



ELISA method for monitoring human serum IgE specific for Cry1Ab introduced into genetically modified corn

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Abstract

Enzyme-linked immunosorbent assay (ELISA) is the most convenient method of monitoring the occurrence of IgE antibodies specific for novel proteins in genetically modified (GM) foods. The levels of IgE specific for a recombinant protein, Cry1Ab, were determined using an ELISA method. A soluble form of the Cry1Ab protein purified from pCold1 vector-transformed *Escherichia coli* pTf16/BL21 was used as the ELISA coating antigen, and 1 M NaCl was used as the washing buffer to remove IgE non-specifically bound to the coated antigen. Sera from 44 patients allergic to major food allergens were obtained, diluted 20-fold, tested, and found no identifiable IgE above background levels. We also tested sera from patients with corn allergy against whole extracts of non-GM and GM-corn (MON 810) using immunoblotting. The staining patterns were similar for the two types of corn. These results indicate that significant levels of IgE antibodies specific to Cry1Ab were not found in the sera of Japanese patients with food allergies.

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1. Introduction

Cry proteins are insecticidal toxins produced by *Bacillus thuringiensis*. Some of these proteins have been extensively characterized and used in the field as pest-control agents. The receptors for Cry proteins exist on the intestinal cells of certain insects (Hofmann et al., 1988a,b; Van Rie et al., 1989, 1990; Li et al., 1991; Hodgman and Ellar, 1990; Harvey et al., 1986; Wolfersberger, 1992; Knowles and Dow, 1993). However, mammals do not have Cry receptors (Sacchi et al., 1986). Thus, Cry genes have been widely introduced in the creation of new GM crops.

Predicting potential allergenicity is a major issue in safety assessments of genetically modified (GM) crops. To assess whether a protein may be allergenic, its amino acid sequence should be compared with those of known allergenic proteins (FAO/WHO Expert Consultation, 2001; Gendel, 2002; CODEX, 2003). The outcome of such

comparisons should then be combined with other information on allergenicity, such as the protein's digestibility and its ability to bind to IgE in sera from allergic patients.

Cry1Ab produced by *B. thuringiensis* HD-1 is a Cry protein and is toxic to Lepidoptera insects. Cry1Ab is very rapidly digested in simulated gastric fluid (SGF) (Okunuki et al., 2002). Based on the above-mentioned criteria for safety assessments, Cry1Ab is unlikely to induce food allergy. However, several GM crops containing the Cry1Ab gene, like Bt11 and Mon810, have been developed. Therefore, it is important to confirm that allergic patients' sera do not contain IgE that is cross-reactive with Cry1Ab. Moreover, monitoring the occurrence of IgE reactivity with Cry1Ab is an important component of post-marketing surveillance to assure the safety of GM food.

In a previous paper, we modified a conventional enzyme-linked immunosorbent assay (ELISA) method to test human patient sera for specific IgE. In this paper, using our improved ELISA method, we tested 44 serum samples from allergic patients to determine whether the sera contained IgE reactive with Cry1Ab.

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2. Method

2.1. Cloning and expression of Cry1Ab

Genomic DNA was extracted from the GM corn, Bt11. The Cry1Ab gene introduced into Bt11 was amplified by means of polymerase chain reaction (PCR). The amplicon was subcloned into a plasmid vector, pT7-Blue-T vector (Merck, Darmstadt, Germany), and the nucleotide sequence (Fischhoff and Perlak, 1999) was confirmed. To prepare an antiserum, the Cry1Ab gene was subcloned into pET-16b (Merck), and BL21-CodonPlus (DE3) RIL (Stratagene, La Jolla, CA, USA) cells were transformed. Cultures to express the recombinant protein were performed according to the manufacturer's instructions. The proteins in the inclusion bodies were separated using SDS-PAGE, and Cry1Ab was recovered from the gel slice. Rabbits were then immunized with the recombinant protein, and antiserum was obtained. To prepare the ELISA antigen, the amplicon was subcloned into pCold1 DNA (Takara, Ohtsu, Japan) and transformed into pTf16/BL21 (Takara). The recombinant protein was then expressed according to the manufacturer's instruction manual. The fusion peptide was added to Cry1Ab at the N-terminus. The fusion peptide consists of 16 amino acid residues and includes His tag.

