4[b]				2		1	2
STI	9[b]							
Sum of labs in agreement[b]	61 (out of 81 possible)							
Percent agreement	75%							

Results of the number of laboratories reporting the indicated time (minutes) as the last observed time-point for each protein at pH 2.

[a] Ovomucoid (OVM) was not included in this evaluation due to the interference caused by the lysozyme contamination and the poor resolution of the full-length protein.

[b] The maximum number of laboratories in agreement regarding the last time each specific protein, or fragments of the specific protein were visible was summed in each experiment to calculate the percent agreement based on 81 (nine laboratories times 9 proteins).

(Collins and Fine, 1981; Crevieu-Gabriel et al., 1999). The pH in the lumen of the human stomach is typically between pH 1 and 2 under fasting conditions and increases above pH 5 during a meal, although there is considerable intra- and inter-individual variation (Evans et al., 1988; Lindahl et al., 1997; Russell et al., 1993). In an attempt to account for this pH range in the in vitro pepsin digestion assay, participants at a recent scientific advisory panel of the (FAO/WHO (2001)) recommended performing the pepsin digestion assay at pH 2.0. Results from the current study indicate that there was no appreciable difference in the time to disappearance of the full-length protein or protein fragments at pH 1.2 or 2.0. However, there was slightly greater consistency among laboratories at pH 1.2 than pH 2.0, perhaps because of differential buffering capacities of the test proteins, or solutions that might raise the reaction conditions beyond the optimal activity for pepsin. This result, together with the existence of an historical database of the in vitro digestive fate of proteins tested at pH 1.2 (Astwood et al., 1996; Fu et al., 2002; Kenna and Evans, 2000; Okunuki et al., 2002) as recommended by the (US Pharmacopoeia (1995, 2000)), suggests that pH 1.2 be utilized for analyses of protein digestibility by pepsin.

4.2. Effects of the gel electrophoresis system on detection of proteins and protein fragments

Tris–glycine gels are based on the traditional Laemmli buffer system (Laemmli, 1970), while Tricine gels are considered more appropriate for the resolution of low molecular weight proteins and peptides (Schagger and von Jagow, 1987). Results from this study indicate that the interpretation of test protein digestibility by the in vitro pepsin digestion assay could be affected by the gel electrophoresis system chosen to analyze experimental results. Difficulties in interpretation were most often encountered when the migration of the test protein was in close proximity to pepsin. The selection of the gel system should take into account the size of the test protein and fragments being evaluated, and may have to be empirically derived to provide optimum resolution between pepsin and the test protein.

4.3. Effects of the gel staining procedure on the pepsin digestion assay

Coomassie brilliant blue R-250 and G-250 stains can detect as little as 0.5 $\mu g/cm^2$ in a gel matrix (Syrovy and

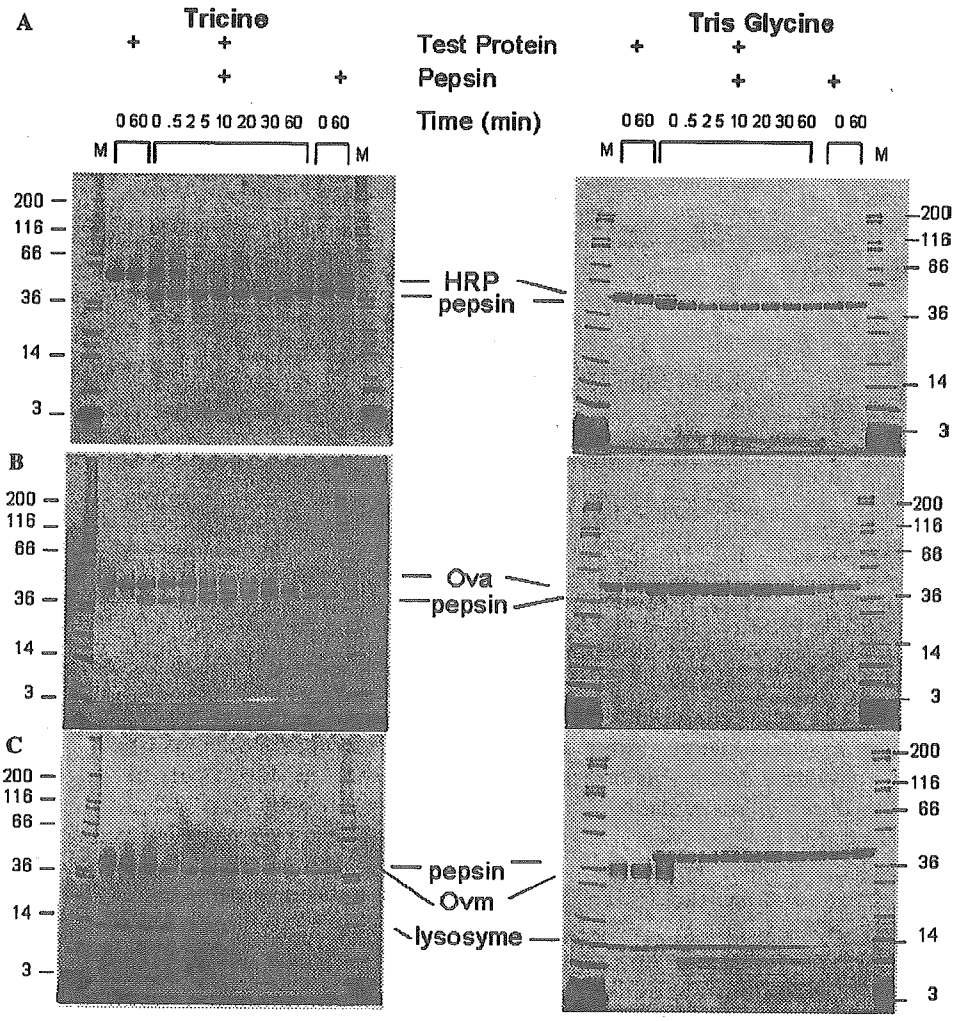

Fig. 3. Choice of gel type (Tricine vs Tris–glycine) can affect interpretation of in vitro pepsin digestion assay results. Digested samples were separated by electrophoresis SDS–polyacrylamide gels (10–20%) prior to fixing and staining. Eight laboratories used Tricine gels, one laboratory used Tris–glycine gels. Images of selected gels showing Ova (A), Ovm (B), and HRP (C) samples digested in pH 2.0 pepsin buffer.

Hodny, 1991). The dye binds primarily to basic and aromatic amino acid residues, especially arginine (Compton and Jones, 1985). Staining with colloidal Coomassie brilliant blue has been reported to detect a minimum of 0.1 ng of protein, which is slightly more sensitive than staining with other forms of Coomassie brilliant blue or methods employing other dyes (Syrovy and Hodny, 1991). In this study there were differences noted in the detection of proteolytic fragments of Ara h 2, BSA, HRP, PAT, RUBISCO, and Ova, as well as minor differences in the detection of full-length ConA and Ova between laboratories. These variations in detection may be due, in part, to differences in staining procedures used by individual laboratories, the method of fixing the proteins prior to staining, the method and time of destaining, or image capture and processing. As demonstrated in this study, either staining system could be used effectively.

4.4. Reproducibility of the assay, full-length proteins

In this study there was 91% agreement across all laboratories on the final time point at which the full-length protein was detected for all proteins tested at pH 1.2 and 77% agreement on all proteins tested at pH 2.0. Most of the differences noted were the result of laboratories reporting slightly different times at which the full-length protein was last detected. For example, at pH 1.2 seven laboratories reported that the ConA protein was last detected at the 30-min time point, while two labs reported it last detected at the 60-min time point. Similar small differences were noted when the assay was performed at pH 2.0. However, there was slightly more variation in the results when the assay was performed at this pH. It should be noted that none of the variation between participants was the result of widely disparate results of protein digestibility.

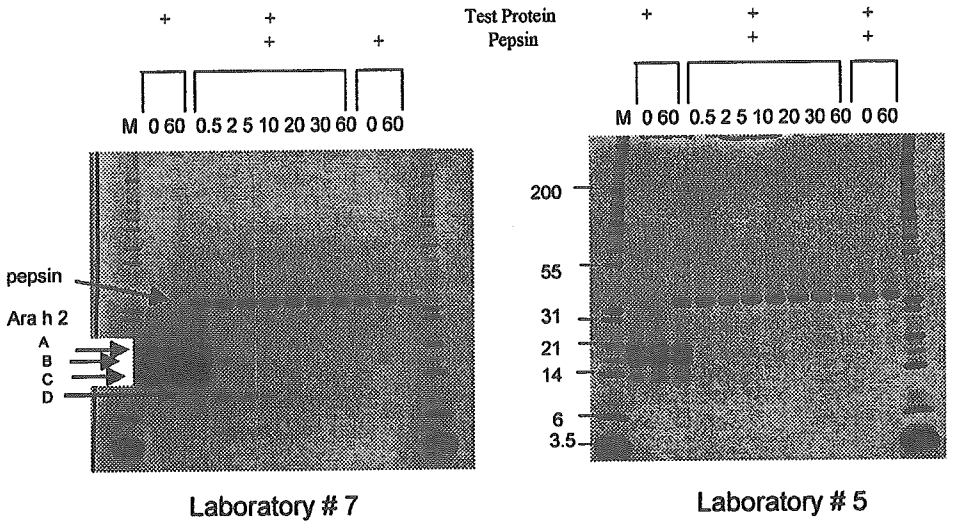

Fig. 4. Pepsin resistant fragments of Ara h 2. Seven of nine laboratories detected at least one fragment produced from proteolysis of Ara h 2 by pepsin. The left panel represents the gel from laboratory #7 for the digestion products of Ara h 2. Full-length Ara h 2 is represented by bands A and B. Band C is a contaminant. The dominant fragment (band D) was approximately 10 kDa, with the identity confirmed (data not shown). Laboratory #5 was one of the two laboratories that did not detect any fragment of Ara h 2 at any time point in the digestion assay. Both laboratory #5 and #7 fixed the gels in TCA prior to staining with Coomassie brilliant blue R-250.

One difference between this study and previous studies was noted for the peanut allergen Ara h 2. The rapid degradation of full-length Ara h 2 as observed in this study is similar to observations made by Fu et al. (2002) who showed that Ara h 2 was degraded within 0.5 min in a simulated gastric fluid containing pepsin. However, these results differ from those observed by both Sen et al. (2002) and Astwood et al. (1996) who showed that native Ara h 2 was resistant to digestion by pepsin for 20 and 60 min, respectively. One possible explanation for this discrepancy may be due to differences in the preparation of Ara h 2. The Ara h 2 for this study was prepared in the presence of dithiothreitol, a strong reducing agent, while the purification method used in the study by Astwood et al. (1996) was not reported. Sen et al. (2002) showed that the disulfide bonds of Ara h 2 contribute significantly to its overall structure and stability and if disrupted, as with a strong reducing agent, lead to its rapid degradation by GI enzymes including pepsin. Theses differences demonstrate the need to consider whether the test protein is present in the native conformation or a denatured form.

