papaya, DNA fragments were amplified by PCR using the following primer set. Forward primer: 5′-GACATCTCCA-CTGACGTAAGGG-3′ (p324). Reverse primer: 5′-CTATCR-CTCTCTCCAGTTTTTG-3′ (p323).

DNA Sequencing The PCR-amplified DNA fragments were extracted from the agarose-gel and purified using a QIAquick PCR purification kit (QIAGEN). The DNA fragments were directly sequenced from both strands using forward and reverse primers with an ABI PRISM 3700 DNA analyzer and Terminator v3.1 Cycle Sequencing Kit (Life Technologies), according to the manufacturer's instructions. Nucleotide sequences were analyzed using Lasergene version 7.2 software (DNASTAR Inc., Madison, WI, U.S.A.).

Real-Time PCR Real-time PCR assays were performed using the ABI PRISM™ 7900 Sequence Detection System (Life Technologies). The 25 μl reaction mixture consisted of 2.5 μl sample DNA solution (25 ng), 12.5 μl Universal Master Mix^{R} (Life Technologies), 0.8 μM forward and reverse primers, and 0.1 μM probe. The PCR conditions were as follows: 2 min at 50 °C, 95 °C for 10 min followed by 50 cycles of 15 s at 95 °C and 1 min at 60 °C. GM papaya was detected using the following primers and probe. Forward primer: 5′-GATCCCCGGGTGGTCAGT-3′ (YK-1F). Reverse primer: 5′-CCGGTATCCACAGCTTCATTTT-3′ (YK-1R). Probe: 5′-FAM-AGACGCCATGGAAGG-MGB-3′ (YK-P).

For detecting the papaya endogenous internal control gene, *chymopapain* (*Chy*; GenBank accession No.: AY803756), we designed the following primers and probe referring to published report.[15] Forward primer: 5′-CCATGCGATCCTC-CCA-3′ (Q-Chy-1F2). Reverse primer: 5′-CATCGTAGCCA-TTGTAACACTAGCTAA-3′ (Q-Chy-2R). Probe: 5′-FAM-TTCCCTTCAT(BHQ1)CCATTCCCACTCTTGAGA-3′ (Q-Chy-P). Black-hole quencher 1 (BHQ1) was labeled for Q-Chy-P at the underlined thymidine in the nucleotide sequence.

All primers and probes were diluted with an appropriate volume of distilled water, and stored at −20 °C until use. Results were analyzed using SDS 2.1 sequence detection software (Life Technologies) for ABI PRISM™ 7900 Sequence Detection System.

Real-Time PCR Data Analysis Typically, the baseline was set to cycles 3 through 15. The ΔRn threshold for plotting the cycle threshold (Ct) values was set to 0.2 during exponential amplification. Reactions with Ct values of less than 48 and exponential amplification plots were scored as positive. If the Ct value could not be obtained, the reaction was

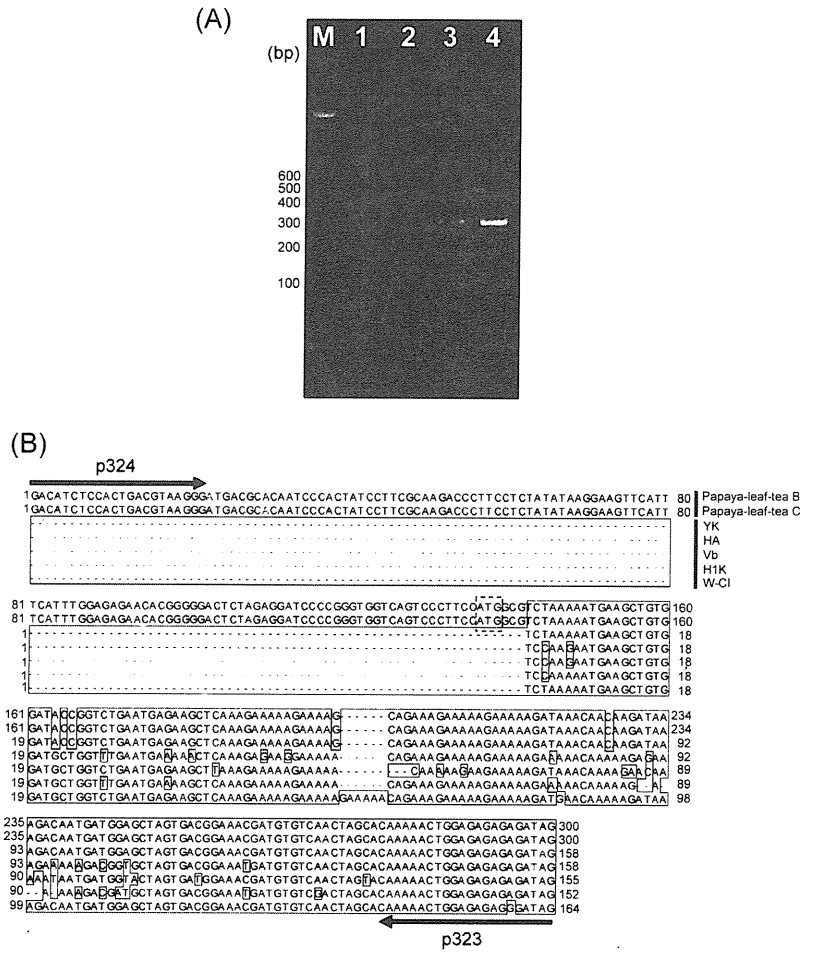

Fig. 1. PCR Targeting Construct Specific Sequence of PRSV-Resistant GM Papaya

(A) DNA templates (lane 1, non-GM papaya (Sunset); lane 2, papaya-leaf-tea A; lane 3, papaya-leaf-tea B; lane 4, papaya-leaf-tea C) were used for the PCR test using the p324 and p323 primer set. The PCR-amplified products were run on a 3% (w/v) agarose-gel. Lane M, 100-bp DNA ladder marker (B) Nucleotide sequence alignment of PRSV CP cloned from YK, HA, Vb, H1K, W-CI strains and the PCR products obtained using papaya-leaf-tea B and C. Homologous sequences were boxed. The initiation codon for the GM papaya was boxed with a dashed line. Numerals beside the sequence indicate the numbers of nucleotides from the 5′ terminus.

scored as negative. Reactions with Ct values of less than 48, but without exponential amplification as judged by visual inspection of the respective ΔRn plots and multi-component plots were scored as negative.