2.2. Purification of Cry1Ab

pTf16/BL21 transformed with pCold1 DNA/Cry1Ab was grown, and the expression of Cry1Ab was induced by 0.1 mM IPTG (isopropyl-1-thio- β -D-galactopyranoside). The cells were then collected by centrifugation and washed in 25% sucrose, 10 mM Tris-HCl (pH 8.0), and 1 mM EDTA. The cell pellet was resuspended in 50 mM of sodium phosphate and 300 mM of NaCl (pH 7.0) containing protease inhibitors and was then disrupted by sonication. The suspension was centrifuged, and the supernatant was recovered. The lysate was applied to a chelate column (TALON Cell-Thru; Clontech, Palo Alto, CA, USA) to absorb histidine-tagged Cry1Ab. The eluted fraction was concentrated with Centricon YM-30 (Millipore, Bedford, MA, USA), then separated through Superdex 200 10/300 GL (Amersham Biosciences) in phosphate-buffered saline (PBS, pH 7.0). Cry1Ab was detected at each purification step using Western blotting with the rabbit polyclonal antiserum described above.

2.3. Serum samples

Serum samples were obtained between 2003 and 2004 from 36 Japanese patients with food allergies who were positive for allergen-specific IgE when examined using the Immuno-CAP (Pharmacia Diagnostic, Uppsala, Sweden) method and who clinically exhibited allergic symptoms. Eight sera commercially available from corn allergic patients in the United States (Plasma Lab International, Everett, WA, USA) purchased on 2000 were also used. Information about the serum samples are shown in Table 1. Sera from 10 healthy volunteers (specific IgE-negative) in Japan were used as a control group. Informed consent was obtained from all the food allergic patients and healthy volunteers. This study was approved by the Institutional Review Board of the National Institute of Health Sciences.

2.4. ELISA

Assay plates (96 wells) were coated with purified Cry1Ab [0.2 μ g/50 μ L 50 mM sodium carbonate buffer (pH 9.6)/well] at 4 °C overnight. After washing with PBS containing 0.05% Tween 20 (PBS-T), the plates were blocked with PBS containing 0.1% casein for 2 h at room temperature, washed with PBS-T, and incubated with serum samples diluted (1/20) in 0.1% casein-PBS at 4 °C overnight. Then, the wells were washed with PBS-T containing 1 M-NaCl, as described previously (Takagi et al., 2006). The wells were treated with peroxidase-conjugated goat anti-human IgE antibodies (Nordic Immunology, 1:1000) in 0.1% casein-PBS for 1 h at room temperature and were washed. A substrate solution was added (TMB

reagent; BD Biosciences, San Diego, CA) and the color development was done for 5 min. The colorimetric intensity ($OD_{450} - OD_{570}$) was measured according to the manufacturer's protocol. Control was measured with buffer only. The control value was already subtracted from the values shown in Fig. 2. The experiment was carried out in duplicate. We judged the sample to be ELISA-positive when the absorbance for a patient's serum sample was greater than the value of the average +5 SD of the control sera (Yagami et al., 1998).

For the inhibition assay, the serum samples (diluted 1:20) were pre-incubated overnight at 4 °C with or without purified Cry1Ab prior to being added to the wells.

Specificity of ELISA with Cry1Ab as coating antigen was confirmed by binding monoclonal anti-Cry1Ab antibody 3A11CS (Walschus et al., 2002).

2.5. Western blot analysis of serum IgE added to GM or non-GM corn extracts

GM corn (MON 810) and non-GM corn were purchased at the same time. Both corns were grown in Ohio, US and were genetically closely related.

Corn powder was made from GM corn and non-GM corn. The corn powder (5 g) was stirred in 20 ml PBS at 4 °C overnight. The suspension was then centrifuged at 100,000g for 1 h at 4 °C. The supernatant was collected and used as an extract. The protein concentration of the extract was determined using a BCA protein assay kit (Pierce, Rockford, IL, USA).

Each corn extract (1 mg of protein) was separated using an SDS-PAGE 2 D-gel (4–20% acrylamide). Then, the proteins were transferred onto a nitrocellulose membrane. The membrane was blocked in 0.5% casein dissolved in PBS. The membrane was cut vertically (0.3 cm), and each piece was incubated with 4- or 40-times diluted sera for 1 h and then overnight at 4 °C. After washing with PBS-T, the membranes were incubated with 500-times diluted peroxidase-conjugated donkey anti-human IgE for 1.5 h at room temperature. After washing, the membranes were stained with a substrate solution (Immunostain HRP-100; Konica, Tokyo, Japan).