4.5. Reproducibility of the assay-protein fragments

In this study there was 80% agreement on the last time point that protein fragments were detected across all laboratories for all proteins tested at pH 1.2 and 75% agreement on all proteins tested at pH 2.0. Consensus between laboratories on the final time point at which protein fragments were detected was not as high as that observed for the full-length protein. Possible explanations for differences in the detection of protein fragments could be differences in gel staining methods and gel electrophoresis system as described above. In addition, variation was due in part to the detection in some laboratories of very small protein fragments. For example, 5/9 labs reported detecting protein fragments for RUBISCO. The protein fragments had migrated very close to the buffer front for those labs reporting their detection (<3000 MW) and could either be masked by the dye front, or have migrated out of the gel.

4.6. Biological relevance of the assay

The rationale for using pepsin-resistance as part of a safety assessment of novel proteins has been challenged in the absence of significant modification(s) to the assay (Burnett et al., 2002; FAO/WHO, 2001; Houben et al., 1997). Reluctance to support pepsin-resistance in the safety assessment of proteins has been due to the lack of reproducible and consistent correlation between pepsin-resistance and allergenicity. While there are reports in the literature which demonstrate that many major food allergens are resistant to pepsin digestion (Asero et al., 2001; Astwood et al., 1996; Murtach et al., 2002; Tanaka et al., 2002), some recent studies report pepsin resistant proteins that are not allergenic (for example, ConA and Zein), as well as pepsin sensitive proteins that are allergenic [for example, shrimp tropomyosin and casein (Fu et al., 2002)]. The absence of a standardized digestibility protocol has contributed to inconsistencies in predicting allergenicity based on resistance of proteins to pepsin digestion. Therefore, the standardization of a digestibility protocol becomes essential to the utility of pepsin-resistance in the safety evaluation of proteins.

Urisu et al. (1999) and Yamada et al. (2000) demonstrated that pepsin-resistant fragments of ovomucoid bind IgE and that the binding correlates well with the pattern of persistent allergy to egg white proteins. Other studies have also tested the IgE binding capacity of the residual fragments following pepsin digestion of allergens and found that they bind IgE, or that they contain major IgE binding domains (Maleki et al., 2000; Sen et al., 2002; Tanaka et al., 2002), or epitopes. (Budd et al., 1983; Lorusso et al., 1986). Small protein fragments, between 1500 and 3500 MW, may not be able to elicit allergic symptoms as there are theoretical limits to the size of a peptide that can simultaneously bind to two IgE molecules attached to FcεRI on mast cells and induce mast cell degranulation (Bannon et al., 2002; Lack et al., 2002). Given the fact that pepsin-resistant proteins or pepsin-resistant fragments may have the potential to bind IgE, identifying this property is useful in the safety assessment process. It must be recognized, however, that this property is not absolutely predictive of allergenic potential.

The *Codex Alimentarius* Commission (2003) recommends a "weight of evidence" approach for the assessment of potential allergenicity of proteins introduced into crops through recombinant-DNA technology, and this approach includes an in vitro pepsin digestion assay such as the one described in this study. The results obtained in this study showed consistency between laboratories for individual proteins, lending support for the inclusion of this type of assay in the Codex approach. Further data regarding the predictive value of pepsin digestibility to human food allergy may be obtained using this protocol with additional allergenic and non-allergenic proteins.

References

Asero, R., Mistrello, G., Roncarolo, D., de Vries, S.C., Gautier, M.-F., Ciurana, C.L.F., Verbeek, E., Mohammadi, T., Knul-Brettvlova, V., Akkerdaas, J.H., Bulder, I., Aalberse, R.C., van Ree, R., 2001. Lipid transfer protein: a pan-allergen in plant-derived foods that is highly resistant to pepsin digestion. Int. Arch. Allergy Immunol. 122, 20–32.

Astwood, J.D., Fuchs, R.L., 1996. Allergenicity of foods derived from transgenic plants. Monogr. Allergy 32, 105–120.

Astwood, J.D., Leach, J.N., Fuchs, R.L., 1996. Stability of food allergens to digestion in vitro. Nat. Biotech. 14, 1269–1273.

Bannon, G.A., Goodman, R.E., Leach, J.N., Rice, E., Fuchs, R.L., Astwood, J.D., 2002. Digestive stability in the context of assessing the potential allergenicity of food proteins. Comments Toxicol. 8, 271–285.

Besler, M., Steinhart, H., Paschke, A., 2001. Stability of food allergens and allergenicity of processed foods. J. Chromatogr. B Biomed. Sci. Appl. 756 (1-2), 207–228.

Buchanan, B.B., Adamidi, L., Lozano, R.M., Yee, B.C., Momma, M., Kobrehel, K., Ermel, R.W., Frick, O.L., 1997. Thioredoxin-linked mitigation of allergic responses to wheat. PNAS 94, 5372–5377.

Budd, T.W., Kuo, C.Y., Cazin, J., Yoo, T.J., 1983. Allergens of Alternaria: further characterization of a basic allergen fraction. Int. Arch. Allergy Appl. Immunol. 71 (1), 83–87.

Burnett, G.R., Wickham, M., Fillery-Travis, A., Robertson, J.A., Belton, P.S., Gilbert, S.M., Tatham, A.S., Shewry, P.R., Mills, E.N.C., 2002. Interaction between protein allergens and model gastric emulsions. Biochem. Soc. Trans. 30 (6), 916–918.

Codex Alimentarius Commission, 2003. Alinorm 03/34: Joint FAO/WHO Food Standard Programme, Codex Alimentarius Commission, Twenty-Fifth Session, Rome, Italy 30 June–5 July, 2003. Appendix III, Guideline for the conduct of food safety assessment of foods derived from recombinant-DNA plants and Appendix IV, Annex on the assessment of possible allergenicity, pp. 47–60.

Collins, J.F., Fine, R., 1981. The enzymatic digestion of elastin at acidic pH. Biochim. Biophys. Acta 657 (1), 295–303.

Compton, S.J., Jones, C.G., 1985. Mechanism of dye response and interference in the Bradford protein assay. Anal. Biochem. 151 (2), 369–374.

Crevieu-Gabriel, I., Gomez, J., Caffin, J.P., Carre, B., 1999. Comparison of pig and chicken pepsins for protein hydrolysis. Reprod. Nutr. Dev. 39 (4), 443–454.

del Val, G., Yee, B.C., Lozano, R.M., Buchanan, B.B., Ermel, R.W., Lee, Y.M., Frick, O.L., 1999. Thioredoxin treatment increases digestibility and lowers allergenicity of milk. J. Allergy Clin. Immunol. 103, 690–697.

Evans, D.F., Pye, G., Bramley, R., Clark, A.G., Dyson, T.J., Hardcastle, J.D., 1988. Measurement of gastrointestinal pH profiles in normal ambulant human subjects. Gut 29 (8), 1035–1041.

FAO/WHO, 2001. Evaluation of allergenicity of genetically modified foods. Report of a Joint FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology. January 22–25, 2001. Rome, Italy.

Fu, T.J., 2002. Digestion stability as a criterion for protein allergenicity assessment. Ann. N. Y. Acad. Sci. 964, 99–110.

Fu, T.J., Abbott, U., Hatzos, C., 2002. Digestibility of food allergens and non-allergenic proteins in simulated gastric and intestinal fluids—a comparative study. J. Agric. Food Chem. 50, 7154–7160.

Gu, X., Beardslee, T., Zeece, M., Sarath, G., Markwell, J., 2000. Identification of IgE-binding proteins in soy lecithin. Int. Arch. Allergy Immunol. 126, 218–225.

Honma, K., Kohno, Y., Saito, K., Shimojo, N., Horiuchi, T., Hayashi, H., Suzuki, N., Hosoya, T., Tsunoo, H., Niimi, H., 1996. Allergenic epitopes of ovalbumin (OVA) in patients with hen's egg allergy: inhibition of basophil histamine release by haptenic ovalbumin peptide. Clin. Exp. Immunol. 103 (3), 446–453.

Houben, G.F., Knippels, L.M.J., Penninks, A.H., 1997. Food allergy: predictive testing of food products. Environ. Toxicol. Pharmacol. 4, 127–135.

IUIS, Allergen Nomenclature, 1994. Allergen Nomenclature, by the International Union of Immunological Societies Allergen Nomenclature Sub-Committee. (Current listing at http://www.allergen.org/List.htm.) Bulletin of the World Health Organization. 72(5):796–806.

Jarvinen, K.M., Chatchatee, P., Bardina, L., Beyer, K., Sampson, H.A., 2001. IgE and IgG binding epitopes on α-lactalbumin and β-lactoglobulin in cow's milk allergy. Int. Arch. Allergy Immunol. 126, 111–118.

Kawashima, T., Taniai, M., Usui, M., Ando, S., Kurimoto, M., Matuhasi, T., 1992. Antigenic analysis of Sugi basic protein by monoclonal antibodies: II. Detection of immunoreactive fragments in enzyme-cleaved Cry j 1. Int. Arch. Allergy Immunol. 98 (2), 118–126.

Kenna, J.G., Evans, R.M., 2000. Digestibility of proteins in simulated gastric fluid. The Toxicologist 54 (1), Abstract 666.

Lack, G., Chapman, M., Kalsheker, N., King, V., Robinson, C., Venables, K., 2002. Report on the potential allergenicity of

genetically modified organisms and their products. Clin. Exp. Allergy 32, 1131–1143.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophageT4. Nature 227 (259), 680–685.

Lindahl, A., Ungell, A.L., Knutson, L., Lennernas, H., 1997. Characterization of fluids from the stomach and proximal jejunum in men and women. Pharm. Res. 14 (4), 497–502.

Lorusso, J.R., Moffat, S., Ohman, J.L., 1986. Immunologic and biochemical properties of the major mouse urinary allergen (Mus m 1). J. Allergy Clin. Immunol. 78 (5), 928–937.

Maleki, S.J., Kopper, R.A., Shin, D.S., Park, C.W., Compadre, C.M., Sampson, H., Burks, A.W., Bannon, G.A., 2000. Structure of the major peanut allergen Ara h 1 may protect IgE-binding epitopes from degradation. J. Immunol. 164 (11), 5844–5849.

Marquez, U.M.L., Lajolo, F.M., 1981. Composition and digestibility of albumin, globulins, and glutelins from phaseolus vulgaris. J. Agric. Food Chem. 29, 1068–1074.

Mendez, A., Vargas, R.E., Michelangeli, C., 1998. Effects of concanavalin A, fed as a constituent of Jack Bean (Canavalia ensiformis L.) seeds, on the humoral immune response and performance of broiler chickens. Poultry Sci. 77, 282–289.

Metcalfe, D.D., Astwood, J.D., Townsend, R., Sampson, H.A., Taylor, S.L., Fuchs, R.L., 1996. Assessment of the allergenic potential of foods from genetically engineered crop plants. Crit. Rev. Food Sci. Nutr. 36 (S), 165–186.

