

Immunoproteomic and Two-Dimensional Difference Gel Electrophoresis Analysis of *Arabidopsis* Dehydration Response Element-Binding Protein 1A (*DREB1A*)-Transgenic Potato

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To produce crops that are more tolerant to stresses such as heat, cold, and salt, transgenic plants have been produced those express stress-associated proteins. In this study, we used immunoproteomic and two-dimensional difference gel electrophoresis (2D-DIGE) methods to investigate the allergenicity of transgenic potatoes expressing *Arabidopsis* *DREB1A* (dehydration responsive element-binding protein 1A), driven by the *rd29A* promoter or the 35S promoter. Immunoproteomic analysis using sera from potato-allergic patients revealed several immunoglobulin E (IgE)-binding protein spots. The patterns of protein binding were almost the same between transgenic and non-transgenic potatoes. The IgE-binding proteins in potato were identified as patatin precursors, a segment of serine protease inhibitor 2, and proteinase inhibitor II by matrix assisted laser desorption/ionization-time of flight (MALDI-TOF) MS/MS. 2D-DIGE analysis revealed several differences in protein expression between non-transgenic potato and transgenic potato; those showing increased expression in transgenic potatoes were identified as precursors of patatin, a major potato allergen, and those showing decreased expression in transgenic potatoes were identified as lipoxygenase and glycogen (starch) synthase. These results suggested that transgenic potatoes may express slightly higher levels of allergens, but their IgE-binding patterns were almost the same as those of control 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.

Key words transgenic crop; dehydration responsive element-binding protein 1A; potato; allergenicity; proteome; two-dimensional difference gel electrophoresis

To increase the quality and yield of crops, transgenic plants have been developed that show enhanced resistance to insects or herbicides, or improved growth and nutrient status. In addition, transgenic plants expressing proteins associated with stress responses are being developed in an effort to produce crops with greater resistance to stresses such as cold, heat, and salt.¹⁾ Genetic engineering of a transcriptional factor may affect expressions of multiple genes, therefore, there may be unintended effects on protein expression levels in transgenic plants. For appropriate safety assessment, therefore, it is important to evaluate the proteomics of transgenic plants, particularly with respect to allergenicity.

Even after the establishment of the 2003 Codex guidelines for methods to assess safety of foods derived from transgenic organisms, there is still controversy regarding methods used to test their allergenicity.²⁾ Allergenicity of foods derived from transgenic organisms has been evaluated using immunological methods such as immunoblots, histamine release assays, skin prick tests, and oral exposure.^{3,4)} Immunoproteomic analysis of allergens with sera from allergic patients is a useful method for comprehensively comparing unknown allergens as well as known allergens in extracts of foods derived from transgenic organisms. Previously, we reported application of the immunoproteome to assess allergenicities of *GH-1* (growth hormone 1)-transgenic amago salmon⁵⁾ and enhanced green fluorescent protein (EGFP)-transgenic chicken meat.⁶⁾ The two-dimensional difference

gel electrophoresis (2D-DIGE) method, a gel-based proteomic technology, is useful for evaluating relative differences in protein expression, if any, between non-transgenic and transgenic crops. The CyDye DIGE fluors used to label samples are spectrally resolvable, size- and charge-matched fluorescent dyes.^{7,8)} The method enables analysis of one or more samples on the same gel and is highly sensitive, and has a wide range of detection.

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.^{9–11)} Several cases of potato allergies have been reported,^{12–16)} and a major potato allergen that causes hypersensitive reactions has been identified as patatin (Sola t 1).¹⁷⁾ Potato allergy is considered to be cross-reactive with latex or pollens, and their cross-reactive fruit and vegetables.^{18–20)} 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.²¹⁾ These allergens make up approximately 70–80% of total soluble potato proteins.²²⁾

In this study, we investigated the allergenicity of stress-tolerant transgenic potatoes^{23,24)} expressing the *Arabidopsis* *DREB1A* (dehydration responsive element-binding protein 1A) gene, a transactivational factor against environmental stresses.^{25,26)} *DREB1A* is a transcriptional factor which ex-

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presses in response to cold and binds to drought responsive *cis*-acting elements termed the dehydration-responsive element (DRE) involved in the induction of *rd29A* expression.²⁷⁾ Several stress-related genes were identified as downstream genes of DREB1A.^{28,29)} Introduction of *AtDREB1A* gene may affect expressions of multiple genes including stress-related genes, therefore, proteomic analysis can be useful for safety assessment of *AtDREB1A*-transgenic potato evaluating comprehensive protein expressions in addition to endogenous allergen expressions.