2.6. Digestibility test

Escherichia coli lysate was prepared from pTf16/BL21 transformed with pCold1/Cry1Ab in the absence of protease inhibitors. The lysate (780 μ g protein/260 μ L) was mixed with 390 μ L of 1.667 $\times$ SGF (1 $\times$ SGF: 0.32% pepsin (Sigma, St. Louis, MO, USA), 0.03 M NaCl, and 0.084 M HCl (pH 1.2)) and was incubated at 37 °C. A 65- μ L aliquot was then taken and added to 22.5 μ L of 0.2 M Na₂CO₃ and 22.5 μ L of 5 $\times$ Laemmli SDS-PAGE sample loading buffer (40% glycerol, 5% 2-mercaptoethanol, 10% SDS, 0.33 M Tris, 0.05% bromophenol blue, pH 6.8). The mixture was boiled and separated using SDS-PAGE. The proteins were transferred onto nitrocellulose. Cry1Ab remaining after digestion was visualized using immunoblotting with the monoclonal antibody 3A 11CS (Walschus et al., 2002).

In addition, an extract of the GM corn, Mon810, was produced as described above and was subjected to a digestibility test.

3. Result

3.1. Expression and purification of Cry1Ab

Bacillus thuringiensis originally biosynthesizes Cry1Ab as a 130-kDa protoxin that is proteolytically digested to yield a 68-kDa active form. The truncated gene had been modified for expression in plants and was introduced in the creation of GM-corn. In the present study, the 1.8-kb Cry1Ab gene was obtained from a GM corn strain (Bt 11). The obtained sequence was identical to the reference sequence (Fischhoff and Perlak, 1999).

Table 1
Serum samples from patients

Serum No.	Anti-Cry1 Ab IgE-ELISA (OD _{450–570})	Food allergen (Immuno-CAP score)
1	0.128	Egg white (3), OVM (3), milk (2), buckwheat (3), crab (3), prawn (3)
2	0.204	Egg white (3), wheat (5)
3	0.098	Cheese (3), egg white (4), milk (3), wheat (2)
4	0.115	Egg white (3), OVM (4), milk (3), mites (5)
5	0.036	Sesame (5), buckwheat (5), egg white (3), wheat (5), peanut (5), soybean (4)
6	0.091	Egg white (6), cocksfoot (5), cedar (5), Japanese cypress (3)
7	0.349	Egg white (2), OVM (3), salmon caviar (3), milk (3), casein (3), cheese (3)
8	0.081	Rice (4), tomato (4), mackerel (6), spinach (3), carrot (2), potato (3), sardine (5), chicken (4), flatfish (5), salmon (3), egg white (6), milk (6), wheat (6), sesame (6)
9	0.221	Mites (6), egg white (4), animal dander (4), milk (2)
10	0.104	Wheat (3), peanut (3), cheese (6), OVM (5), egg white (4), milk (6)
11	0.385	Egg white (4), OVM (4), wheat (2), rice (2), sesame (3), buckwheat (2), tuna (3), prawn (4), crab (4)
12	0.233	Mites (4), egg white (5), milk (6), wheat (3), peanut (3), casein (5), OVM (5)
13	0.087	Milk (6), cheese (5), wheat (4), rice (2), egg white (2), peanut (2), soybean (3)
14	0.071	OVM (4), cocksfoot (4), cedar (2), mites (3), animal dander (2), egg white (4), milk (4), wheat (2), peanut (3), casein (4)
15	0.166	OVM (3), egg white (3), buckwheat (4)
16	0.112	+amylase (2), egg white (4), milk (5), wheat (4)
17	0.199	OVM (6), cocksfoot (3), hogweed (3), cedar (4), house dust (2), Alternaria (3), egg white (6), milk (2), wheat (3), sesame (4), buckwheat (3), peanut (4)
18	0.162	house dust (2), animal dander (3), egg white (6), milk (6), wheat (6), corn (2), rice (4), soybean (5), melon (2), banana (3)
19	0.281	Animal dander (4), egg white (6), milk (3), wheat (3), sesame (2), soybean (3), mackerel (2), horse mackerel (2)
20	0.061	Egg white (6), milk (4), cod (2), wheat (6), rice (5), soybean (6), pork (2), beef (2), tuna (2), salmon (2), chicken (3), horse mackerel (2), sardine (2)
21	0.09	OVM (5), mites (5), animal dander (5), egg white (5), milk (2), wheat (4)
22	0.095	
23	0.058	Egg white (3), egg yolk (3), milk (4)
24	0.12	Egg white (5), soybean (4), potato (5), milk (2)
25	0.072	Egg white (3), peanut (3), milk (3), egg yolk (2), OVM (3)
26	0.042	Peanut (2), soybean (2), buckwheat (2), wheat (4), egg yolk (3), OVM (5), egg white (5)
27	0.137	Mites (2), house dust (2), cedar (4), apple (3), Japanese cypress (2), white birch (4)
28	0.384	Peanut (3), mites (3), house dust (3)
29	0.32	Mites (6), egg white (3), peanut (5), sauid (3), prawn (3)
30	0.106	Barley (3), rice (3), mackerel (2), egg white (6), milk (3), soybean (4), grapefruit (3)
31	0.051	house dust (3), kiwi (3), egg white (3)
32	0.116	Egg white (4), OVM (4)
33	0.055	Egg white (3), milk (3), peanut (3)
34	0.067	Egg white (6), milk (6), OVM (6)
35	0.117	Mites (3)
36	0.068	Cod (3), crab (3), prawn (3)
37	0.043	Corn (4)
38	0.075	Corn (3)
39	0.08	Corn (3)
40	0.047	Corn (4)
41	0.045	Corn (3)
42	0.163	Corn (3)
43	0.037	Corn (3)
44	0.115	Corn (3)