Murtach, G.J., Dumoulin, M., Archer, D.B., Alcocer, M.J., 2002. Stability of recombinant 2 S albumin allergens in vitro. Biochem. Soc. 30 (6), 913–915.

Nielson, S.S., 1988. Degradation of bean proteins by endogenous and exogenous proteases-a review. Cereal Chem. 65, 435–442.

Okunuki, H., Teshima, R., Shigeta, T., Sakushima, J., Akiyama, H., Goda, Y., Toyoda, M., Sawada, J., 2002. Increased digestibility of two products in genetically modified food (CP4-EPSPS and CryIAb) after preheating. J. Food Hyg. Soc. Japan 43, 68–73.

Russell, T.L., Berardi, R.R., Barnett, J.L., Dermentzoglou, L.C., Jarvenpaa, K.M., Schmaltz, S.P., Dressman, J.B., 1993. Upper gastrointestinal pH in seventy-nine healthy, elderly, North American men and women. Pharm. Res. 10 (2), 187–196.

Schagger, H., von Jagow, G., 1987. Tricin–sodium dodecyl sulfate–polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal. Biochem. 166 (2), 368–379.

Sen, M.M., Kopper, R., Pons, L., Abraham, E.C., Burks, A.W., Bannon, G.A., 2002. Protein structure plays a critical role in peanut allergen stability and may determine immunodominant IgE-binding epitopes. J. Immunol. 169, 882–887.

Syrovy, I., Hodny, Z., 1991. Staining and quantification of proteins separated by polyacryloamide gel electrophoresis. J. Chromatogr. 569, 175–196.

Tanabe, S., Kobayashi, Y., Takahata, Y., Morimatsu, F., Shibata, R., Nishimura, T., 2002. Some human B and T cell epitopes of bovine serum albumin, in the major beef allergen. Biochem. Biophys. Res. Commun. 293, 1348–1353.

Tanaka, K., Matsumoto, K., Akasawa, A., Nakajima, T., Nagasu, T., Iikura, Y., Saito, H., 2002. Pepsin-resistant 16-kDa Buckwheat protein is associated with immediate hypersensitivity reactions in patients with Buckwheat allergy. Int. Arch. Allergy Immunol. 129, 49–56.

Thompson, C.J., Movva, N.R., Tizard, R., Carmeir, R., Davies, J.E., Lauwereys, M., Botterman, J., 1987. Characterization of the herbicide resistance gene, bar, from *Streptomyces hygroscopicus*. EMBO J. 6, 25219–25223.

Urisu, A., Yamada, K., Tokuda, R., Ando, H., Wada, E., Kondo, Y., Morita, Y., 1999. Clinical significance of IgE-binding activity to enzymatic digests of ovomucoid in the diagnosis and the prediction of the outgrowing of egg white hypersensitivity. Int. Arch. Allergy Immunol. 120, 192–198.

US Pharmacopoeia, The National Formulary, 1995. USP XXIII, NF XVIII, US Pharmacopoeia Convention, Inc., Mack Printing Co., Easton, PA, p. 2053.

US Pharmacopoeia, The National Formulary, 2000. USP XXIV, NF XIX, US Pharmacopoeia Convention, Inc., Mack Printing Co., Easton, PA, p. 2235.

Yamada, K., Urisu, A., Kakami, M., Koyama, H., Tokuda, R., Wada, E., Kondo, Y., 2000. IgE-binding activity to enzyme-digested ovomucoid distinguishes between patients with contact urticaria to egg with and without overt symptoms on ingestion. Allergy 55, 565–569.

Zikakis, J.P., Rzucidlo, S.J., Biasotto, N.O., 1977. Persistence of bovine milk xanthine oxidase activity after gastric digestion in vivo and in vitro. J. Dairy Sci. 60, 533–541.

Original

A Histochemical Method Using a Substrate of β-Glucuronidase for Detection of Genetically Modified Papaya

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A histochemical assay for detecting genetically modified (GM) papaya (derived from Line 55-1) is described. GM papaya, currently undergoing a safety assessment in Japan, was developed using a construct that included a β-glucuronidase (GUS) reporter gene linked to a virus coat protein (CP) gene. Histochemical assay was used to visualize the blue GUS reaction product from transgenic seed embryos. Twelve embryos per fruit were extracted from the papaya seeds using a surgical knife. The embryos were incubated with the substrate 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc) in a 96-well microtiter plate for 10–15 hours at 37℃. Seventy-five percent of GM papaya embryos should turn blue theoretically. The histochemical assay results were completely consistent with those from a qualitative polymerase chain reaction (PCR) method developed by this laboratory. Furthermore, the method was validated in a five-laboratory study. The method for detection of GM papaya is rapid and simple, and does not require use of specialized equipment.

Key words: genetically modified papaya; recombinant DNA; GUS assay; PCR; detection method; embryo

Introduction

In recent years, there has been great progress in food biotechnology, including transgenic crop breeding and genetic modification for food production. The Ministry of Health, Labor and Welfare (MHLW) in Japan announced a mandatory safety assessment of genetically modified (GM) crops and processed foods containing GM ingredients. Since April 1, 2001, any GM foods which have not been authorized must not be imported or sold in Japan. Therefore, qualitative detection methods for regulated unauthorized GM foods were required. We reported the detection methods for GM soybean[1], GM maize[2)-4)], GM potato (NewLeaf Plus, New Leaf Y)[5),6)] and GM papaya[7] (Line 55-1 or its derivatives) using qualitative polymerase chain reaction (PCR) methods. Since qualitative PCR methods involve several steps and are costly for routine analyses, a rapid method to detect GM foods was sought.

GM papaya (Line 55-1) has not yet been authorized in Japan. The GM papaya expresses the *Papaya ringspot virus* (PRSV) coat protein (CP), which confers tolerance to PRSV, neomycin phosphotransferase II (NPTII), and β-glucuronidase (GUS)[8]. The GUS gene, which was derived from *E. coli*, encodes the enzyme β-glucuronidase. Jefferson *et al.* developed a histochemical assay for GUS expression in transformed plants using the enzyme activity of GUS towards 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc) as a substrate[9]. In the present study, we describe a rapid and

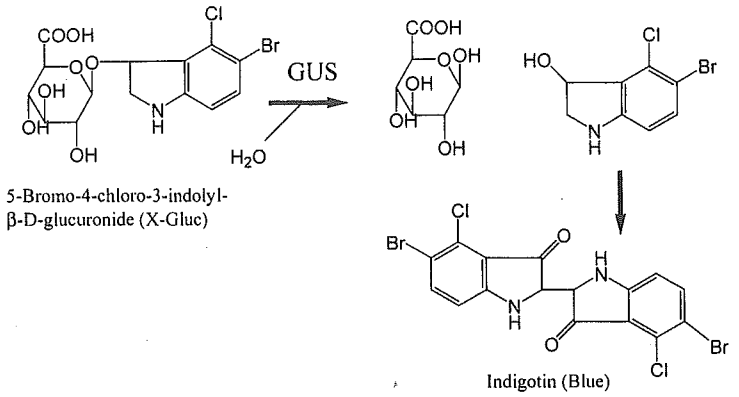

Fig. 1. Reaction between the GUS enzyme and X-Gluc

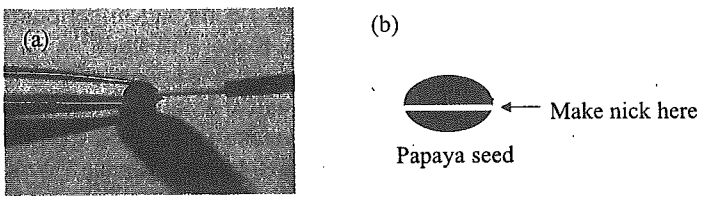

Fig. 2. Procedure for removing the seed coat
(a) Photograph of cutting the papaya seed to make the nick. (b) Schematic structure of the papaya seed cut for removing the seed coat.

simple method for the qualitative detection of GM papaya (Line 55-1 derivatives) using the GUS assay. The method was validated in a five-laboratory study.

Materials and Methods

Samples

Seeds from non-genetically modified (non-GM) papaya (Kapoho) and GM papaya (Rainbow, an F_1 hybrid of the PRSV CP gene homozygous Line 55-1× Kapoho) were obtained from the Ministry of Health, Labor and Welfare (MHLW) in Japan. The fruit and the seeds were stored at room temperature, 4°C, or −20°C for one week to 6 months.

Reagents

4-Bromo-5-chloro-3-indolyl-β-D-glucuronide (X-Gluc) and 4-bromo-5-chloro-3-indolyl-β-D-glucuronide cyclohexylammonium salt were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Agarose and a DNA marker (Cat. No. 3407A) were purchased from Takara Shuzo Co., Ltd. (Kyoto, Japan). DNA polymerase (AmpliTaq Gold) was purchased from Applied Biosystems (Foster City, CA, USA). All other reagents were of special grade. Water was purified using a Millipore Milli-Q Synthesis A 10 super water purifier and then autoclaved at 121℃ for 20 minutes.

Preparation of reagents

A freshly prepared 200 mmol/L sodium phosphate buffer (pH 7.0) was used for each test. A 20 mg sample of X-Gluc powder was transferred into a microcentrifuge tube (1.5 mL) and dissolved in 1 mL of dimethylformamide (X-Gluc solution). The X-Gluc solution was stored at −20℃. The substrate solution was prepared by adding the X-Gluc solution to 200 mmol/L sodium phosphate buffer (pH 7.0) to give a final concentration of 1 mmol/L. The solution was thoroughly mixed using a vortex mixer (MY-51, Yamato Co., Ltd., Tokyo, Japan). The substrate solution was prepared following excision of the embryos.

Procedure for identifying genetically modified papaya (Line 55-1 derivatives)

A 50 μL aliquot of 200 mmol/L sodium phosphate buffer (pH 7.0) was added to each well in a 96-well microtiter plate. The required number of wells for the assay is the number of papayas×12 since 12 embryos per papaya were tested.