We carried out 1D- and 2D-immunoblotting using sera from patients with potato allergies. Some potato proteins that bound to immunoglobulin E (IgE) were identified by mass spectrometry. Finally, we compared protein expression between transgenic and non-transgenic potatoes using 2D-DIGE methods, to detect unintended changes in protein expression in transgenic potatoes.

MATERIALS AND METHODS

Potatoes and Potato Extracts Three lines of transgenic potatoes expressing the *AtDREB1A* gene were generated as described previously.^{23,24)} 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 phosphate buffered saline (PBS) or urea buffer (7 M urea, 2 M thiourea, 30 mM Tris, 4% CHAPS, pH 8.5) with a Polytron homogenizer (Kinematica, Inc., NY, U.S.A.). The mixture was centrifuged at 9000g 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, U.S.A.), and the extract was stored at -80 °C until use.

Sera We used sera from 14 potato-allergic patients; 4 from Japan, and 10 from the United States. All patients were positive for potato-specific IgE (Immuno-CAP scores 3–5). Table 1 summarizes background information on these potato-allergic patients. 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.

1D-Immunoblot Analysis with Patients' Sera We conducted 1D-immunoblots as described previously.⁶⁾ Briefly, PBS-extracted proteins from non-transgenic (NT) and transgenic (rd29A, 35S) potatoes were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10–20% acrylamide gel. Separated proteins were then transferred onto 0.22- μ m nitrocellulose membranes (S&S, Dassel, Germany). The membranes were incubated with 0.5% casein-PBS for 2 h at room temperature (RT), then with sera from potato-allergic patients (diluted 1:10 with 0.1% casein-PBS) for 1 h at RT and overnight at 4 °C. After washing, membranes were incubated with horseradish peroxidase (HRP)-linked anti-human IgE (1:500 dilution, Nordic Immunology, Tilburg, Netherlands) for 1 h at RT, and color was developed with Konica Immunostain (Konica Minolta, Tokyo, Japan).

2D-PAGE and Immunostaining with Patients' Sera

We conducted 2D-PAGE and immunostaining as described previously.⁶⁾ Briefly, potato proteins (25 μ g) were applied to an Immobiline Drystrip (pH 3–10, 13 cm long, GE Healthcare), and first-dimensional isoelectric focusing (IEF) was performed at 20 °C up to 10000 Vh with an Ettan IPGphor 3 isoelectric focusing system (GE Healthcare). After cysteine reduction and alkylation, second-dimensional SDS-PAGE was performed on an acrylamide gel (DRC, Co., Ltd., Tokyo, Japan). The separated proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories, Hercules, CA, U.S.A.), and whole proteins were labeled with Cy5 mono-reactive dye (GE Healthcare, PA25001). After washing to remove the unbound Cy5, the membrane was immunostained with sera from potato-allergic patients (1:100 dilution), and HRP development was performed with enhanced chemiluminescence (ECL) plus Western Blotting Detection Reagent (GE Healthcare, 457 nm/520BP40). Fluorescence images were acquired with a Typhoon9400 variable image analyzer (GE Healthcare).

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 μ g 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% [v/v] glycerol, 2% [w/v] SDS) containing 0.5% (w/v) dithiothreitol (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 Co., Ltd., Tokyo, Japan). Fluorescence images were acquired 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).

The fluorescence intensity of each protein spot was normalized to that of the internal standard. We calculated normalized values for protein spots from five tubers of each transgenic potato (rd29A and 35S) and five non-transgenic potatoes (NT). Differences among these values were tested by one-way ANOVA followed by Dunnett-tests, and $p < 0.05$ was considered to indicate a significant difference. The "ratio" denotes the mean value of a relative normalized protein spot from transgenic potatoes divided by that of a protein spot from non-transgenic potatoes (transgenic/non-transgenic). The spots showing ratios > 2 (i.e., significantly different expression in transgenic vs. non-transgenic potatoes) are listed in Tables 3 and 4.