RESULTS AND DISCUSSION

Detection of Unauthorized GM Papaya To investigate the contamination with unauthorized GM papaya in commercially processed products, containing papaya as a major ingredient, in Japan, we used genomic DNA purified from the papaya-leaf-tea products as a template for the PCR test. The forward primer (p324) was designed to hybridize in the cauliflower mosaic virus (CaMV) 35S promoter sequence, which is the most common promoter used in the transformation of papaya for various GM papaya traits,[2] and the reverse primer (p323) was designed in the highly conserved sequence of the CP gene, which is cloned from various strains of PRSV (GenBank accession no.: YK, X97251; HA, S46722; Vb, AF243496.1; H1K, AF196839.1; W-CI, AY027810.2). Electrophoresis of the PCR products using p324 and p323 primers showed a single band of about 300 bp in length using DNA purified from two of the three papaya-leaf-tea products (papaya-leaf-tea B and C) (Fig. 1A). The DNA purified from non-GM papaya (Sunset) as a control and papaya-leaf-tea A generated no PCR products with the identical length. Direct sequence analysis of the PCR product and BLASTn analysis indicated that the 3′ end sequence was identical to the CP gene in a Taiwan isolate of PRSV (PRSV YK strain)[16] (Fig. 1B). Furthermore, the multiple cloning site (containing restriction sites for *Bam*HI and *Nco*I) and two amino acid mutations (methionine and alanine) between the CaMV 35S promoter and the N-terminus of CP gene were detected (Fig. 2A). According to the literature,[17] the design of this transgenic vector construct was identical to that of the GM papaya, which was generated to resist infection of the PRSV YK strain. These results suggest that the papaya-leaf-tea products were contaminated with the unauthorized GM papaya (PRSV-YK).

Development of a Construct-Specific Detection Method for PRSV-YK In order to qualitatively detect PRSV-YK in processed products, containing papaya as a major ingredient, with high specificity and sensitivity, we designed specific primers and a probe for a real-time PCR assay producing a short amplicon (57 bp), based on the detected transgenic construct sequence. The forward (YK-1F) and the reverse (YK-1R) primers were designed in the region between the transgenic vector backbone and the CP gene sequence. The probe

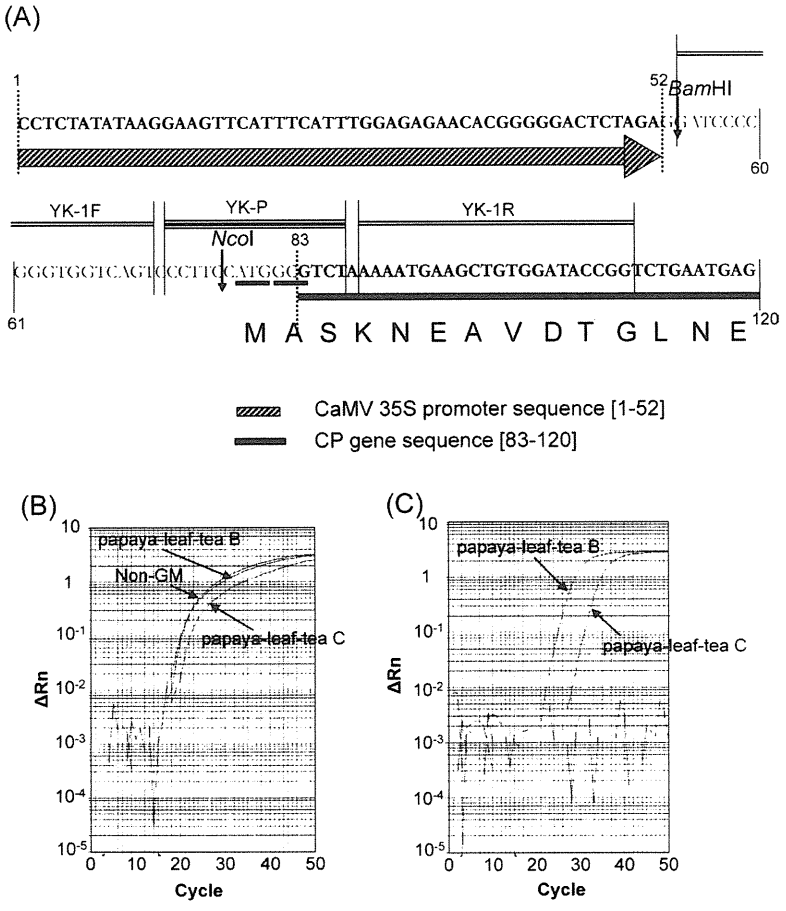

Fig. 2. Detection of PRSV-YK Using Real-Time PCR

(A) A fragment of the transgenic vector construct sequence was obtained and restriction sites were marked by vertical arrows. Design of the primers (YK-1F and YK-1R) and the probe (YK-P) for detecting construct-specific sequence of PRSV-YK is indicated by lines above the sequence. Numerals indicate the numbers of nucleotides from the 5′ terminus. (B) Endogenous *Chy* detection using a primer set (Q-Chy-1F2 and Q-Chy-2R) and probe (Q-Chy-P) (C) PRSV-YK detection using a primer set (YK-1F and YK-1R) and probe (YK-P). The threshold value was set at 0.2. Positive amplification curves are designated by arrows.

(YK-P) was designed on the site of the initiation codon of the CP gene (Fig. 2A).