The fresh papaya was cut in half lengthwise and a dark brown seed was picked at random. The viscous outer seed coat was removed with a surgical knife on a glass plate. A lengthwise cut was then made on the seedcoat in a straight line with a surgical knife. The tip of the knifepoint was placed into the cut to dislodge the seed coat, and the white endosperm enclosing the embryo was removed (Fig. 2). Careful examination of the endosperm revealed a transverse white line indicating the embryo. The endosperm was slit lengthwise adjacent to the white line. The freed embryo was carefully extracted with forceps, examined for nicks, and placed in the buffer in the microtiter plate (Fig. 3). For reaction with X-Gluc, the embryos should not be injured to prevent false-positives. Embryos from non-transgenic papaya can turn blue if the embryos are cut or broken. Twelve embryos were extracted from each test fruit. Seeds without an endosperm or seeds with

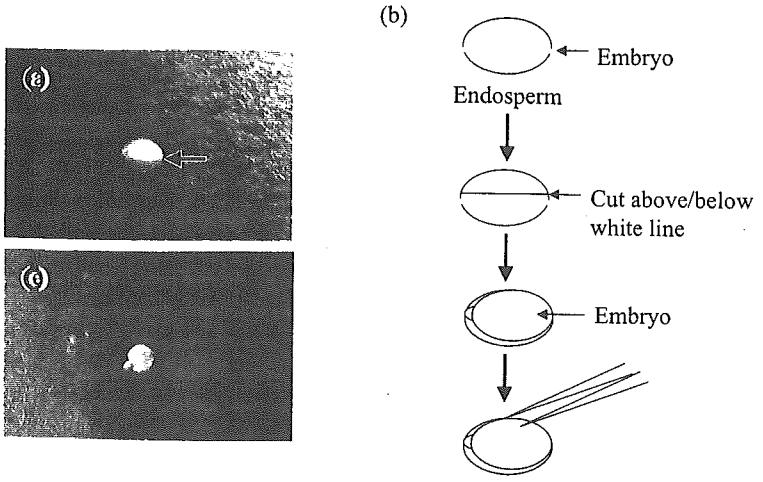

Fig. 3. Procedure for extracting embryo from the endosperm
(a) Photograph of endosperm after removing seed coat. (b) Scheme for extracting embryo from the endosperm. (c) Photograph of extracted embryo. The arrow indicates the white line.

white seed coats were not used. After placing all of the embryos in the wells, the sodium phosphate buffer was replaced with 50 μL of the substrate solution (1 mmol/L X-Gluc in 200 mmol/L sodium phosphate buffer, pH 7.0). The 96-well microtiter plate was placed under vacuum for 15 minutes using an aspirator to improve substrate infiltration into the tissue. The 96-well microtiter plate was sealed and incubated at 37℃ in an incubator (BNA-111; ESPEC Co., Ltd., Osaka, Japan) for 10–15 hours. After incubation, the reaction was stopped by adding 50 μL of 70% ethanol to each well. Finally, the embryos that were stained blue were counted and the percentage that expressed GUS was calculated using the following formula:

The percentage of embryos expressing GUS = the number of embryos that turned blue/12×100.

Assessment of the results

If the seed samples were from Rainbow papaya, a self-pollinated fruit, an average of 75% of the F_2 embryos was expected to turn blue (9/12 embryos). Embryos from non-transgenic papaya do not turn blue. Since the sample size was small, the percentage of GUS-expressing embryos was expected to vary. When the percentage expressing GUS was more than 33.3% (*i.e.*, more than 4 embryos turned blue), the sample was regarded as positive. When GUS expression was less than 25% (*i.e.*, less than 3 embryos turned blue), the sample was regarded as negative.

Qualitative PCR method

The qualitative PCR method was carried out according to the previous report[7]. The seeds were removed from the papaya, and the flesh was cut into 10 mm cubes, and freeze-dried. The freeze-dried flesh was ground using a mixing mill (SCM-40A, Shibata Co., Ltd., Tokyo, Japan). DNA was extracted using a silica-gel membrane-type kit (QIAGEN DNeasy Plant Mini) with some method modifications. An 80 mg sample of

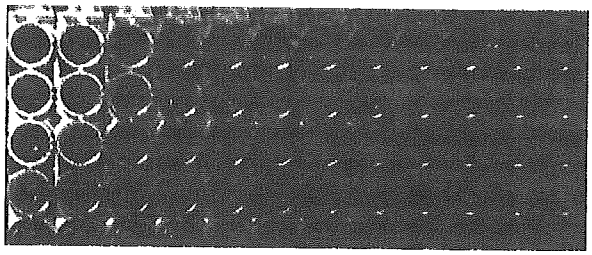

Fig. 4. Typical result of GUS method using papaya embryos

ground sample was transferred into a 2 mL microcentrifuge tube. A 400 μL aliquot of AP1 buffer previously warmed to 65℃ and 4 μL of RNase A were added to the tube, which was then incubated at 65℃ for 15 minutes. AP2 buffer (195 μL) was added, and the mixture was cooled on ice for 5 minutes and centrifuged at 10,000×*g* for 5 minutes at room temperature. The supernatant was applied to a QIAshredder spin column, and the column centrifuged for 2 minutes at 10,000×*g* at room temperature. The eluate was transferred to a 2 mL microcentrifuge tube. The eluate was added to the centrifuge tube with 1.5 times the volume of the AP3 buffer/ethanol mixture and the mixture was stirred for 10 seconds using a vortex mixer. A 500 μL aliquot of the mixture was applied to the mini spin column and the column was centrifuged at 10,000×*g* at room temperature for 5 minutes. The eluate was discarded. 500 μL aliquots of the remaining mixture were applied to the same column and the column was centrifuged, discarding the eluate after each centrifugation. The procedure was repeated until all of the mixture had been applied. A 500 μL aliquot of the AW buffer was added to the column and the column was centrifuged at 10,000×*g* at room temperature for 5 minutes. The eluate was discarded. The AW buffer was added to the column and the procedure was repeated. After the eluate was discarded, the column was centrifuged at 10,000×*g* or more for 15 minutes to dry it. The column was connected to the kit's centrifuge tube and 50 μL of

previously warmed water was added to the column. The column was allowed to equilibrate for 5 minutes and then centrifuged at 10,000×*g* for 1 minute to elute the DNA. Water was added and the same procedures were repeated. The solution of combined eluates was used as the DNA sample stock solution.

The PCR reaction mixture was prepared in a PCR reaction tube. The reaction volume of 25 μL contained 25 ng of genomic DNA, 0.2 mmol/L dNTP, 3 mmol/L $MgCl_2$, 0.2 μmol/L of 5′ and 3′ primers[8] and 0.625 units of Taq DNA polymerase. Reactions were buffered with PCR buffer II (Applied Biosystems, Foster City, CA, USA). Amplification was performed in a thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) according to the following PCR step-cycle program: pre-incubation at 95℃ for 10 minutes, denaturation at 95℃ for 0.5 minutes, annealing at 60℃ for 0.5 min, and extension at 72℃ for 0.5 minutes. The cycle was repeated 40 times followed by a final extension at 72℃ for 7 minutes.

After PCR amplification, agarose gel electrophoresis of the PCR reaction product was carried out according to the previous report[1)-7)]. Samples of 7.5 μL of each PCR reaction mixture were electrophoresed at constant voltage (100 V) on a 4% agarose gel in the TAE buffer solution (40 mmol/L Tris–HCl, 40 mmol/L acetic acid, and 1 mmol/L EDTA, pH 8.0). After electrophoresis, the gel was stained in the TAE buffer solution with 0.5 μg/mL ethidium bromide for 30 min and de-stained in distilled water for 30 min. The gel was photographed with a Chemi-lumi Image Analyser (Chemi-lum Image Analyser with "Diana" system as the analytical software, Raytest, Germany).

Inter-laboratory validation

Inter-laboratory validation was performed in five laboratories including the National Institute of Health Sciences (NIHS). We requested all participants to follow the test protocol described above. The materials, reagents, the test protocol, and three blind samples (GM papaya seeds and non-GM papaya seeds) were supplied by the NIHS. Additional reagents and materials were supplied by participating laboratories. The data were compared and analyzed statistically.

Results and Discussion

Examination of GUS detection method conditions for detecting GM papaya

The GUS assay has been used worldwide to identify transgenic plants expressing the β-glucuronidase transgene. Crops awaiting approval in Japanese safety assessments, for example, GM papaya expressing GUS gene, could be monitored with a histochemical assay. Adoption of a GUS assay protocol would simplify identification of GM papaya by replacing the PCR method. Figure 1 shows the GUS reaction. When X-Gluc is added to plant tissues expressing GUS, X-Gluc is de-esterified by GUS and the indoxyl derivative monomer is released. The blue indigotin dye (insoluble in water) is generated by oxidative polymerization of these monomers. Seed embryos of GM papaya that contain the GUS gene would turn blue. A rapid detection method for GM papaya, developed using the GUS assay of embryos, was based on a protocol prepared by the United States Department of Agriculture (USDA)[*9]. The procedure for extracting embryos from seed is shown in Figs. 2 and 3. According to the protocol prepared by the USDA, we have to excise 36 embryos from the seeds.

To judge the result rapidly, we proposed to take only 12 embryos from the seeds and to test 12 embryos because the embryo extraction from the seeds takes the longest time during the procedure. Although it takes a few hours to excise 36 embryos from the seeds, it should take only an hour to excise 12 embryos. If we test 12 embryos, the possibility of misjudgment (false-positive or -negative) in the GM papaya could be 1/416667. Thereafter, we tested only 12 embryos from each papaya.

We examined the conditions for the reaction between the expressed GUS of the embryo and the substrate X-Gluc. We found that incubation at 37℃ for 10–15 hours with 1 mmol/L X-Gluc gave the clearest blue on the embryos from the GM papaya versus white on the embryos from the non-GM papaya. When we incubated the embryos from non-GM papaya for more than 15 hours, some embryos appeared slightly blue. Therefore, incubation for longer than 15 hours can result in false positives. Figure 4 shows typical results of the method following the protocol described. Only the embryos from the GM papaya turned blue, while none of the embryos from non-GM papaya did.

Effects of the storage conditions of GM papaya on the proposed method

Since storage conditions of the GM papaya could affect GUS activity in the seeds, the effects of storage conditions were examined (Table 1). GM and non-GM papayas, stored from one week to six months at 4℃ or −20℃, were tested. Non-GM papayas were never GUS-positive. Furthermore, GM papayas stored for up to three weeks at 4℃ and three months at −20℃ were correctly identified. However, embryos from the seeds of GM papaya stored for six months at −20℃ never stained blue using this method.

Comparative study of the proposed method and qualitative PCR method

Previously, we reported the detection of recombinant DNA from GM papaya using a qualitative PCR method[7]. The qualitative PCR method and the proposed method for detecting GM papaya were compared. Fifty-two papaya samples were investigated using both the proposed method and the qualitative PCR method. The results from the proposed method were completely

[*9] Letter from the Commodities Branch to the Papaya Administrative Committee COM-01-205

Table 1. Effects of Temperature and Period for Storage on the GUS Activity

Temperature		Period of storage						
		0 day	1 w	2 w	3 w	4 w	6 w	6 m
RT	non-GM	0.0	0.0	–	–	–	–	–
	GM	69.4	79.2	–	–	–	–	–
4℃	non-GM		0.0	0.0	0.0	–	–	–
	GM		72.2	63.9	88.9	–	–	–
−20℃	non-GM		0.0	0.0	0.0	0.0	0.0	0.0
	GM		72.2	70.9	79.2	91.7	79.2	0.0

Unit: %; $n=2$–6; w: weeks; m: months; –: not tested; 0 day: The result on the day we received the papayas.