In-Gel Digestion and Protein Identification by Matrix Assisted Laser Desorption/Ionization-Time of Flight

(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, U.S.A.] and protease max [Promega, Madison, WI, U.S.A.]). 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, U.S.A.). All product ions were submitted to a computer database search analysis with the Mascot MS/MS ion search (Matrix Science Inc., MA, U.S.A.) by using the NCBI nr database (all entries) or the potato expressed sequence tag (EST) database.

RESULTS

Detection of IgE-Binding Proteins in Potatoes by 1D-Immunoblotting Using Potato-Allergic Patients' Sera

We obtained approximately 6 mg of soluble proteins from 1 g potato tubers (both non-transgenic and transgenic). The proteins in the non-transgenic (NT) and transgenic (rd29A, 35S) potato were separated by 1D-SDS-PAGE and stained with Coomassie Brilliant Blue (CBB). Patterns of proteins were very similar between non-transgenic and transgenic potatoes (Fig. 1a).

To identify the proteins that bound to IgE, we conducted 1D-immunoblotting of non-transgenic and transgenic potato extracts with serum from each individual potato-allergic pa-

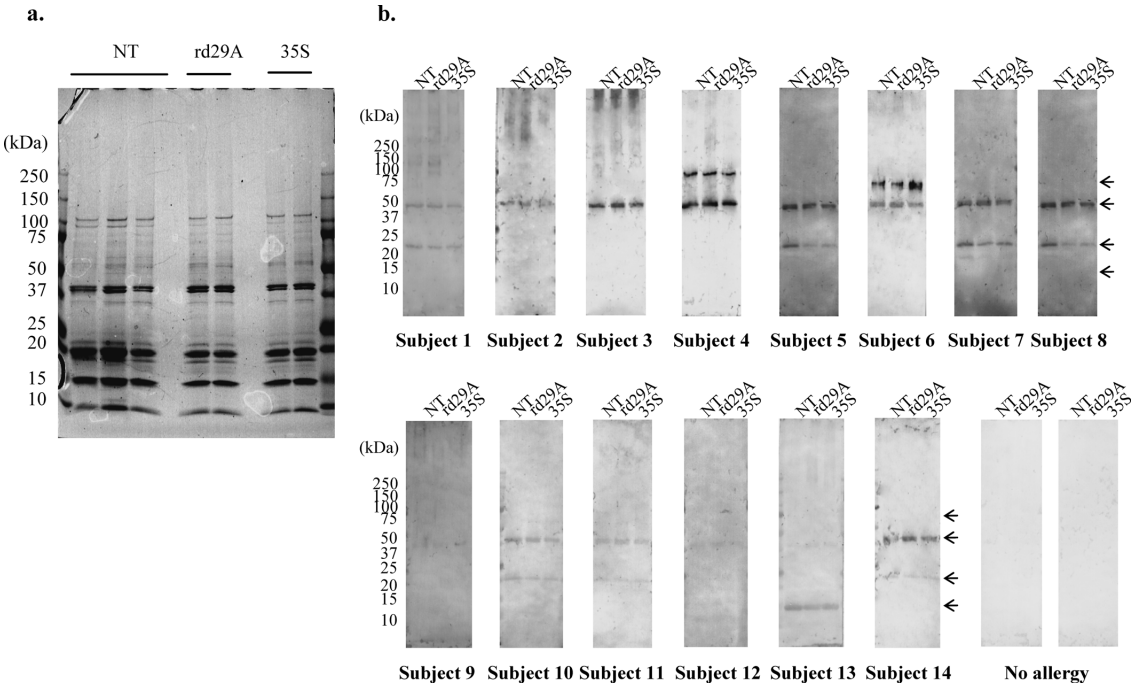


Fig. 1. 1D-Immunoblots of Non-transgenic and Transgenic Potato Extracts with Potato-Allergic Patients' Sera

Non-transgenic (NT) and transgenic (rd29A, 35S) potato extracts were separated by SDS-PAGE and (a) stained with CBB or (b) immunoblotted with potato-allergic patients' sera. Sera from allergy-free subjects were used as negative controls. Arrows indicate IgE-binding proteins. Molecular weights are shown at left.