Since the forward primer sequence for detecting the papaya endogenous internal control gene, *Chy*, had an unintentional error of a single nucleotide sequence in the previous report[15] (according to personal communication), we used the right sequence for the forward primer (Q-Chy-1F2), the reverse primer (Q-Chy-2R) and the probe (Q-Chy-P). The real-time PCR assay for PRSV-YK detection confirmed that the papaya-leaf-tea products B and C were positive for PRSV-YK, producing Ct values of 25.93 and 31.88 with a threshold value of 0.2, respectively. Endogenous *Chy* detection was positive for all samples, with the papaya leaf-tea product B, C and the non-GM papaya (Sunset) producing Ct values of 21.55, 23.82 and 21.45, respectively, with a threshold value of 0.2 (Figs. 2B, C). The copy numbers of PRSV-YK construct and *Chy* sequence were calculated from Ct values using standard curves which were generated using the positive control plasmid. Papaya-leaf-tea products B and C contained 1 copy of PRSV-YK construct sequence in 27 copies and 167 copies of *Chy* sequence, respectively (data not shown). Because the genetic background of PRSV-YK used in the processed papaya products was unknown, estimation of the content of PRSV-YK in a papaya product was not possible. The non-template control and the genomic DNA derived from other crops, such as maize, rice, soybean, flax and canola, gave no amplification signals in the PRSV-YK and the endogenous *Chy* detection systems (data not shown). These results indicated that the developed method is specific for detecting PRSV-YK.

In the present study, as a result of monitoring processed products, which included papaya as a major ingredient, for contamination with unauthorized GM papaya, we found a transgenic vector construct for expression of the CP gene, which was cloned from the YK strain, in papaya-leaf-tea products. The design of a part of the transgenic vector construct was identical to the one reported in 1996.[17] We also detected PRSV-YK contamination in 1 out of 7 products of papaya jam and 2 out of 3 products of papaya pickles in real-time PCR test for PRSV-YK detection (data not shown). The origin of the GM papaya contamination in the papaya products in Japan remains to be clarified. Furthermore, we successfully developed a construct-specific real-time PCR detection method for PRSV-YK. Further studies are required to determine the detection limits, and whether the method can be used for detection in other commercially processed products containing papaya as a major ingredient.

Acknowledgments We are grateful to the Hawaii Papaya Industry Association for providing Sunset as a reference material. We thank Prof. Yoshihiro Ozeki, Dr. Satoshi Futo, and Dr. Hiroyuki Haraguchi for their useful suggestions. This study was partly supported by Grants from the Ministry of Health, Labour, and Welfare of Japan and from the Japanese Health Sciences Foundation.

REFERENCES

1) Yeh S., Bau H., Cheng Y., Yu T., Yang J., *Proc. Int. Symp. Biotech.*, **461**, 321—328 (1998).
2) Tecson Mendoza E. M., C Laurena A., Botella J. R., *Biotechnol. Annu. Rev.*, **14**, 423—462 (2008).
3) Akiyama H., Watanabe T., Wakui C., Chiba Y., Shibuya M., Goda Y., Toyoda M., *Shokuhin Eiseigaku Zasshi*, **43**, 301—305 (2002).
4) Akiyama H., Sugimoto K., Matsumoto M., Isuzugawa K., Shibuya M., Goda Y., Toyoda M., *Shokuhin Eiseigaku Zasshi*, **43**, 24—29 (2002).
5) Akiyama H., Sakata K., Kondo K., Tanaka A., Liu M. S., Oguchi T., Furui S., Kitta K., Hino A., Teshima R., *J. Agric. Food Chem.*, **56**, 1977—1983 (2008).
6) Matsuoka T., Kawashima Y., Akiyama H., Miura H., Goda Y., Kusakabe Y., Sebata T., Isshiki K., Toyoda M., Hino A., *Shokuhin Eiseigaku Zasshi*, **41**, 137—143 (2000).
7) Matsuoka T., Kuribara H., Akiyama H., Miura H., Goda Y., Kusakabe Y., Isshiki K., Toyoda M., Hino A., *Shokuhin Eiseigaku Zasshi*, **42**, 24—32 (2001).
8) Matsuoka T., Kuribara H., Sucfuji S., Miura H., Kusakabe Y., Akiyama H., Goda Y., Isshiki K., Toyoda M., Hino A., *Shokuhin Eiseigaku Zasshi*, **42**, 197—201 (2001).
9) Akiyama H., Sasaki N., Sakata K., Ohmori K., Toyota A., Kikuchi Y., Watanabe T., Furui S., Kitta K., Maitani T., *J. Agric. Food Chem.*, **55**, 5942—5947 (2007).
10) Nakamura K., Akiyama H., Yamada C., Satoh R., Makiyama D., Sakata K., Kawakami H., Mano J., Kitta K., Teshima R., *Biol. Pharm. Bull.*, **33**, 532—534 (2010).
11) Goda Y., Asano T., Shibuya M., Hino A., Toyoda M., *Shokuhin Eiseigaku Zasshi*, **42**, 231—236 (2001).
12) Yamaguchi A., Shimizu K., Mishima T., Aoki N., Hattori H., Sato H., Ueda N., Watanabe T., Hino A., Akiyama H., Maitani T., *Shokuhin Eiseigaku Zasshi*, **47**, 146—150 (2006).
13) Wakui C., Akiyama H., Watanabe T., Fitch M. M., Uchikawa S., Ki M., Takahashi K., Chiba R., Fujii A., Hino A., Maitani T., *Shokuhin Eiseigaku Zasshi*, **45**, 19—24 (2004).
14) "Notification 658," Food Safety Commission of Japan, 9 July, 2009.
15) Guo J., Yang L., Liu X., Zhang H., Qian B., Zhang D., *J. Agric. Food Chem.*, **57**, 6502—6509 (2009).
16) Wang C. H., Yeh S. D., *Arch. Virol.*, **142**, 271—285 (1997).
17) Cheng Y., Yang J., Yeh S., *Plant Cell Rep.*, **16**, 127—132 (1996).