Table 2. Result of Inter-laboratory Validation for GUS Method (5 Labs)

Lab. No.	non-GM	GM-1	GM-2
1	0.0	58.3	66.7
2	0.0	75.0	50.0
3	0.0	58.3	66.7
4	0.0	66.7	83.3
5	0.0	83.3	91.7
Average percentage of GUS-expressing embryos	0.0	68.3	71.7
The percentage of correct results	100 (5/5)*	100 (5/5)*	100 (5/5)*

Unit: %

Asterisks indicate the ratio of the number of labs that submitted correct data to the number of participating labs.

consistent with those using the qualitative PCR method (data are not shown). The qualitative PCR method required three days to analyze the recombinant DNA from the papaya. On the other hand, results from the proposed method took approximately one day. Therefore, the proposed method is much faster than the qualitative PCR method.

Inter-laboratory validation of the proposed method with 5 labs

To verify the reproducibility and reliability of the proposed method, we evaluated the method in an inter-laboratory study using blind samples. Five laboratories participated in the study. All participants received the protocol, reagents, and three tubes of seeds from different papayas. Table 2 shows the results of the validation study. These results show no misjudgments of any of the blind samples tested in the five laboratories and suggest that the proposed method is suitable for detection of GM papaya (55-1 derivative).

A more rapid method is used by researchers and papaya growers in Hawaii (M. Fitch, personal communication). The fresh or dried seed are cut in half crosswise, deliberately injuring the embryo. The embryo is removed from one or both halves of the endosperm and placed directly in the well of the microtiter plate containing the X-Gluc solution. The injured embryos are allowed to incubate for 30 minutes at 37℃ and blue embryos are counted after 30 minutes. The shorter Hawaii protocol has not been compared in multiple blind tests nor compared with PCR assays, however, the results appear to be acceptable for research purposes.

In conclusion, the proposed method using the GUS assay is rapid, simple, and reproducible for the detection of GM papaya and does not require specialized equipment or complicated procedures. The method should be useful in monitoring imported papaya for the presence of GM fruits.

Acknowledgments

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References

1) Matsuoka, T., Kawashima, Y., Akiyama, H., Miura, H., Goda, Y., Sebata, T., Isshiki, K., Toyoda, M., Hino, A., A detection method for recombinant DNA from genetically modified soybeans and processed foods containing them. Shokuhin Eiseigaku Zasshi (J. Food Hyg. Soc. Japan), **40**, 149–157 (1999).
2) Matsuoka, T., Kawashima, Y., Akiyama, H., Miura, H., Goda, Y., Kusakabe, Y., Sebata, T., Isshiki, K., Toyoda, M., Hino, A., A method for detecting recombinant DNA from genetically modified maize. Shokuhin Eiseigaku Zasshi (J. Food Hyg. Soc. Japan), **41**, 137–143 (2000).
3) Matsuoka, T., Kuribara, H., Akiyama, H., Miura, H., Goda, Y., Kusakabe, Y., Isshiki, K., Toyoda, M., Hino, A., A multiplex PCR method for detecting recombinant DNAs from five lines of genetically modified maize. Shokuhin Eiseigaku Zasshi (J. Food Hyg. Soc. Japan), **42**, 24–32 (2001).
4) Matsuoka, T., Kuribara, H., Suefuji, Y., Miura, H., Akiyama, H., Kusakabe, Y., Goda, Y., Isshiki, K., Toyoda, M., Hino, A., A detection method for recombinant DNA from genetically modified maize CBH351. Shokuhin Eiseigaku Zasshi (J. Food Hyg. Soc. Japan), **42**, 197–201 (2001).
5) Akiyama, H., Sugimoto, K., Matsumoto, M., Isuzugawa, K., Shibuya, M., Goda, Y., Toyoda, M., A detection method for recombinant DNA from genetically modified potato (NewLeaf Plus potato) in snacks. Shokuhin Eiseigaku Zasshi (J. Food Hyg. Soc. Japan), **43**, 24–29 (2002).
6) Akiyama, H., Watanabe, T., Wakui, C., Chiba, Y., Shibuya, M., Goda, Y., Toyoda, M., A detection method for recombinant DNA from genetically modified potato (NewLeaf

Y potato). Shokuhin Eiseigaku Zasshi (J. Food Hyg. Soc. Japan), **43**, 301–305 (2002)
7) Goda, Y., Asano, T., Shibuya, M., Hino, A., Toyoda, M., Detection of recombinant DNA from genetically modified papaya. Shokuhin Eiseigaku Zasshi (J. Food Hyg. Soc. Japan), **42**, 231–236 (2001).
8) Fitch, M. M. M., Manshardt, R. M., Gonsalves, D., Slightom, J. L., Sanford, J. C., Virus resistant papaya plants derived from tissues bombarded with the coat protein gene of *Papaya ringspot virus*. Bio/Technology, **10**, 1,466–1,472 (1992).
9) Jefferson, R. A., Kavanagh, T. A., Bevan, M. W., GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. The EMBO Journal, **6**, 3,901–3,907 (1987).

Frequency of Mutations of the Transgene, which might Result in the Loss of the Glyphosate-Tolerant Phenotype, was Lowered in Roundup Ready® Soybeans

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Polymerase chain reaction (PCR) primers were prepared to amplify the DNA fragment between the genomic DNA sequence adjacent to the 5′-integration site of Roundup Ready® (RR) soybeans neighboring the transgene and the parts of the coding region of the transgene, together with the primer set for the internal host gene, the α' subunit of β-conglycinin storage protein gene (*Cong* gene). Using the primers for the transgene and *Cong* gene, the DNA fragments were amplified from the individual genomic DNAs prepared from 72 samples of RR soybean isolated from imported soybean seeds labeled "not segregated." Although the frequency of alterations of the nucleotide sequences in both the transgene and *Cong* gene were almost the same, the mutations that caused alterations to the amino acid sequence were more highly repressed in the transgene than in the *Cong* gene. In the nucleotide sequence upstream of the coding region of the transgene, the number of alterations of the nucleotide in the proximal promoter region was smaller than that in the further upstream region, suggesting that the mutants missing or being weak glyphosate-tolerance by an alteration of the critical nucleotide sequences in the promoter or coding region might be discarded artificially. It is supposed that the selective bias on the transgene might be extremely high, which indicates that the nucleotide sequence of the transgene might be stable and maintained in inbred RR soybean lines.

Key words —— genetically modified crop, genetically modified organism, mutation, Roundup Ready soybean, soybean, transgene

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INTRODUCTION

It has been suspected that the transgene might be a "hot spot" for mutations in the genetically modified (GM) organisms, but current research into commercially important GM crops has not yet indicated if this suspicion is correct. It is true that some reports showed instability of the transgenes caused by rearrangements of transforming DNA in regenerated calli and plantlets just after transformation.[1,2] However, such instability should be prevented to establish commercial GM cultivars. The transgenes should also be inherited correctly and in a stable and predictable manner over successive generations. Field trial experiments showed a stable inheritance of the phenotype in the progeny of GM crops, and in other experiments the stability of the transgenes in individual plants of the progeny of GM crops were found using a Southern blot analysis.[3–5] These results suggested that recombination or rearrangement of the transgenes required to drastically alter their structure did not occur, or occurred to only a limited extent, in the progeny of GM crops. However, the alteration, substitution or deletion of transgenes at the nucleotide sequence level has not yet been investigated in individual plants of the progeny of GM crops.

We isolated and identified the genomic sequence adjacent to the 5′-integration site of the transgene of Roundup Ready® (RR) soybeans.[6] Using this sequence, we prepared the primer pair amplified for

the proximal insertion region, the cauliflower mosaic virus 35S promoter (designated as 35S promoter) and part of the petunia chloroplast transit peptide (*CTP*) gene in the RR soybean. We also constructed the primers for the promoter and coding regions of the soybean α' subunit of β conglycinin storage protein genes (designated *Cong* genes below)[7,8] as controls of the internal host gene. β conglycinin is a major component of seed storage proteins in the soybean (up to 30% of total seed protein) and is composed of three subunits: α, α' and β. Their high expression ensures the synthesis and accumulation of storage protein and affects the nutritive quality of seed crops. As such, these genes might have been maintained by breeders. Using the primers for the transgene and internal gene, DNA fragments were amplified with the genomic DNA prepared from 72 individual RR soybeans isolated from batches of imported soybeans with an "unsegregated" label from U.S.A. that had been sampled by the quarantine station of the Ministry of Health, Welfare and Labor (MHWL). The nucleotide sequences of the amplified DNA fragments using these primer pairs were determined, and the mutation rates of the nucleotide and deduced amino acid sequences in the transgene were analyzed in comparison with the internal *Cong* genes.

MATERIALS AND METHODS

Preparation of Genomic DNA from Soybean and Identification of the RR Soybean —— Soybean (*Glycine max*) seeds imported from U.S.A. labeled as "not segregated" and sampled by the quarantine station of the MHLW were used in this study. The genomic DNAs were prepared from individual seeds of several sampled batches using a modified CTAB method.[9] In order to identify which genomic DNA preparations were derived from individual RR soybean seeds, the genomic DNAs were subjected to a qualitative PCR using the primers for the nucleotide sequences of the 35S promoter and 5-enol-pyruvylshikimate-3 phosphate synthase (*EPSPS*) gene.[9]

PCR Amplification of the Genomic DNA Prepared from RR Soybeans —— In order to isolate the DNA fragments that corresponded to the proximal insertion region-35S promoter-*CTP* (accession number I07318), the primer pair of CHM1, 5'-ACTGAAAAATTCAGAACCTTGTGC-3', and CTP-R, 5'-TTCAAAACCAACATAGAATTTGCTG-3', was prepared. The other pair, C-1, 5'-GCTGATCAGGATCGCCGCGTC-3', and C-2R, 5'-GAATGGACGTGGCTGCTCACC-3', was prepared to isolate the fragments that corresponded to the proximal promoter and coding region of the *Cong* gene (accession number M13759). A DNA extract (4 μl) of 100 ng prepared above was added to a reaction mixture that consisted of 1 × LA-PCR buffer (TaKaRa Bio Inc., Shiga, Japan), 2.5 mM $MgCl_2$, 0.25 mM dNTP and 0.5 μM of the primers. The mixture was made up to a total volume of 20 μl with water, and the equal volume of mineral oil was added on the mixture. The tubes of the reaction mixture were set on the PCR machine (MiniCycler™, MJ Japan Ltd., Tokyo, Japan) and incubated at 98°C for 1 min, after which the temperature was lowered to 92°C. The cap of the tube was opened and 0.25 units (1 μl) of LA *Taq* DNA polymerase (TaKaRa Bio Inc.) were added to the bottom of the reaction mixture using a pipette. The cap was closed and PCR proceeded for 35 cycles of denaturing at 92°C for 30 sec, annealing at 52°C for 45 sec and extension at 72°C for 1 min. Following this, the mixture was incubated at 72°C for 10 min, and then cooled at 4°C. PCR products were separated by 1.5% agarose gel electrophoresis and the DNA fragments were subcloned into the Bluescript SK+ plasmid. T7 and T3 primers were used to determine the double strand nucleotide sequences using LI-COR 4000 (Aloka Co., Ltd., Tokyo, Japan) or ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems Japan Ltd., Tokyo, Japan).