Sample numbers 1–36 were from Japanese patients. Sample numbers 37–44 were purchased from Plasma Lab.

The correlation of Cap score and specific IgE concentration are as follows (2, 0.70–3.5; 3, 3.5–17.4; 4, 17.5–49.9; 5, 50.0–99.9; 6, >100 IU/ml). All the sera were derived from the patients who diagnosed as food allergy and exhibited allergic symptoms.

Usually, the production of a soluble form of truncated Cry protein is difficult in *E. coli*. However, a satisfactory amount was expressed in a soluble form using pCold1 DNA as a vector and BL21-overexpressing chaperone (pTf16/BL21) as a host. Under these conditions, Cry1Ab was detected as a 70-kDa protein using Western blotting and an antiserum against Cry1Ab.

Purification of Cry1Ab from *E. coli* was done as described in the Section 2. In the first step, a chelate column (TALON CellThru) was used (Fig. 1, lane 3). Then, the eluted fraction was further purified using Superdex 200 10/300 GL. In total, 31 µg Cry1Ab was obtained from one liter of culture. As shown in Fig. 1 (lane 4), the resultant fraction was homogenous on SDS-PAGE when visualized by Co-

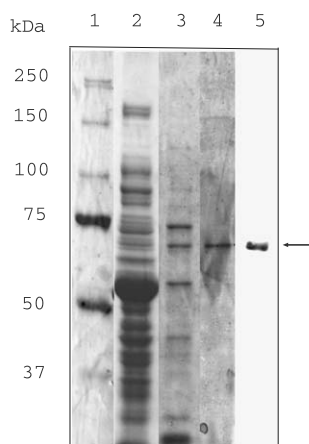


Fig. 1. Purification of Cry1Ab expressed in *E. coli*. Lane 1, molecular weight marker; lane 2, non-binding fraction of chelate (TALON CellThru) column; lane 3, binding fraction of TALON CellThru; lanes 4 and 5, fractions of Superdex 200 10/300 GL. Lanes 1–4, stained with CBB; lane 5, stained with Western blotting using antiserum against Cry1Ab. Cry1Ab is indicated with an arrow.

massie brilliant blue (CBB) staining. The purified protein was confirmed to be Cry1Ab using Western blotting and a polyclonal antibody to Cry1Ab (lane 5).