Proteomic and Allergenomic Analyses of Rice and Potato Proteins

Reiko TESHIMA[1]*, Rika NAKAMURA[1], Rie SATOH[1,2]
[1]*Natl.Inst. Health Sci.,Tokyo158-8501, JAPAN*
[2]*NARO Food Res. Inst., Tsukuba 305-8642, JAPAN*
**Corresponding author: rteshima@nihs.go.jp*

Abstract

Proteomic technology has great potential to identify relevant proteins in foods derived from plant. Recently, new strategies, referred to as "allergenomics", have allowed the characterization of allergenic proteins. In this presentation, we used two-dimensional diference gel electrophoresis (2D-DIGE) and immunoproteomic method for proteomic and allergenomics analyses, and compared for differences of protein expression between transgenic and non-transgenic rice and potato.

Introduction

As a result of advances in molecular biology, crops that are adapted to poor conditions (such as heat, cold and salt) and that are abundant for nutrients are being developed by using transgenic techniques. The Codex Alimentarius Commission states that safety assessments of genetically modified (GM) foods need to include an investigation of tendencies to provoke allergy that might result from the gene insertion (ftp://ftp.fao.org/es/esn/food/guide_plants_en.pdf). Comprehensive analyses, such as by metabolomics and proteomics, may be useful tools for detecting unexpected effects in GM foods. Actually, the development of a comprehensive analysis procedure in recent years has made it possible to detect the allergenic components that had never been detected before (Batista et al., 2007; Nakamura et al., 2009, 2010a).

In this presentation, we introduced two-dimensional difference gel electrophoresis (2D-DIGE) and immunoproteomic method for proteomic and allergenomic analyses, and compared the diffrences of protein expression between transgenic and non-transgenic plants by using a model transgenic rice with high-level tryptophan accumulation (Tozawa et al., 2001; Wakasa et al., 2006), and transgenic potatoes expressing Arabidopsis DREB1A (dehydration responsive element-binding protein 1A), driven by the rd29A promoter or the 35S promoter(Behnam et al., 2006, 2007). In allergenomic analyses, we used sera from rice- or potato-allergic patients and performed comprehensive probing of IgE binding proteins, which are candidates for allergens in rice and potato.

Materials and Methods

Potatoes and Potato Extracts

Three lines of transgenic potatoes expressing the AtDREB1A gene were generated as described previously (Behnam et al., 2006, 2007). In two of the lines the transgene was driven by the rd29A promoter (D163, D164), and in the other line the transgene was driven by the cauliflower mosaic virus 35S promoter (35S). To prepare the potato extract, 1 g potato tuber slices was ground in PBS or urea buffer (7 M urea, 2 M thiourea, 30 mM Tris, 4% CHAPS, pH 8.5) with a Polytron homogenizer (Kinematica, NY, USA). The mixture was centrifuged at 9,000 g for 10 min, and then the supernatant was filtered through a Dismic-45 filter (Advantec Toyo Kaisha Ltd., Tokyo, Japan) and used as the potato extract. The protein concentration was measured with a BCA assay Kit (Pierce, IL, USA), and the extract was stored at –80 °C until use.

Rice and Rice extracts

Mature brown seeds of non-transgenic rice (Oryza sativa cv. Nipponbare) (WT) and OASA1D transgenic rice line HW5 (HW5) harvested from an isolated field were used in the experiments. Ten grains of brown rice were pulverized with a Multi Beads Shocker (Yasui Kikai, Osaka, Japan) at 2,500 rpm for 1 min. Salt-soluble proteins were extracted with 3 mL of 1M NaCl with a Protease Inhibitor Cocktail (Sigma-Aldrich Corp., St. Louis, MO, USA) by shaking overnight at 4°C with a Micro Tube Mixer (Taitec, Saitama, Japan). The solutions obtained from the seeds containing salt-soluble proteins of each variety were then centrifuged twice at 9,300 g for 10 min at 4°C. The protein concentration of the supernatant was determined with a BCA Protein Assay Reagent Kit (Thermo Fisher Scientific, MA, USA). The protein solutions were stored in aliquots at -80°C until used.

Patients' sera

We used sera from 27 rice-allergic and 14 potato-allergic patients; 20 from Japan, and 21 from the United States. All patients were positive for rice-specific IgE (Immuno-CAP scores 2-5) or potato-specific IgE (Immuno-CAP scores 3-5). Sera from patients without allergies were used as negative controls. Informed consent was obtained from all patients and volunteers. Our study was approved by the Ethics Review Committee of the National Institute of Health Sciences.

2D-DIGE

Urea-extracted proteins from five tubers of non-transgenic (NT) and transgenic (rd29A, 35S) potatoes were used for 2D-DIGE. Protein concentrations were measured with a 2D-Quant Kit (GE Healthcare), and equal quantities of proteins from transgenic and non-transgenic potatoes were mixed and used as an internal standard. The internal standard was labeled with Cy2, and potato samples were labeled with Cy3 or Cy5, according to manufacturer's protocol. Cy-labeled proteins (25 μg proteins from each sample) were mixed together and applied to an Immobiline Drystrip (pH 3–10 NL, 13 cm long, GE Healthcare), and the drystrip was rehydrated overnight at 20 °C. First-dimensional IEF was performed at 20 °C under the following conditions: 300 V for 4 h; 1000 V for 1 h; 8000 V for 4 h. The strips were equilibrated for 20 min in equilibration solution (100 mM Tris-HCl, pH 8.0, 6 M urea, 30% glycerol, 2%SDS) containing 0.5%DTT, and then in equilibration solution containing 4.5% (w/v) iodoacetamide. Second-dimensional SDS-PAGE was performed at 220 V for 3 h on acrylamide gels (DRC, Tokyo, Japan). Fluorescence images were acquired with a Typhoon9400 variable image with a Typhoon9400 variable image analyzer (GE Healthcare). Fluorochromes were detected as follows; Cy2 with a 488 nm bandpass (520BP40) filter, Cy3 with a 532 nm bandpass (580BP30) filter, and Cy5 with a 630 nm bandpass (670BP30) filter. The spots were identified and matched using the Decyder software version 6 (GE Healthcare).