Table 1. Information about Potato-Allergic Patients' Sera

Subject No. ^{a)}	Specific IgE to potato (UA/ml)	Immuno-CAP class	Specific IgE to major allergens (CAP class)
1	61	5	Egg white 84.3 (5) Soybean 37.6 (4) Gelatin <0.34 (0) Milk 1.16 (2)
2	16.9	3	Tomato 10.7 (3) Sesame 12.8 (3) Soybean 9.17 (3)
3	100	6	Egg white >100 (6) Wheat >100 (6) Shrimp 69.5 (5) Spinach 19.4 (4)
4	—	4	OVM 39.1 (4) Mite 1 (5) Dog epiderm (2) Egg white (6) Wheat (5) Sesame (4) Apple (3) etc.
5	5.7	3	Mite 1.2 (2) Animal epiderm 0.4 (1) Sesame 6.4 (3) Greenpea 4.8 (3) Peanut 5.9 (3) Soybean 5.5 (3) etc.
6	8.5	3	Mite 0.5 (1) Sesame 8.5 (3) Greenpea 3 (2) Peanut 5.9 (3) Soybean 4.6 (3) Whitepea 4.6 (3) etc.
7	16.4	3	Mite 3.1 (2) Sesame 16.4 (3) Greenpea 12.5 (3) Peanut 15.6 (3) Soybean 14.9 (3) Whitepea 16.2 (3) etc.
8	61	5	Egg white 1.5 (2) Sesame 11.8 (3) Greenpea 6.9 (3) Peanut 12.2 (3) Soybean 10.2 (3) Whitepea 11.9 (3) etc.
9	7.86	3	<i>D. pteronyssinus</i> 23.3 (4) Cat epiderm 3.21 (2), Horse dander 1.01 (2), Dog dander 0.86 (2) Soybean 6 (3) etc.
10	8.55	3	Soybean 8.69 (3) Carrot 9.72 (3) Rice 11.3 (3) Bee 11.1 (3) Latex 10.4 (3) etc.
11	16.7	3	Sesame 20.8 (4) Peanut 22 (4) Soybean 16.4 (3) Carrot 18.8 (4) Strawberry 17.6 (4), etc.
12	3.52	3	<i>D. pteronyssinus</i> 2 (2) Cat epiderm 1.44 (2), Dog dander 1.67 (2) Egg white 0.1 (0) Sesame 3.7 (3), etc.
13	4.54	3	Egg white 18.1 (4) Sesame 16.9 (3) Buckwheat 16.1 (3) Pea 23.1 (4) Peanut 15 (3) Soybean 68.5 (5), etc.
14	11.8	3	Peanut 15.7 (3) Soybean 7.80 (3) Carrot 10.9 (3) Wheat 12.9 (3) Onion 13.6 (3) Corn 7.14 (3) Latex 8.14 (3) etc.

a) Subjects 1 to 4 were from Japan, and the others were from U.S.A.

tient. Table 1 shows the potato-specific Immuno-CAP scores of the 14 subjects of this study. As shown in Fig. 1b, IgE in the serum of every patient bound to a 42 kDa protein, *i.e.*, a protein with the same molecular weight as that of patatin, a major allergen of potato. Other proteins that bound to the sera of potato-allergic patients had molecular weights of 14 kDa (subject 13), 21 kDa (subjects 1, 5, 7, 8, 10, 11, 14), and 75 kDa (subject 4). There were no clear differences in the patterns of IgE-binding proteins between non-transgenic potatoes and transgenic potatoes.

Detection and Identification of IgE-Binding Protein Spots by 2D-PAGE To detect qualitative differences in allergens between non-transgenic and transgenic potato proteins, we conducted 2D-immunoblot analysis with patients' sera and then compared 2D-immunostaining patterns between the non-transgenic and the transgenic potato proteins. Non-transgenic and transgenic potato extracts were separated by 2D-PAGE and transferred to PVDF membranes, and then whole proteins on the membrane were labeled with Cy5 dye. The membranes were then incubated with patients' sera, and the IgE-binding protein spots were detected with ECL plus.

Figure 2 shows representative images of both non-transgenic (a and c) and transgenic (b and d) potato proteins immunostained with serum from subject 4. Protein spots that bound to IgE are shown by arrows in Fig. 2. None of the IgE-binding protein spots showed clear qualitative differences, such as changes in isoelectric point (pI) or MW, between the non-transgenic and transgenic potatoes. In addition, there were no clear differences between non-transgenic and transgenic potatoes in terms of immunoblotting patterns of proteins that bound with other patients' sera (subjects 13 and 14; data not shown).