3.2. ELISA

ELISA using purified Cry1Ab as a coating antigen was performed, and the presence of Cry1Ab-specific IgE was determined. All the values for the 44 serum samples from allergic patients were less than the average + 5 SD of the values from 10 healthy subjects. The distribution of the ELISA results is shown in Fig. 2. The distributions of the control group and the patients group were similar. The three serum samples with the highest ELISA values were further examined using ELISA inhibition. Even when the serum samples were pre-incubated with a higher concentration of the soluble antigen (1 µg/mL), almost the same amount of IgE was bound to the coating antigen (data not

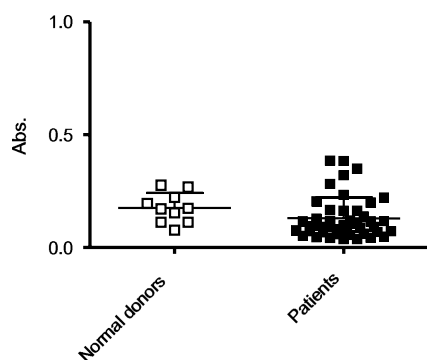


Fig. 2. Data obtained from 54 serum samples analyzed using our improved IgE-ELISA method. Serum samples from 44 allergic patients and 10 healthy volunteers were diluted (1:20) and assayed for anti-Cry1Ab IgE using the improved ELISA method. The average (horizontal long line) and standard deviation (upper short line) are indicated for each group.

shown). IgE specific to Cry1Ab was not found in the sera from food allergic patients.

3.3. Western blot analysis of GM or non-GM corn extracts using corn-specific IgE from allergy patients

Two patient sera, P1 and P2 that were positive for corn-specific IgE (19.3 and 18.5 IU/mL, respectively) were used for immunoblotting. Several proteins in the corn extracts were recognized by the IgE from the corn-allergic patients (Fig. 3). However, the staining patterns were virtually the same for the GM and the non-GM corn extracts. IgE from serum P2 strongly reacted with a protein of about 11 kDa in a similar manner in both the GM and the non-GM corn extracts. No band was detected when healthy control serum was used for the immunoblotting (data not shown).

3.4. Digestibility test

When Cry1Ab in corn extracts from Mon810 or Bt11 was examined in a digestibility test, it was no longer detected after 0.5 min of digestion. Cry1Ab degraded very rapidly. When Cry1Ab in *E. coli* was tested, it also was rapidly digested, with a half-life of 0.3 min. Smaller fragments derived from Cry1Ab digestion were not found.

4. Discussion

GM crops authorized for food use in Japan include 74 varieties of 7 plants (as of January 2006). In most of these GM crops, CP4-EPSPS (5-enolpyruvyl-shikimate-3-phosphate synthase from *Agrobacterium* sp. strain CP4), PAT (phosphino-thricin acetyltransferase), or *B. thuringiensis* insecticidal toxins have been introduced. At present, no food allergies induced by these proteins have been reported. However, it is important to monitor for the unintended occurrence of allergenic reactions to these proteins. Previously, we reported an ELISA-based method for monitoring the allergenicity of three recombinant proteins derived from transgenes in GM crops: CP4-EPSPS, Cry9C, and PAT. The monitoring of serum IgE specific for Cry1Ab has not yet been reported. Therefore, serum samples obtained from an allergic patient population were tested for Cry1Ab-specific IgE.

ELISA for serum Cry1Ab-specific IgE requires a soluble form of Cry1Ab. In obtaining a soluble form, several combinations of vectors and host *E. coli* strains were preliminarily compared. For example, pET-16b, pET-41a and pGEX-6P-1 vectors were examined, but these vectors only expressed a small amount of soluble Cry1Ab. Then, pCold1 DNA vector and *E. coli* hosts overexpressing chaperones were tried, and a sufficient amount of Cry1Ab was expressed in soluble form when pTf16/BL21 was used as a host.

Previously, we reported an improved ELISA method for monitoring specific IgE in human sera (Takagi et al., 2006). In this method the non-specific binding of IgE to antigen