In-Gel Digestion and Protein Identification by MALDI-TOF-MS/MS

The spots of interest were excised from 2D-gels, destained, dried, and in-gel digested for 2 h at 37°C in trypsin solution (30 μg/mL Trypsin Gold-Mass Spec Grade [Promega, Madison, WI, USA] and protease max [Promega, Madison, WI, USA]). The digested peptides were mixed with α-cyano-4-hydroxycinnamic acid (α-CHCA, Sigma Aldrich), and their MS spectra and MS/MS fragment ion mass were determined with a 4800 MALDI TOF/TOF Analyzer (Applied Biosystems, CA, USA). All product ions were submitted to a computer database search analysis with the Mascot MS/MS ion search (Matrix Science, MA, USA) by using the NCBInr database (all entries) or the potato expressed sequence tag (EST) database as described in previous paper (Teshima et al., 2010).

Results

Allergenomics of rice

It is known that allergenicity of rice is partly dependent on globulin and albumin fraction proteins (Shibasaki et al, 1979). Urisu et al. found that 22 proteins extracted from rice with 1M NaCl exhibited binding activity with immunoglobulin E (IgE) in rice allergic patients' sera, and that a16kDa protein and a 33kDa protein were the major allergens (Urisu et al., 1991). The 16kDa allergen is a spheroidal protein that is homologous to α-amylase / trypsin-inhibitor family proteins and contains 10 cysteine residues (Matsuda et al., 1988; Izumi et al., 1992, 1999; Adachi et al., 1993). The 33kDa allergen has been identified as a novel type of plant glyoxalase I (Usui et al., 2001). A 26kDa protein, an α-globulin purified from salt-soluble proteins, has also been shown to be a rice allergen (Limas et al., 1990).

In our experiments, multiple spots containing known and novel IgE-binding proteins were detected among the salt-soluble proteins of non-transgenic rice by 2D immunoblotting . Five kind of IgE-binding proteins were detected, two known allergen family proteins (RAG 2 and glyoxalase 1) and three other IgE-binding proteins, a 19kDa, 52kDa, and 63kDa globulin-like protein, and revealed no significant differences in expression of these proteins between the transgenic (HW5) and non-transgenic (WT, Nipponbare) rice (Satoh et al, 2011). In Fig 1 of this presentation, we showed a typical Cy-dye image of IgE-binding proteins of 2D-PAGE using 14cm x 14cm SDS--PAGE gel and summarized the IgE-binding proteins.

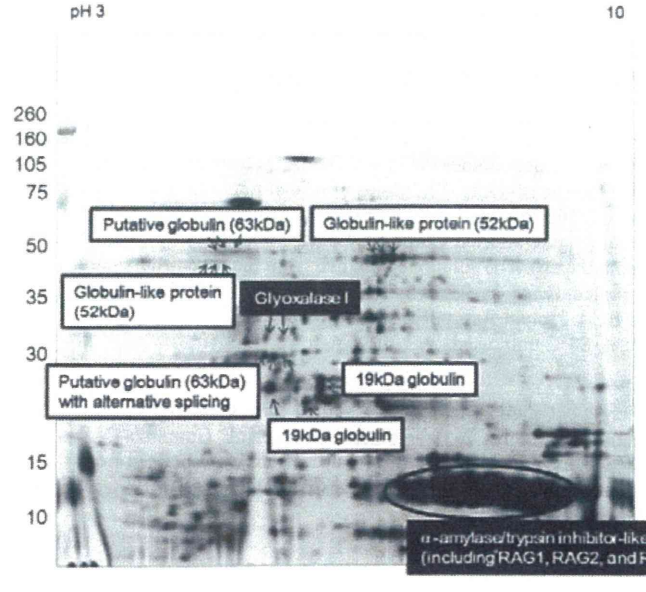

Fig.1. Comprehensive identification of allergens (Cy-dye image of IgE-binding proteins of 2D-PAGE) Drystrip(pH3-10NL, 13cm) was used for 1st dimention IEF and 10-20% gradient gel (14 x14cm) was used for 2nd dimention SDS-PAGE.

Gi number of putative globulin (63kDa), globulin-like proteins (52kDa), 19kDa globulin, glyoxalase I are as follows: gi41469581, gi115455865, gi20159 and gi16580747. As for α-amylase/trypsin inhibitor-like protein family, RAG2(encoded by the gene locus Os07g0214300 derived from RAP-DB, http://rapdb.dna.affrc.go.jp/), and other RAs(rice seed allergenic proteins) which are encoded by a multigene family that consists of at least 4 genes (Adachi et al, 1993) seem to be included. Because RAs are highly homologous with each other (Adachi et al., 1993), the rabbit anti-RAG2 antibody seems to be detect RA family proteins widely.

Proteomics and allergenomics of potato

Potato (Solanum tuberosum) is a staple food that is consumed worldwide. Various transgenic potatoes have been developed and their food safety has been assessed using serological and proteomic techniques. Several cases of potato allergies have been reported (Castells et al, 1986, Jeannet-Peater et al., 1999, Beausoleil et al., 2001, De Swert et al, 2007, de Lagran et al, 2009), and a major potato allergen that causes hypersensitive reactions has been identified as patatin (Sola t 1) (Schmidt et al., 2002). Potato allergy is considered to be cross-reactive with latex or pollens, and their cross-reactive fruit and vegetables. Other potato allergens, cathepsin D-, cysteine-, and serine protease inhibitors all of which are members of the trypsin inhibitor family, have been designated as Sola t 2, Sola t 3, and Sola t 4, respectively (Seppala et al., 2001). These allergens make up approximately 70–80% of total soluble potato proteins.

From allergenomics analysis, proteins from 13 IgE-binding spots were excised from the gel, digested with trypsin, and analysed by MALDI-TOF-MS/MS. The IgE-binding proteins in potato were identified as patatin precursors, a segment of serine protease inhibitor 2, and proteinase inhibitor II (Nakamura et al., 2010b). As for patatin precursors, two proteins which have different gi number (gi 73426683 (MW 41.2kDa/pI 4.9) and gi 73426671 (MW42.6k/pI 5.49) were identified from different two spots. These proteins showed >80% homology to Sola t 1, but their MW and pI were slightly different, suggested that they were isotypes of Sola t 1.