To identify IgE-binding protein spots, we carried out 2D-immunostaining with sera from subjects 4, 13, and 14. Sera from these patients were selected because they showed the characteristic IgE-binding pattern in 1D-immunoblotting (Fig. 1b). Several protein spots with molecular weights of approximately 42 kDa appeared at slightly different MW and pI (Figs. 3a–c).

The IgE-binding protein spots Nos. 1–13 in Fig. 3 were excised from the gel, digested with trypsin, and analyzed by MALDI-TOF-MS/MS. This resulted in identification of four proteins matching the peptides from these spots. The results of the database search are shown in Table 2. Spots 3 and 4,

which reacted with all sera from potato-allergic patients, showed homology to patatin precursors of *Solanum tuberosum* cv. Kuras. The proteins in spots 3 and 4 showed >80% homology to Sola t 1, but their MW and pI were slightly different, suggesting that they are isoforms of Sola t 1. A segment of serine protease inhibitor 2 was identified from the IgE-binding protein spots, although the proteins in the spots showed lower molecular weights than that of the mature protein. The database search of spot 13 revealed two proteins with significant homology; proteinase inhibitor II, which is homologous to serine protease inhibitor 1, and an aspartic protease inhibitor, which is the potato allergen Sola t 4. No proteins in the NCBI database matched to peptide masses from spots 5 and 6; therefore, we used the MS/MS data obtained from these spots to search the potato EST (Expressed Sequence Tag) database. This revealed that spots 5 and 6

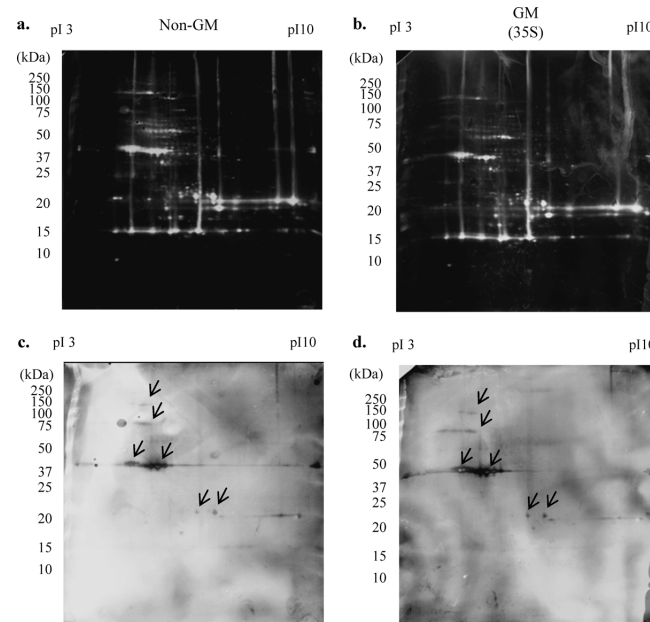


Fig. 2. 2D-Immunoblot Patterns of Non-transgenic and Transgenic Potato Extracts with Serum from Subject 4

Non-transgenic (NT) and transgenic (35S) potato extracts were separated by IEF followed by SDS-PAGE, and proteins were blotted onto PVDF membrane. Whole proteins were labeled with Cy5 (a, b) and immunoblotted with serum from subject 4 and then with HRP-labeled anti-human IgE antibody. IgE-binding protein spots were detected with ECL plus (c, d). Arrows indicate the spots compared between non-transgenic and transgenic potato.

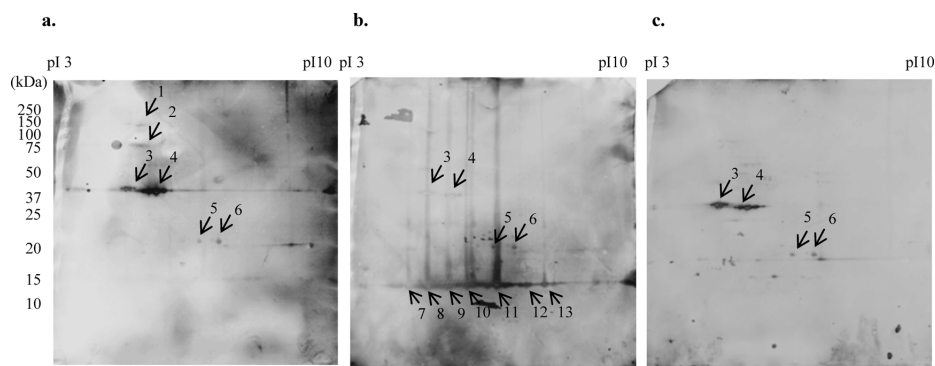


Fig. 3. 2D-Immunoblot Patterns of Non-transgenic Potato Extracts with Serum from Individual Subjects