RESULTS AND DISCUSSION

It has been suspected that the transgene(s) introduced into GM organisms are unstable and variable compared to the internal host genes. However, the neutral theory of molecular evolution[10] tells us that the rate of mutation, such as point mutations of nucleotide sequences, must occur equally in the whole genome of all organisms. As a result, elite organisms will be selected and prosper against environmental pressure. If the natural theory is applicable to GM crops, the rate of mutation of the transgene should be the same as that of internal host genes. However, the rate of substitutions and deletions of nucleotide and amino acid sequences of the transgene has not been investigated in detail. Here, the frequency of mutations of the transgenes and the internal host gene, *Cong* gene, was investigated in the individual genomes of 72 RR soybeans by de-

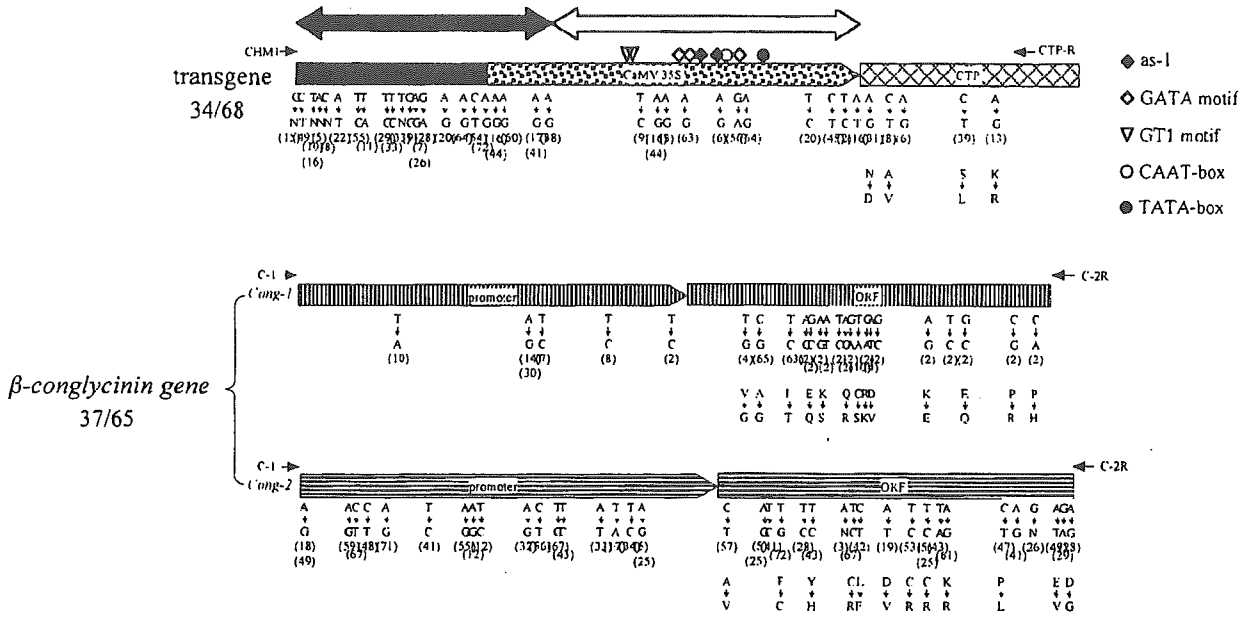

Fig. 1. Point Mutations and Deletions at the Proximal Promoter and Coding Regions of the Transgene and *Cong* Genes in Individual RR Soybeans

Among the 68 and 65 nucleotide sequences that could be determined by double strands, 34 and 37 differences could be observed in the transgene and *Cong* gene, respectively. The number in parentheses indicates the sample number of the individual RR soybeans in which substitutions or deletions of the nucleotide sequences were found. The arrowed upper letters, A, G, C and T, are nucleotides that were substituted to the lower letters. Nucleotides changed to N are point deletions. The lower parts under the coding regions (CTP and ORF) show the substitution of deduced amino acid sequences. The symbols at the upper side of the 35S promoter show the position of the *cis*-elements and CAAT- and TATA-boxes. The region with the open line and double arrowheads is the putative promoter region, while the closed line with double arrowheads indicates the upstream region of the promoter of the transgene.

termination of the nucleotide sequences of PCR amplified DNA fragments using the primer pairs, CHM1 and CTP-R for the transgene and C-1 and C-2R for *Cong* genes. Nucleotide sequences of 68, 15 and 50 of amplified DNA fragments for the transgene, *Cong-1* gene and *Cong-2* gene, respectively, were determined by double strand sequences (Fig. 1). The lengths of the determined sequences cut from the proximal region of the primer sites were 572 bp for the transgene, 604 bp for the *Cong-1* gene and 617 bp for the *Cong-2* gene (Table 1). Point mutations and deletions of 34 and 37 for the fragments derived from the transgene and *Cong* genes, respectively, were identified (Fig. 1). In total, we determined the nucleotide sequences of 38896 bps (68 fragments × 572 bps) and 39910 bps (15 fragments × 604 bps + 50 fragments × 617 bps) for the transgene and *Cong* genes, respectively. This showed that one mutation was found per 1144 bps in the transgene and 1079 bps in the *Cong* genes (Table 1). These results indicated that the mutation rate of the transgene was almost the same as that of the *Cong* genes. The amino acid sequences of the coding region of the transgene and *Cong* genes, 41 amino acids and 97 or 96 amino acids, respectively, were encoded in the fragments. Although only four amino acid substitutions were found in the transgene, 25 substitutions were found in the Cong proteins (Fig. 1). This showed that the substitution of amino acids was found per 697 and 250 amino acid sequences encoded in the transgene and *Cong* genes, respectively (Table 1). Despite almost the same chance to cause base substitutions between the transgene and *Cong* genes, amino acid substitutions were repressed in the transgene compared to those in the *Cong* genes. The repression of the substitution in the amino acid sequences was because the mutations in the nucleotide sequence of the coding region of the transgene occurred in the third letters of the codons. If the amino acid sequences of the transgene changed, some of the substitutions would result in a loss of the functions of the transit peptide, CTP. This would lead to a loss of the most important phenotype, glyphosate-tolerance. The loss of this phenotype would mean the loss of the superiority of RR soybeans to non-GM soybeans. Thus, during the development, maintenance and seed production of RR soybean lines, the highest selection pressure should be placed on preventing the loss of the glyphosate-tolerant phenotype. It is thought that the amino acid

Table 1. Frequency of the Nucleotide and Amino Acid Sequences of the Transgene and *Cong* Gene in Individual RR Soybeans

	Number of confirmed nucleotide sequences	Number of nucleotide sequences (bp)	Total number of nucleotide sequences (bp)	Total number of mutation points	Average length of appearance of the mutation (bp/one mutation)
Mutation on the nucleotide sequence					
Transgene	68	572	38896	34	1144
Cong-1 gene	15	604	39910	37	1079
Cong-2 gene	50	617			
	Number of confirmed nucleotide sequences	Number of deduced amino acid sequences	Total number of amino acid sequence	Total number of amino acid substitutions	Average length of the appearance of the mutation (amino acids/one substitution)
Mutation on the amino acid sequence					
Transgene	68	41	2788	4	697
Cong-1 gene	15	97	6255	25	250
Cong-2 gene	50	96			

sequence of the transgene might be tend to be more stable but less change than other genes.

An objection could be raised that the transgene is a new gene, which was introduced into the soybean genome about a decade ago.[3] However, *Cong* genes are old internal host genes that exist in the elite soybean inbred line(s) established several decades ago. Alterations of nucleotide and amino acid sequences have accumulated in *Cong* genes of the inbred lines, which were introduced into the original glyphosate-tolerant soybean by a backcross during breeding to develop the Roundup Ready® soybean. Cong proteins are storage proteins in soybean seeds and some of the alterations in the amino acid sequences do not cause lethal mutations. As such, alterations might have accumulated in the soybean genome (Fig. 1). The above results should be confirmed by future experiments to investigate the mutation rate in the transgene after several decades.

It is remarkable that the alteration of the nucleotide sequences of the promoter region of the *Cong-1* gene might be reduced in comparison with the coding region of the *Cong-1* gene and the promoter region of the *Cong-2* gene (Fig. 1). This reduction might prevent the loss of promoter activity in the *Cong-1* gene, which suggests that expression of the *Cong-1* gene might be required to produce Cong-1 protein as a storage protein in soybean seeds, even if several amino acid sequences of Cong-1 protein have been altered. Therefore, selection pressure by breeders might be placed on the promoter region, and not on the coding region, of the *Cong-1* gene, whereas it might be placed equally on both regions of the *Cong-2* gene. When the nucleotide sequence of the soybean genomic DNA and 35S promoter region was divided into the proximal promoter region (shown by the open line with double arrowheads in Fig. 1) and its upstream region (closed line with double arrowheads in Fig. 1), the number of mutation points in each region was found to be completely different (12 in about 255 bps and 25 in about 210 bps for the proximal and upstream regions, respectively). That is, the nucleotide sequence of the proximal promoter region was less mutated and more stable than the 5′ upstream region. The transgene in the original glyphosate-tolerant soybean should have a homologous nucleotide sequence. Divergence of the nucleotide sequences was detected in the progeny of the RR soybean. The 35S promoter is the most popular and common promoter to drive transgenes in transgenic plants because its expression is very high, but it is less tissue or organ specific in many plant species.[11] Several *cis*-elements important for its expression have been revealed.[12] These include as-1[13] and the GT-1[14] and GATA motifs.[15] All these elements were located in the proximal promoter region (see Fig. 1). If the point mutation occurred in the *cis*-element that included the essential boxes for transcription factors, *e.g.*, CAAT and TATA, it should stop or reduce promoter activity, which would result in a loss or reduction of the glyphosate-tolerant phenotype. Therefore, breeders should continually select highly tolerant line(s) during the reproduction of seeds to keep the nucleotide sequence of the important region of the 35S promoter. Although the region noticed here was a homologous nucleotide sequence to the original, the speed and rate of mutations were different between the proximal promoter and its upstream regions. Selective pressure (artificial inbreeding bias) from the developer and pro-

ducer of the seeds might lead to the nucleotide sequence of the important region for strong promoter activity being kept and not as greatly mutated than the upstream region of the promoter. This might ensure a high expression of the transgene, and perhaps result in phenotypes derived from it, glyphosate tolerance being kept and maintained as in the Roundup Ready® soybeans that are commercially superior to non-GM soybeans.

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Takeshi Ogasawara and Yukie Chikagawa contributed equally to this report as the first author.