As for 2D-DIGE analyses, five independent non-transgenic and transgenic potato tuber samples were used. The fluorescence intensity (F.I.) of each spot was normalized to that of the internal standard, and the differences in protein expression levels are expressed as the ratio of the F.I. of the transgenic spot to the F.I. of non-transgenic spot (transgenic /non-transgenic). Fig. 2 shows representative Cy images of NT, rd29A and 35S potato tuber samples.

From a total of 700 potato protein spots, 46 spots (rd29A) and 16 spots (35S) showed more than 2-fold differences in protein expression compared with that in non-transgenic potatoes (Dunnett test, $p<0.05$). Proteins expressed differently in rd29A potato and 35S potato are shown in Fig. 2, and the numbers beside the circles correspond to spot numbers in Tables 3 and 4 of previous paper (Nakamura et al., 2010b).

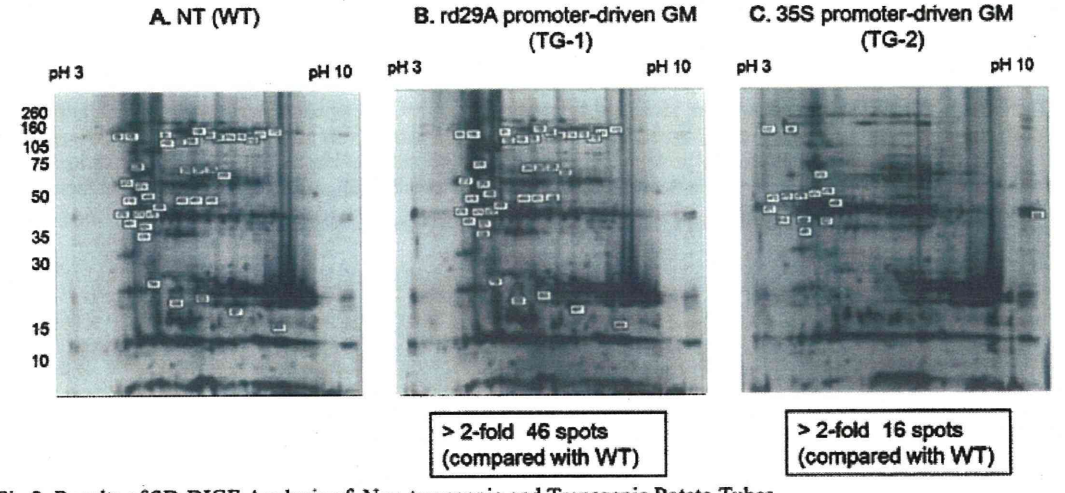

Fig.2. Results of 2D-DIGE Analysis of Non-transgenic and Transgenic Potato Tubes

Each extract from non-transgenic (NT) and transgenic (rd29A, 35S)potato tubes was pre-labeled with Cy dyes and separated by 2D-PAGE. Representative fluorescence images of (A) NT , (B)rd29A, and (C) 35S potato extracts are shown. Normalized fluorescence value of circled spots showed significant difference (ratio>2 or <-2) between non-transgenic and transgenic potatoes by Dunnett test ($p<0.05$). Number beside the circles correspond to spot numbers shown in Table 3 and 4 of our previous paper (Nakamura et al., 2010b), and 46 spots (rd29A) and 16 spots (35S) showed more than 2-fold differences in protein expression compared with that in non-transgenic potatoes.

The proteins in spots of interest were identified by MALDI-TOF-MS/MS. The proteins showing increased expression in rd29A potatoes and 35S potatoes were identified as precursors of patatin (spot No.418, 465, 475, 476, 513,539), a major potato allergen. The expression of patatin with lower pI value (gi 73426683) seemed to be increased in transgenic potato. The expression levels of other IgE-binding spots in transgenic potato did not show significant differences ($p>0.05$) compared with their expression in non-transgenic potatoes. Proteins showing

decreased expression in transgenic potatoes were identified as lipoxygenase and glycogen (starch) synthase (Nakamua et al., 2010b). Other protein spots were unable to be identified because of insufficient peptide.

Most of the spots showing increases were identified as patatin precursors. Patatin is a storage protein with lipid acyl hydrolase activity and a common protein family in potato varieties (Stupar et al., 2006, Ali et al., 2007). It has been reported that expression of patatins is affected by various factors, including tuber size and storage conditions (Stupar et al., 2006; Lehesranta et al., 2006). The transgenic potato tubers used in this study tended to be smaller than non-transgenic potato tubers, because introduction of AtDREB1A suppresses tuber growth. In addition, different potato genotypes, even among common cultivars, have significant variation in quantity and isoforms under altered developmental stages and organs, and also under different physiological and environmental conditions (Blundy et al., 1991, Liu et al., 1991).

While substantial equivalence concept is the primary aspect to follow the comparison of transgenic crops with their non-transgenic counterparts in food safety, consideration should be also made whether the variation is within a common variety in the same way as the consequence of breeding practice at a decision-making process. Further elucidation shall be made on genotype by environment interactions which commonly occur in any plant species. The environmental variation on transgenic potatoes, should be examined more in food safety aspects as crops could be grown under different conditions, with respect to variation of protein expressions in potato tubers whether transgenic individuals could have significant deviation on their expression beyond the variation of their non-transgenic comparators (Arvanitoyannis et al., 2008).

In vitro mast cell activation studies are useful to understand the biological significance of differences in expression of allergenic proteins in plants. Recently, we developed an in vitro cell activation system using a humanized FcεRI-expressing rat mast cell line (RS-ATL8) (Nakamura et al., 2010). As a preliminary study, we determined the challenge-required (threshold) amounts of potato protein from extract of a non-transgenic and two transgenic tubers. The amounts of protein required to activate RS-ATL8 cells sensitized with a potato-allergic patient's serum were the same for the three tubers (unpublished data). Further analyses using different potato tubers and different patients' sera should be conducted to confirm the biological significance of differences in protein expression with respect to allergenicity. And further research is required to confirm that the differences observed in this study are due to gene transfection, rather than environmental factors.