Non-transgenic potato extract was separated by IEF followed by SDS-PAGE. Proteins were blotted onto PVDF membrane and immunoblotted with serum from individual potato-allergic subjects, and then with HRP-labeled anti-human IgE antibody. Figure shows ECL plus images of immunoblot with sera from (a) subject 4, (b) subject 13, and (c) subject 14. Numbered spots were excised from the gel for peptide identification by MALDI-TOF-MS/MS.

Table 2. Results of Protein Identification of IgE-Binding Spots by MALDI-TOF-MS/MS

Spot No.	Protein hits	gi:	MW (kDa)/pI	Mowse score	Sequence coverage	Matched peptides
3	Patatin precursor (<i>Solanum tuberosum</i>)	73426683	41.224/4.9	580	25%	7
4	Patatin precursor (<i>Solanum tuberosum</i>)	73426671	42.559/5.49	683	35%	9
5, 6	FSPM2252 potato tuber cDNA library <i>Solanum tuberosum</i> cDNA clone FSPM2252, mRNA sequence (EST)	222415233				
7—12	(Segment 1 of 2) serine protease inhibitor 2 (PSPI-21) (PSPI-21-5.2)	21362951	16.252/4.93	207	42%	4
13	Proteinase inhibitor II (<i>Solanum tuberosum</i>)	21522	17.561/6.77	347	20%	6
	Aspartic proteinase inhibitor (<i>Solanum tuberosum</i>)	21413	24.124/7.51	111	13%	2

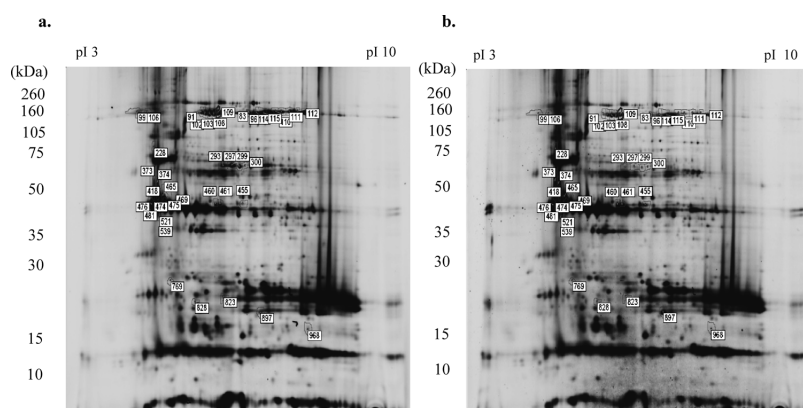


Fig. 4. Results of 2D-DIGE Analysis of Non-transgenic and Transgenic Potato Tubers

Each extract from non-transgenic (NT) and transgenic (rd29A, 35S) potato tubers was pre-labeled with Cy dyes and separated by 2D-PAGE. Representative fluorescence images of (a) NT and (b) rd29A potato extracts are shown. Normalized fluorescence value of circled spots showed significant difference (ratio >2 or <-2 , in Table 3) between non-transgenic and transgenic potatoes by Dunnett test ($p<0.05$). Numbers beside the circles correspond to spot numbers in Tables 3 and 4.

Table 3. Results of Protein Identification of Increased Protein Spot in Transgenic Potatoes^{a)}