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REFERENCES

1) Register, J. C., Peterson, D. J., Bell, P. J., Bullock, W. P., Evans, I. J., Frame, B., Greenland, A. J., Higgs, N. S., Jepson, I. and Jiao, S. (1994) Structure and function of selectable and non-selectable transgenes in maize after introduction by particle bombardment. *Plant Mol. Biol.*, **25**, 951–961.
2) Kohli, A., Griffiths, S., Palacios, N., Twyman, R. M., Vain, P., Laurie, D. A. and Christou, P. (1999) Molecular characterization of transforming plasmid rearrangements in transgenic rice reveals a recombination hotspot in the CaMV 35S promoter and confirms the predominance of microhomology mediated recombination. *Plant J.*, **17**, 591–601.
3) Padgette, S. R., Kolacz. K. H., Delannay, X., Re, D. B., LaVallee, B. J., Tinius, C. N., Rhodes, W. K., Otero, Y. I., Barry, G. F., Eichholts, D. A., Peschke, V. M., Nida, D. L., Taylor, N. B. and Kishore, G. M. (1995) Development, identification, and characterization of a glyphosate-tolerant soybean line. *Crop Sci.*, **35**, 1451–1461.
4) Delannay, X., Bauman, T. T., Beighley, D. H., Buettner, M. J., Coble, H. D., DeFelice, M. S., Derting, C. W., Diedrick, T. J., Griffin, J. L., Hagood, E. S., Hancock, F. G., Hart, S. E., LaVallee, B. J., Loux, M. M., Lueschen, W. E., Matson, K. W., Moots, C. K., Murdock, E., Nickell, A. D., Owen, M. D. K., Paschall, E. H., Prochaska, L. M., Raymond, P. J., Reynolds, D. B., Rhodes, W. K., Roeth, F. W., Sprankle, P. L., Tarochione, L. J., Tinius, C. N., Walker, R. H., Wax, L. M., Weigelt, H. D. and Padgette, S. R. (1995) Yield evaluation of a glyphosate-tolerant soybean line after treatment with glyphosate. *Crop Sci.*, **35**, 1461–1467.
5) Armstrong, C. L., Parker, G. B., Pershing, J. C., Brown, S. M., Sanders, P. R., Duncan, D. R., Stone, T., Dean D. A., DeBoer, D. L., Hart, J., Howe, A. R., Morrish, F. M., Pajeau, M. E., Petersen, W. L., Reich, B. J., Rodriguez, R., Santino, C. G., Sato, S. J., Schuler, W., Sims, S. R., Stehling, S., Tarochione, L. J. and Fromm, M. E. (1995) Field evaluation of European corn borer control in progeny of 173 transgenic corn events expressing and insecticidal protein from *Bacillus thuringiensis*. *Crop Sci.*, **35**, 550–557.
6) Ogasawara, K. (2005) Development of detection method for genetically modified food. In *Ph D thesis of Tokyo University of Agriculture and Technology*, Tokyo, Japan.
7) Doyle, J. J., Schuler, M. A., Godette, W. D., Zenger, V., Beachy, R. N. and Slightom, J. L. (1986) The glycosylated seed storage proteins of *Glycine max* and *Phaseolus vulgaris*. Structural homologies of genes and proteins. *J. Biol. Chem.*, **261**, 9228–9238.
8) Yoshino, M., Kanazawa, A., Tsutsumi, K., Nakamura, I. and Shimamoto, Y. (2001) Structure and characterization of the gene encoding alpha subunit of soybean β-conglycinin. *Genes Genet. Syst.*, **76**, 99–105.
9) Yamaguchi, H., Sasaki, K., Kidachi, Y., Shirama, K., Kiyokawa, S., Ryoyama, K., Matsuoka, T., Hino, A., Umetsu, H. and Kamada, H. (2000) Detection of recombinant DNA in genetically modified soybeans and tofu. *Jpn. J. Food Chem.*, **7**, 112–116.
10) Kimura, M. (1983) *The neutral theory of molecular evolution*. Cambridge University Press, London.
11) Bevan, M. W., Manson, S. E. and Goelet, P. (1985) Expression of tobacco mosaic virus coat protein by a cauliflower mosaic virus promoter in plants transformed by *Agrobacterium*. *EMBO J.*, **4**, 1921–1926.
12) Odell, J. T., Nagy, F. and Chua, N.-H. (1985) Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter. *Nature* (London), **313**, 810–812.
13) Lam, E., Benfey, P. N., Gilmartin, P. M., Fang, R. X. and Chua, N.-H. (1989) Site-specific mutations alter in vitro factor binding and change promoter expression pattern in transgenic plants. *Proc. Natl. Acad. Sci. U.S.A.*, **86**, 7890–7894.
14) Lam, E. and Chua, N.-H. (1990) GT-1 binding site confers light responsive expression in transgenic tobacco. *Science*, **248**, 471–474.
15) Lam, E. and Chua, N.-H. (1989) ASF-2: A factor that binds to the cauliflower mosaic virus 35S promoter and a conserved GATA motif in *Cab* promoters. *Plant Cell*, **1**, 1147–1156.

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Construction of ELISA System to Detect NPTII Protein in Genetically Modified Foods

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Abstract

An ELISA detection system specific for neomycin phosphotransferase II (NPTII) protein was constructed using the antibodies against overexpressed NPTII protein in recombinant *Escherichia coli*. NPTII protein in genetically modified (GM) tobacco plants could be qualitatively detected using this system; however, non-specific detection in normal tobacco, soybean, corn and potato was not observed. These results suggest that the constructed ELISA system can monitor NPTII protein in GM foods.

Key words: genetically modified (GM) food, neomycin phosphotransferase II (NPTII), ELISA

I. Introduction

GM foods are being circulated throughout the world and therefore, the importance of their safety assessment was proposed. The safety assessment criteria vary among different countries. In Japan, the stability of the inserted DNA, potential toxicity and allergenicity of an expressed protein, secondary effects due to gene expression etc., have to be assessed according to the safety assessment, which is mandatory required by the Food Sanitation Law.

NPTII catalyzes the ATP-dependent phosphorylation of the 3'-hydroxyl group in the aminohexose region of certain aminoglycosides, including neomycin and kanamycin. The gene coding NPTII (*npt*II) has been used as a selectable marker to distinguish GM organisms. The *npt*II is contained in various kinds of vector plasmids and is transferred to cells with the objective gene(s). In some GM foods, that have already been approved in Japan, namely, corn, potato, sugar beat, cotton, the *npt*II has been used as a marker gene during the preparation of the recombinant. However, it is not removed and is expressed in cells after the foods are commercialized. Hence, it is necessary to evaluate the *npt*II during the safety assessment of GM foods similar to the assessment of genes that confer resistance to herbicides, insects, viruses, etc. There have been no reports regarding the adverse effects of either NPTII or the *npt*II on humans, animals or the environment (Miki and McHugh, 2004). It has also been mentioned that the *npt*II has been appropriately evaluated, and there are no safety concerns for the time being (Food Safety Commission Decision, 2004). In future, the marker gene should be removed for consumer acceptance based on the strategies for creating marker-free transgenic plants (Miki and McHugh, 2004). On the other hand, it is necessary that the safety of the *npt*II or the NPTII protein is continuously monitored to avert unintended or unexpected effects.

In this paper, in order to monitor the stability of the NPTII protein and the expression of the *npt*II, an ELISA detection system was constructed. The antibodies for the ELISA system were prepared from the NPTII protein that was overexpressed in recombinant *E. coli*. The constructed ELISA system could qualitatively detect the NPTII protein expressed in a genetically modified tobacco plant.

II. Materials and Methods

1. Construction of NPTII expression vector

The strategy for the construction is shown in Fig. 1. The *npt*II was amplified using the primer pair of 5'-CATATGATTGAACAAGATGGATT-3' and 5'-

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TCAGAAGAACTCGTCAAGAAGGCGAT-3'. pBI121 plasmid DNA was used as the template DNA. The reaction mixture for PCR consisted of 1x PCR buffer (TaKaRa Bio Inc., Shiga, Japan), 0.25 mM dNTP (TaKaRa Bio Inc.), 0.2 μM primers, 1 unit of rTaq DNA polymerase (TaKaRa Bio Inc.), and 0.1 μg template DNA. The mixture was made up to 25 μl with water. The tube of the reaction mixture was set on the thermal cycler (TP-400, TaKaRa Bio Inc.) and PCR was carried out as follows: 30 cycles of denaturation at 94°C for 30 s, annealing at 54°C for 30 s, and extension at 72°C for 1 min. Then, the mixture was incubated at 72°C for 10 min, and then cooled at 4°C. The amplified DNA fragment was subcloned into the pCR®2.1-TOPO® plasmid (Invitrogen, Tokyo, Japan). M13 reverse and forward primers were used to determine the double strand nucleotide sequences using Gene Rapid (Amersham Pharmacia Biotech, Tokyo, Japan).

The *npt*II that was subcloned into the pCR®2.1-TOPO® plasmid was digested with *Nde*I and *Xho*I restriction enzymes (Nippon Gene Co. Ltd., Tokyo, Japan). The fragment was ligated with the expression vector pET14b (Novagen, Merck Co. Ltd., Tokyo, Japan); it was then digested with *Nde*I and *Xho*I restriction enzymes. Ligation mixture consisted of a solution from Ligation high™ (Toyobo Co. Ltd., Osaka, Japan) and all the fragments. The mixture was incubated at 16°C for 30 min. An aliquot of the mixture was used for transformation to BL21(DE3) competent cells (Novagen, Merck Co. Ltd.). The transformant was screened on LB medium containing ampicillin. Screened *E. coli* consisting of appropriate plasmids were used for expression of the NPTII protein. The *npt*II on the plasmid was sequenced with a T7 promoter primer and a T7 terminator primer using Gene Rapid.

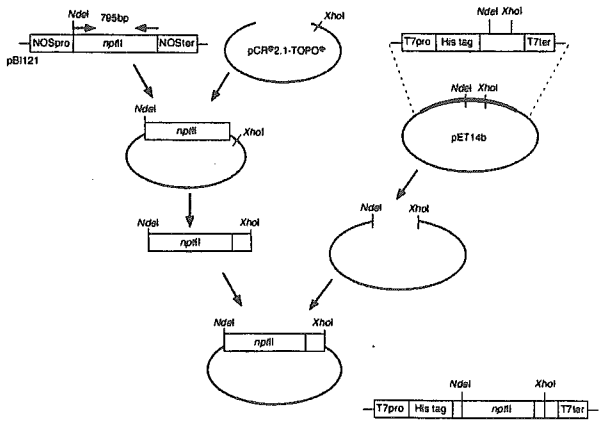

Fig. 1 Strategy for the construction of the *npt*II-pET14b vector.
The PCR amplified *npt*II was inserted in to the pCR®2.1-TOPO® vector. The insert in the *npt*II-pCR®2.1 plasmid was digested with *Nde*I and *Xho*I restriction enzymes. The fragment was inserted in to the pET14b plasmid that was digested with the same enzymes.