Conclusion

Multiple spots containing known and novel IgE-binding proteins were detected among the salt-soluble proteins of non-transgenic rice by 2D immunoblotting. Five kind of IgE-binding proteins were detected, two known allergen family proteins (RAG 2 and glyoxalase 1) and three other IgE-binding proteins, a 19kDa, 52kDa, and 63kDa globulin-like protein, and revealed no significant differences in expression of these proteins between the transgenic and non-transgenic rice.

The IgE-binding proteins in potato were identified as patatin precursors, a segment of serine protease inhibitor 2, and proteinase inhibitor II. Among them, expression of precursors of patatin was slightly increased in transgenic potatoes. Further research on changes in protein expressions in response to environmental factors is required to confirm whether the differences observed in this study are due to gene transfection, rather than environmental factors.

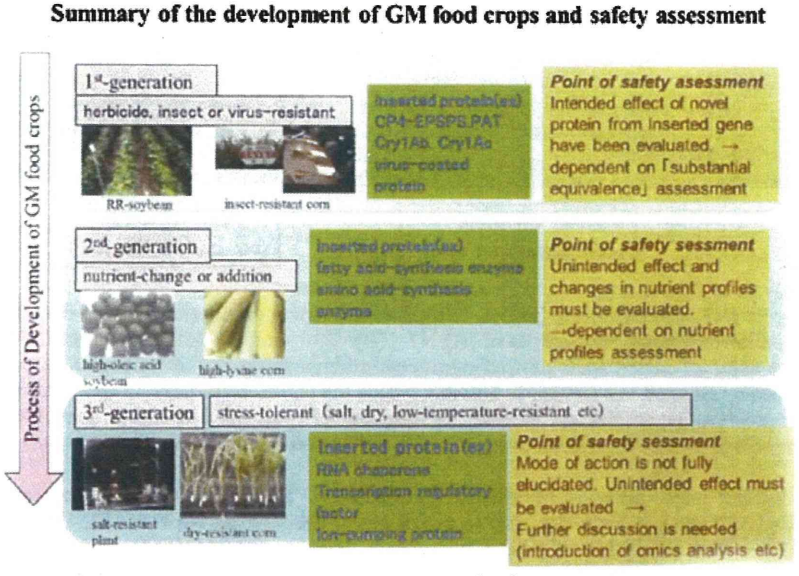

Fig. 3. Summary of the development of GM crops and safety assessment

We summarized the safety assessment of genetically modified (GM) food crops in Fig. 3. Until now, most commercial GM foods are categorized in 1st generation GM food, like herbicide, insect or virus resistant. In that cases, as intended effect of novel protein from inserted gene have been evaluated, safety assessment has been done according to substantial equivalence concept which follows the comparison of transgenic crops with their non-transgenic counterparts in food safety. Nowadays 2nd generation (nutrient-changed or added) and 3rd generation(stress (salt, dry, low temperature etc) tolerant) GM crops are developing in worldwide. In that cases, as mode of action is not fully elucidated, comprehensive analyses, such as by metabolomics and proteomics, may be useful tools for detecting unexpected effects in GM crops, and consideration should be also made whether the variation observed in GM crops is within a common variety in the same way as the consequence of breeding practice at a decision-making process.

Recently, proteomic and allergenomic technology have been greatly improved. Such comprehensive analyses seems to be useful tools for identifying relevant proteins in foods derived from plant, and also for safety assessment of newly-developed GM foods.

References

Adachi T, Izumi H, Yamada T, Tanaka K, Takeuchi S, Nakamura R, Matsuda T (1993) Gene structure and expression of rice seed allergenic proteins belonging to the a-amylase/trypsin inhibitor family. Plant Mol. Biol., 21, 239-248

Ali A., Javad L.,Pak. J. (2007) Study on variation of potato varieties using electrophoretic tuber storage proteins.Biol. Sci., 10, 3195-3199

Arvanitoyannis IS, Vaitsi O, Mavromatis A (2008) Potato: a comparative study of the effect of cultivars and cultivation conditions and genetic modification on the physico-chemical properties of potato tubers in conjunction with multivariate analysis towards authenticity.Crit. Rev. Food Sci. Nutr., 48, 799-823

Batista R, Martins I, Jeno P, Ricardo C P, Oliveira M M (2007) A proteomic study to identify soya allergens - the human response to transgenic versus non-transgenic soya samples. Int. Arch. Allergy Immunol. 144, 29-38.

Beausoleil JL, Spergel JM, Pawlowski NA (2001) Anaphylaxis to raw potato. Ann. Allergy Asthma Immunol., 86, 68-70

Behnam B, Kikuchi A, Celebi-Toprak F, Yamanaka S, Kasuga M, Yamaguchi-Shinozaki K, Watanabe KN (2006) The Arabidopsis DREB1A gene driven by the stress-inducible rd29A promoter increases salt-stress tolerance in proportion to its copy number in tetrasomic tetraploid potato (Solanum tuberosum). Plant Biotechnol., 23, 169-177

Behnam B, Kikuchi A, Celebi-Toprak F, Kasuga M, Yamaguchi-Shinozaki K, Watanabe KN (2007) Arabidopsis rd29A::DREB1A enhances freezing tolerance in transgenic potato. Plant Cell Rep., 26, 1275-1282

Blundy KS, Blundy MA, Carter D, Wilson F, Park WD, Burrell MM (1991) The expression of class I patatin gene fusions in transgenic potato varies with both gene and cultivar. Plant Mol. Biol., 16, 153-160

Castells MC, Pascual C, Esteban MM, Ojeda JA (1986) Allergy to white potato. J. Allergy Clin. Immunol., 78, 1100-1104

De Swert LF, Cadot P, Ceuppens JL (2007) Diagnosis and natural course of allergy to cooked potatoes in children. Allergy., 62, 750-757

de Lagrán ZM, de Frutos FJ, de Arribas MG, Vanaclocha-Sebastián F (2009) Contact urticaria to raw potato. Dermatol. Online J., 15, 14