Spot No.	Appearance ^{b)}	Av. ratio ^{c)}	Protein hits	gi:	Theoretical MW (Da)/pI	Mowse score	Sequence coverage	Matched peptides
228	48/69	2	N.D. ^{d)}					
373	42/69	2.25	N.D.					
374	51/69	2.07	N.D.					
418	63/69	2.04	Patatin precursor (<i>Solanum tuberosum</i>)	73426683	41224/4.90	431	38%	10
455	51/69	2.04	Patatin precursor (<i>Solanum tuberosum</i>)	73426671	42559/5.49	197	12%	5
460	45/69	2.12	N.D.					
461	51/69	2.2	N.D.					
465	60/69	2.19	Patatin precursor (<i>Solanum tuberosum</i>)	73426683	41224/4.9	376	29%	8
469	63/69	2.57	Patatin precursor (<i>Solanum tuberosum</i>)	73426671	42559/5.49	161	10%	3
472	42/69	2.08	Patatin precursor (<i>Solanum tuberosum</i>)	73426671	42559/5.49	274	27%	7
474	66/69	2.64	N.D.					
475	69/69	2.54	Patatin precursor (<i>Solanum tuberosum</i>)	73426683	41224/4.90	493	38%	10
476	66/69	2.36	Patatin precursor (<i>Solanum tuberosum</i>)	73426683	41224/4.90	501	38%	10
481	69/69	2.39	N.D.					
513	45/69	2.23	Patatin precursor (<i>Solanum tuberosum</i>)	73426683	41224/4.90	440	38%	10
521	63/69	2.19	N.D.					
539	48/69	2.14	Patatin precursor (<i>Solanum tuberosum</i>)	73426683	41224/4.90	461	38%	10
769	54/69	2.21	N.D.					
897	69/69	2.39	N.D.					

a) The spots in rd29A potato resulted significantly more than 2-fold difference to NT potatoes ($p<0.05$, Dunnett test). b) Number of images in which the spot appeared out of total 69 images (including the internal standard images). c) The ratio of the normalized fluorescence intensity of the rd29A spot to that of the NT spot. d) Not detected.

show homology to a gene fragment in the Potato tuber cDNA library *Solanum tuberosum* cDNA (clone FSPM2252; mRNA sequence gi: 222415233). This fragment has a domain that is homologous to that of members of the soybean trypsin inhibitor (Kunitz) family of protease inhibitors.

2D-DIGE Analysis of Non-transgenic and Transgenic Potato First, threshold value was determined according to

the experimental differences between the same samples labeled with two different fluorescence dyes, Cy3 and Cy5. The Cy-labeled, urea-extracted potato proteins were mixed and separated on a polyacrylamide gel, and approximately 700 spots were detected using DeCyder image software. The ratio of Cy5 intensity to Cy3 intensity was 1.67, at which the difference of ratios of 95% of protein spots were within this

Table 4. Results of Protein Identification of Decreased Protein Spot in Transgenic Potatoes^{a)}

Spot No.	Appearance ^{b)}	Av. ratio ^{c)}	Protein hits	gi:	Theoretical MW (Da)/pI	Mowse score	Sequence coverage	Matched peptides
107	54/69	-2.03	N.D. ^{d)}					
75	42/69	-2.13	N.D.					
83	51/69	-2.08	Lipoxygenase (<i>Solanum tuberosum</i>)	486619	97078/5.43	202	16%	11
91	57/69	-2.53	Lipoxygenase (<i>Solanum tuberosum</i>)	1407701	97085/5.43	237	19%	12
96	48/69	-2.19	Lipoxygenase (<i>Solanum tuberosum</i>)	486619	97078/5.43	186	13%	9
97	60/69	-2.21	N.D.					
99	60/69	-2.29	N.D.					
102	45/69	-2.53	Lipoxygenase (<i>Solanum tuberosum</i>)	486619	97078/5.43	129	10%	7
103	69/69	-2.45	Lipoxygenase (<i>Solanum tuberosum</i>)	2558849	97176/5.47	207	16%	11
104	45/69	-2.36	N.D.					
106	51/69	-2.31	Lipoxygenase (<i>Solanum tuberosum</i>)	486619	97078/5.47	254	22%	15
108	69/69	-2.5	Lipoxygenase (<i>Solanum tuberosum</i>)	2558849	97078/5.47	207	16%	11
109	57/69	-2.55	Lipoxygenase (<i>Solanum tuberosum</i>)	486619	97078/5.45	243	10%	7
110	57/69	-2.3	N.D.					
111	54/69	-2.17	N.D.					
112	54/69	-2.16	N.D.					
114	69/69	-2.23	Lipoxygenase (<i>Solanum tuberosum</i>)	486619	97078/5.43	134	14%	11
115	57/69	-2.33	Lipoxygenase (<i>Solanum tuberosum</i>)	486619	97078/5.43	115	12%	8
117	57/69	-2.96	N.D.					
118	60/69	-2.26	N.D.					
293	66/69	-2.1	Glycogen (starch) synthase (<i>Solanum b</i>)	21471	67160/6.92	367	27%	15
297	63/69	-4.09	Starch (bacterial glycogen) synthase (<i>Slb</i>)	602594	67176/6.92	480	26%	16
299	60/69	-3.48	Glycogen (starch) synthase (<i>Solanum b</i>)	21471	67160/6.92	360	21%	11
300	54/69	-2.73	Aspartic proteinase inhibitor (<i>Solanum b</i>)	21413	24124/7.51	36	12%	2
823	48/69	-2.37	N.D.					
828	54/69	-2.25	N.D.					
968	63/69	-2.07	N.D.					