2. Preparation of NPTII protein

The screened *E. coli* were incubated in LB liquid medium at 37°C for 2.5 h, and 0.4 mM of IPTG was added after incubation at 37°C for 2 h. The cells were harvested with centrifugation at 3000 rpm for 10 min. The pellet was suspended in Bug Buster™ reagent (Novagen, Merck Co. Ltd.) containing 25 U/ml of Benzonase® nuclease (Novagen, Merck Co. Ltd.), and the mixture was incubated at room temperature for 15 min for protein extraction. The cell debris was removed by centrifugation at 15,000 rpm for 30 min at 4°C. The soluble extract was analyzed using SDS-PAGE and was loaded onto a His-Bind Resin (Novagen, Merck Co. Ltd.) column. The fraction was eluted with elution buffer containing 30, 60, and 90 mM imidazole, and then was analyzed by SDS-PAGE. The obtained fusion protein (His tag and NPTII) was treated with biotinylated thrombin (Novagen, Merck Co. Ltd.), and the His tag was removed from the fusion protein. The biotinylated thrombin was removed using streptavidin agarose beads (Novagen, Merck Co. Ltd.) and the cleaved His tag was removed using the His-Bind Resin column.

3. Preparation of anti-NPTII antibody

Polyclonal anti-NPTII antibodies and peroxidase labeled anti-NPTII antibodies were custom prepared at TaKaRa Bio Inc. Rabbits were used for the preparation of polyclonal antibodies. Prepared antibodies were purified with protein A and peroxidase labeled anti-NPTII antibodies were prepared with periodate.

4. NPTII detection by ELISA

Anti-NPTII polyclonal antibodies were diluted to 10 μg/ml with PBS buffer (8 g/l NaCl, 0.2 g/l KCl, 2.9 g/l Na_2HPO_4 and 0.2 g/l KH_2PO_4), and diluted antibodies were placed in the wells of a 96-well microplate (100 μl/well). The microplate was incubated overnight at 4°C. After the incubation, the antibodies were discarded and the wells were blocked with 200 μl of Block Ace (Dainippon pharmaceutical Co. Ltd., Osaka, Japan). After 1 h incubation at room temperature, the wells were washed 3 times with PBS buffer, and the appropriately diluted sample was placed in the wells (100 μl/well). The microplate was incubated at room temperature for an hour. After the incubation, the sample was discarded, and 2.57 μg/ml peroxidase-labeled antibody was put in the wells (100 μl/well). After incubation for 1 hour at room temperature, the wells were washed 3 times with PBS buffer and TMB substrate (KPL Inc., Maryland, USA) was added in the wells (100 μl/well). After 10 min, the reaction was terminated with 100 μl of 1 M H_3PO_4, and the absorbance was measured at 450 nm using a MTP-300 microplate reader (Corona Electric Co. Ltd., Ibaraki, Japan).

5. Establishment of transgenic tobacco with pBI121

Axenic shoots of tobacco strain SR1 were cultured on a phytohormone-free solid Murashige and Skoog (MS; Murashige and

Skoog 1962) medium under 16 h light/8 h dark conditions at 25°C. Leaves of these plants were cut and cocultured with *Agrobacterium tumefaciens* strain EHA101 harboring pBI121. Infected leaves were cultured on MS solid medium containing 1 mg/l 6-benzylaminopurine (BAP) under the same conditions. After 1 week, the leaves were transferred to solid MS medium containing 1 mg/l BAP and 250 mg/l Claforan® (Hoechst Marion Roussel Ltd., Tokyo, Japan) to eliminate bacteria. Several weeks later, newly formed shoots were transferred to solid MS medium containing 0.1 mg/l kanamycin and from these, transformants were selected. The selected transformants were maintained on phytohormone-free solid MS medium. Using RED Extract-N-Amp™ Plant PCR Kits (Sigma-Aldrich Inc., Missouri, USA), the genomic DNA of the transformants was prepared, and the integrated *npt*II was confirmed using the primer pair of 5'-CGTTCAAAAGTCGCCTAAGGTCAC-3' and 5'-AGCAGCCGATTGTCTGTTGTGC-3'. Total protein in the leaves of the transformants was extracted, and the NPTII protein was analyzed using ELISA.

III. Results and Discussion

1. Expression and purification of NPTII protein

The *npt*II on the pBI121 plasmid was amplified using PCR with the 5'-primer which includes the *Nde*I restriction enzyme recognition sequence (CATATG) and 3'-primer that includes the stop codon (TGA). The PCR product that was cloned into the pCR®2.1-TOPO® vector was digested with the restriction enzymes *Nde*I and *Xho*I. The expression vector pET14b was also digested using the same enzymes. The PCR product was inserted into pET14b and the *npt*II expression plasmid (*npt*II-pET14b) was constructed. The sequence of the cloning site in the *npt*II-pET14b was completely correct (data not shown). *E. coli* BL21(DE3) cells with the *npt*II-pET14b plasmid were precultured for 14 h, and these precultured cells were then inoculated in a fresh medium. After 2.5 h, IPTG was added and the expression of NPTII protein was induced. Soluble protein was extracted and analyzed using electrophoresis, and the 31 kDa expressed NPTII protein was confirmed (Fig. 2). The soluble protein fraction was loaded onto the His-Bind column, and NPTII protein was eluted with 60 mM imidazole (Fig. 3, lane 1). The eluate containing fusion protein was treated with biotinylated thrombin in order to eliminate His tag. After the enzymatic treatment, the used biotinylated thrombin was removed using streptavidin agarose. The thrombin free eluate was reloaded on the His-Bind column to remove His tag. The purified NPTII protein was eluted in the void fraction (Fig. 3, lane 2).

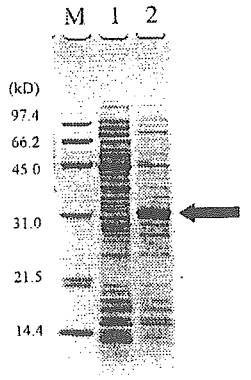

Fig. 2 Electrophoresis of total protein in *E. coli* with the *npt*II-pET14b cultured with (lane 2) or without (lane 1) IPTG. Arrow indicates the induced NPTII protein.
Lane 1 ; *E. coli* containing *npt*II-pET14b plasmid was incubated in LB liquid medium at 37°C for 4.5 h. Lane 2 ; *E. coli* containing *npt*II-pET14b plasmid was incubated in LB liquid medium at 37°C for 2.5 h. After addition of 0.4 mM IPTG, the incubation was continued at 37°C for 2 h.

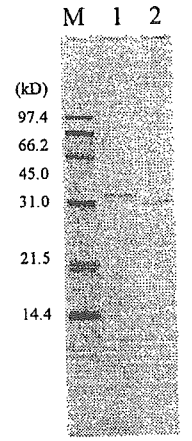

Fig. 3 Electrophoresis of NPTII protein before (lane 1) and after (lane 2) treatment with thrombin.
The fusion protein (His tag and NPTII) was treated with biotinylated thrombin and the His tag was removed from the fusion protein. NPTII protein was purified by using streptavidin agarose beads and the His-Bind column.

2. Confirmation of transformation

Transformation of tobacco with pBI121 was confirmed using PCR, and a 246 base pairs fragment was detected on the agarose gel (Fig. 4, lane 1-3). Since 5'-primer contained the NOS promoter region and the 3'-primer contained *npt*II coding region, the amplified fragment definitely reflects the integrated gene.

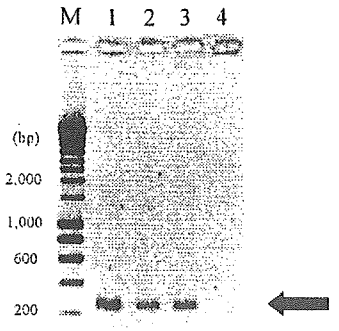

Fig. 4 Electrophoresis of PCR products for confirmation of *npt*II in 3 transformant clones (lane 1-3) and a normal tobacco plant (lane 4). Arrow indicates the amplified fragment. Genomic DNA was extracted using the extraction solution contained in the kit, and the extracts were diluted with the dilution solution in the kit. Diluted DNA solution was mixed with a PCR ready mix from the kit and primer solutions. PCR proceeded for 30 cycles of denaturing at 94°C for 3 min, annealing at 55°C for 1 min, and extension at 74°C for 2 min. Following this, the mixture was incubated at 74°C for 10 min, and then cooled at 4°C. The PCR product was separated by 1.5% agarose gel electrophoresis.

3. NPTII detection by ELISA

The ELISA detection system was constructed with anti-NPTII antibody, which was prepared by TaKaRa Bio Inc. Purified NPTII protein was detected in the range of 5–70 ng/ml (Fig. 5).

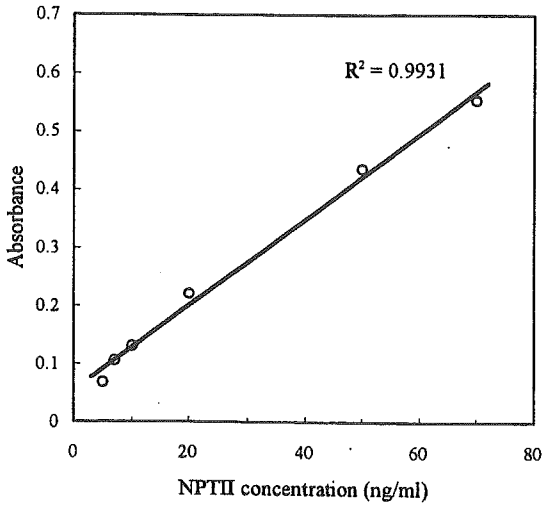

Fig. 5 Correlation between NPTII concentration and absorbance. Absorbance was corrected with blank.

The NPTII protein, which was expressed in tobacco plants transformed with pBI121 plasmid, was also detected with the ELISA system, and non-specific detection was not observed in normal tobacco (data not shown). The content of expressed NPTII protein was estimated to be ca. 0.001% of the fresh weight. The result corresponded to the content of the NPTII protein that was generally expressed in the transgenic plant, from 0.00005 to 0.001% of the fresh weight (Miki and McHugh, 2004). This data suggests that the ELISA system constructed in this study can be used to qualitatively detect the NPTII protein present in GM foods. Furthermore, non-specific detection in some non-GM plants, which the genetically modification has been applied, was examined. Soybean, corn, and potato showed low levels of background as in the case of non-transgenic tobacco. Hence, it is expected that the NPTII protein in GM soybean, corn, and potato, in which the *npt*II has been introduced, could be qualitatively detected.

IV. Acknowledgement

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V. References

1) Miki, B. and McHugh, S.: Selectable marker genes in transgenic plants: applications, alternatives and biosafety. Journal of Biotechnology, 107, 193–232 (2004).
2) Standards for the safety assessment of genetically modified foods (seed plants). Food Safety Commission (2004).
3) Murashige T. and Skoog, F.: A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiologia Plantarum, 15, 473–497 (1962).