Izumi H, Adachi T, Fujii N, Matsuda T, Nakamura,R, Tanaka K, Urisu A, Kurosawa Y (1992) Nucleotide sequence of a cDNA clone encoding a major allergenic protein in rice seeds. Homology of the deduced amino acid sequence with members of alpha-amylase/trypsin inhibitor family. FEBS Lett., 302, 213-216

Izumi, H., Sugiyama, M., Matsuda, T., Nakamura, R.(1999) Structural characterization of the 16-kDa allergen, RA17, in rice seeds. Prediction of the secondary structure and identification of intramolecular disulfide bridges. Biosci Biotechnol. Biochem. 63, 2059-2063

Jeannet-Peater N, Piletta-Zanin PA, Hauser C (1999) Facial dermatitis, contact urticaria, rhinoconjunctivitis, and asthma induced by potato. Am. J. Contact Dermat., 10, 40-42

Lin S-K, Chang M-C, Tsai Y-G, Lur H-S (2005) Proteomic analysis of the expression of proteins related to rice quality during caryopsis development and the effect of high temperature on expression. Proteomics, 5, 2140-2156

Lehesranta SJ, Davies HV, Shepherd LV, Kostinen KM, Massat N, Nunan N, McNicol JW, Kärenlampi SO (2006) Proteomic analysis of the potato tuber life cycle. Proteomics, 6, 6042-6052.

Limas, G.G., Salinas, M., Moneo, I., Fischer, S., Wittmann-Liebold, B., Mendez, E.(1990) Purification and characterization of ten new rice NaCl-soluble proteins: identification of four protein-synthesis inhibitors and two immunoglobulin-binding proteins. Planta 181, 1-9

Liu X. Y., Rocha-Sosa M., Hummel S, Willmitzer L., Frommer W. B. (1991) A detailed study of the regulation and evolution of the two classes of patatin genes in Solanum tuberosum L. Plant Mol. Biol., 17, 1139-1154

Matsuda, T., Sugiyama, M., Nakamura, R., Torii, S. (1988) Purification and properties of an allergenic protein in rice grain. Agric. Biol. Chem. 52, 1465-1470

Nakamura, R., Satoh, R., Nakajima, Y., Kawasaki, N., Yamaguchi, T., Sawada, J., Nagoya, H., Teshima, R.(2009) Comparative study of GH-transgenic and non-transgenic amago salmon (Oncorhynchus masou ishikawae) allergenicity and proteomic analysis of amago salmon allergens. Regul. Toxicol. Pharmacol., 55, 300-308.

Nakamura R, Nakamura R, Nakano M, Arisawa K, Ezaki R, Horiuchi H, Teshima R (2010a) Allergenicity study of f-GFP-transgenic chicken meat by serological and 2D-DIGE analysis. Food Chem. Toxicol., 48, 1302-1310

Nakamura R, Satoh R, Nakamura R, Shimazaki T, Kasuga M, Yamaguchi-Shinozaki K, Kikuchi A, Watanabe KN, Teshima R (2010b) Immunoproteomic and two-dimensional difference gel electrophoresis analysis of Arabidopsis dehydration response element-binding protein 1A (DREB1A)-transgenic potato. Biol. Pharm. Bull., 33, 1418-1425

Nakamura R, Uchida Y, Higuchi M, Nakamura R, Tsuge I, Urisu A, Teshima R (2010) A convenient and sensitive allergy test: IgE crosslinking-induced luciferase expression in cultured mast cells. Allergy, 65, 1266-1273

Satoh R., Nakamura R., Komatsu A., Oshima M., Teshima R. (2011) Proteomic analysis of known and candidate rice allergens between non-transgenic and transgenic plants. Regul. Toxicol. Pharmacol., 59, 437-444

Shibasaki M, Suzuki S, Nemoto H, Kuroume T (1979) Allergenicity and lymphocyte-stimulating property of rice protein. J Allergy Clin. Immunol., 64, 259 265

Schmidt MH, Raulf-Heimsoth M, Posch A (2002) Evaluation of patatin as a major cross-reactive allergen in latex-induced potato allergy. Ann. Allergy Asthma Immunol., 89, 613–618

Seppälä U, Majamaa H, Turjanmaa K, Helin J, Reunala T, Kalkkinen N, Palosuo T (2001) Identification of four novel potato (Solanum tuberosum) allergens belonging to the family of soybean trypsin inhibitors. Allergy, 56, 619 626

Stupar RM, Beaubien KA, Jin W, Song J, Lee MK, Wu C, Zhang HB, Han B, Jiang J (2006) Structural diversity and differential transcription of the patatin multicopy gene family during potato tuber development. Genetics, 172, 1263 1275

Teshima R, Nakamura R, Satoh R, Nakamura R (2010)·2D-DIGE analysis of rice proteins from different cultivars. Regul. Toxicol. Pharmacol., 58 (Suppl), S30-S35:

Tozawa Y, Hasegawa H, Terakawa T, Wakasa K (2001) Characterization of rice anthranilate synthase a-subunit genes OASA1 and OASA2. Tryptophan accumulation in transgenic rice expressing a feedback-insensitive mutant of OASA1. Plant Physiol., 126, 1493-1506

Urisu A, Yamada K, Masuda S, Komada H, Wada E, Kondo Y, Horiba F, Tsuruta M, Yasaki T, Yamada M, Torii S, Nakamura R (1991) 16-kilodalton rice protein is one of the major allergens in rice grain extract and responsible for cross-allergenicity between cereal grains in the poaceae family. Int. Arch. Allergy Appl. Immunol., 96, 244–252

Usui Y, Nakase M, Hotta H, Urisu A, Aoki N, Kitajima K, Matsuda T (2001) A 33-kDa allergen from rice (*Oryza sativa* L. Japonica). J. Biol. Chem., 276, 11376-11381

Wakasa K, Hasegawa H, Nemoto H, Matsuda F, Miyazawa H, Tozawa Y, Morino K, Komatsu A, Yamada T, Terakawa T, Miyagawa H (2006) High-level tryptophan accumulation in seeds of transgenic rice and its limited effects on agronomic traits and seed metabolite profile. J. Exp. Bot., 57, 3069-3078