a) The spots in rd29A potato resulted significantly more than 2-fold difference to NT potatoes ($p < 0.05$, Dunnett test). b) Number of images in which the spot appeared out of total 69 images (including the internal standard images). c) The ratio of the normalized fluorescence intensity of the rd29A spot to that of the NT spot. d) Not detected.

range. Therefore, the threshold ratio was set to 2 to identify proteins expressed differently between transgenic (rd29A and 35S) and non-transgenic (NT) potatoes.

Five independent non-transgenic and transgenic potato tuber samples were used for 2D-DIGE. 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). Figure 4 shows representative Cy images of NT and rd29A 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 are shown in Fig. 4, and the numbers beside the circles correspond to spot numbers in Tables 3 and 4.

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 (Table 3), a major potato allergen. The expression of patatin with lower pI value (corresponding spot 3 in Fig. 3) seemed to be increased in transgenic potato. The expression levels of other IgE-binding spots (Spots 5—13 in Fig. 3) 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 (Table 4). Other protein spots were unable to be identified because of insufficient peptide.

DISCUSSION

Allergenicity testing of foods derived from transgenic organisms is recommended to determine whether they contain new proteins that are homologous to known allergens. In addition, it is advisable to determine the content of known allergens or cross-reactive proteins in foods derived from transgenic organisms, so that we can evaluate whether they are increased or decreased compared with those in foods derived from non-transgenic organisms.

In this study, we used a comprehensive detection method (immunoblotting with sera from allergic patients) and a quantification method (2D-DIGE) to evaluate unintended effects of genetic modification on inherent allergens in potato.

We conducted 1D-immunoblotting with sera from 14 patients (Fig. 1b) and 2D-immunoblotting with serum from individual patients (Fig. 3). These analyses revealed that sera from all potato-allergic patients reacted with patatin, and that serum from some individuals reacted with proteins that were smaller or larger than patatin. The IgE-binding protein spots were identified by MALDI-TOF-MS/MS (Table 2). Mass spectrometry data indicated that the amino acid sequences of proteins from spots 3 and 4 matched those of patatin precursors (86% homology to full sequences), with some differences at the N-terminal. Therefore, it is likely that the proteins in spots 3 and 4 are patatin isotypes. Mature serine protease inhibitor 2 has molecular weight of 20 kDa, but the IgE-binding spots showing homology to this protein (spots 7—12) had lower molecular weights. Proteins in spots 5 and 6 had a molecular weight of 21 kDa, but there were no pro-

teins in the NCBI database that showed homology to them. However, an additional search of an EST database retrieved a gene fragment of potato. Therefore, the EST database is useful for more comprehensive protein identification.

We determined relative differences in the amounts of allergens and other proteins between non-transgenic and transgenic potato using 2D-DIGE analysis. When compared with protein expression in non-transgenic potatoes, more proteins showed altered expression in potatoes containing the rd29A promoter than in those containing the 35S promoter. This result suggests that the *AtDREB1A* gene is expressed at lower levels in 35S potato than in rd29A potatoes (data not shown).

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.^{30,31} It has been reported that expression of patatins is affected by various factors, including tuber size and storage conditions.^{30,32} 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.^{33–35} Patatin variation occurs with germplasm enhancement practice especially with using wild relatives. Furthermore, expression of other potato tuber proteins has been reported to change in response to environmental factors, such as abiotic stresses.^{36,37} 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.^{38,39}

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).⁴⁰ 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 potato-allergic patient's serum (subject 4) 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.

In conclusion, we detected several IgE-binding proteins in transgenic potato by immunoproteomic analysis, and identified these proteins by mass spectrometry. 2D-DIGE analysis revealed several differences in protein expression between *AtDREB1A*-transgenic potato and control potato. Further re-

search is required to confirm that the differences observed in this study are due to gene transfection, rather than environmental factors.

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