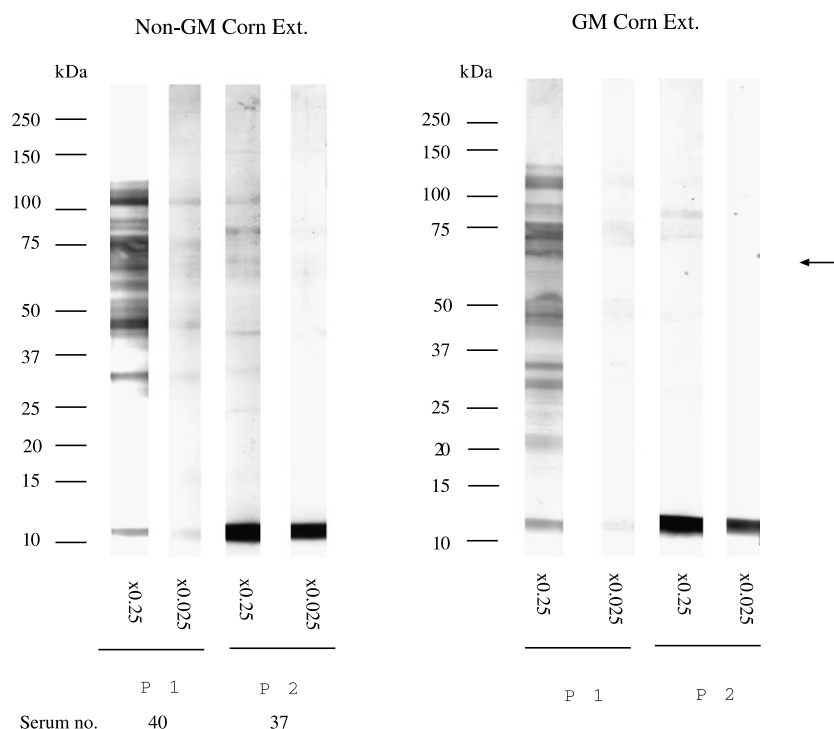


Fig. 3. IgE-Western blotting of sera with GM corn or non-GM corn extracts. Whole extracts from a GM corn strain, Mon810 (Cry1Ab introduced), and a non-GM corn strain were applied to SDS-PAGE and blotted onto nitrocellulose membranes. The membrane pieces were reacted with 4- and 40-fold diluted sera from two patients who are allergic to corn (P1(No.40) and P2(No.37)). The expected location of Cry1Ab is indicated with an arrow.

was reduced by an additional washing using 1 M NaCl. This method is convenient and useful for preliminary screening, for example, when a large number of serum samples are tested. In this study, our improved ELISA method was also used to detect IgE specific for Cry1Ab. As expected, IgE specific for Cry1Ab was not detected.

Eight serum samples used in this assay were purchased from Plasma Lab and were from patients with corn allergy. We purchased the serum samples on 2000 after GM corns started to sell in the US. And one serum sample is from a Japanese patient with corn allergy. The sample was obtained on 2004 after GM corns started to sell in Japan. Because GM corns containing Cry1Ab had been consumed largely in the corn market in the US and in Japan, it is possible to assume that the patients who provided the sera had eaten GM corn containing Cry1Ab. These serum samples seem to be desirable to be tested for IgE specific to Cry1Ab.

The authors think it is preferable to conduct target serum screening when newly produced protein is not known as allergen and specific serum screening is difficult to perform.

If specific serum IgE to a protein were detected, that would not necessarily cause IgE receptors cross-linking on mast cells or basophils, and degranulation of the cells by the stimulation with the protein. When IgE specific to Cry1Ab were detected, further study is needed such as basophile degranulation test, skin test, or oral challenge. Serum IgE test is located in the first step of allergenicity evaluation.

The reactivity of IgE from corn-allergic patients was analyzed using a Western blot analysis of extracts from a GM corn strain, Mon810, and a non-GM corn strain. The resulting staining patterns were virtually the same. A strong 11-kDa band was detected on the Western blot when serum P2, obtained from a corn-allergic patient, was used. Judging from the molecular weight, this band likely corresponds to a major known allergic protein, lipid transfer protein (Pastorello et al., 2003).

Pepsin digestibility of Cry1Ab in *E. coli* lysate, MON810 and Bt11 was examined using simulated gastric fluid (SGF). Cry1Ab was rapidly digested and had a half-life of less than 0.3 min. The high digestibility of Cry1Ab suggests that Cry1Ab expressed in *E. coli* has similar characteristics when it is expressed in GM plants.

Batista et al. (2005) performed a pilot post-marketing study on food allergy to CP4-EPSPS and Cry1Ab and reported that no allergenic responses to the two proteins were observed when skin prick testing and Western blotting was performed. Furthermore, specific IgE for Cry1Ab was not detected in the present study using an improved ELISA method. These studies by us and by Batista et. al. are considered pilot post-marketing studies for GM-food and a large number of serum samples is recommended to be examined in the future.

In conclusion, we have shown that an ELISA method using soluble Cry1Ab antigen is a convenient screening method that can be used prior to confirmatory methods, such as Western blotting or ELISA inhibition tests. In this

pilot study, IgE antibodies to Cry1Ab protein were not detected in sera from Japanese patients with food allergies.

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