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Letter to the Editor

Novel *in vitro* test for pollen-related vegetable/fruit allergy using the EXiLE method

Dear Editor,

Pollen-associated food allergy syndrome (PFS), which is a common problem worldwide, develops when an individual who is sensitized to an inhaled pollen ingests fruits or vegetables that cross-react with the sensitizing pollen allergen.^{1–4} Pathogenesis-related protein 10 (PR-10) and profilin are two major allergens that contribute to this cross-reactivity.² ImmunoCAP and other similar immunochemical methods are used widely in *in vitro* tests to detect serum IgE specific to fruits and vegetables in patients with suspected PFS. However, this method requires allergens to be fixed onto a solid phase, which may cause conformational changes to the allergen structure, particularly in unstable fruit and vegetable allergens. The *in vivo* prick-prick test using fresh fruits or vegetables is the most sensitive test for detecting IgE to food in PFS.^{1,5} However, test results vary depending on the overall condition of the patient, and the number of antigens that can be tested at one time is limited.

We established a novel allergy test based on IgE crosslinking-induced luciferase expression (EXiLE) using a humanized cultured rat mast cell line (RS-ATL8),⁶ which expresses human FcεRI and the nuclear factor of activated T-cell-responsive luciferase reporter gene. After sensitization with diluted patient sera, its stimulation with the addition of a specific allergen can be monitored using the luciferase assay with very high sensitivity.⁷ The use of the EXiLE method is advantageous as it can be performed with intact allergens in the liquid phase. We evaluated whether this method can be used to measure IgE to fresh fruit and vegetable allergens using preserved sera from patients with PFS and assessed the IgE cross-reactivity between pollen and food allergens.

Sera from six patients with PFS were assessed, with a focus on IgE reactivity to fresh apple and tomato as indicators of PR-10⁴ and profilin⁵-related food allergies, respectively. Among the six patients, four (P1, P2, P3, and P4) were allergic to apple and four (P3, P4, P5, and P6) to tomato. These allergies were confirmed based on the patients' medical histories and results of the prick-prick test² using fresh apple or tomato (Supplementary Table 1). Most patients tested negative on the skin prick test using commercial tomato or apple allergen products. We first compared the IgE crosslinking activities induced by commercially available timothy pollen allergen

products or extracts from commercially available birch pollen, commercial tomato or apple allergen products, freshly prepared tomato or apple skin juice, and homemade preserved extracts of whole tomato or apple skin (Supplementary Methods). Five of the six patients exhibited a >1.5-fold increase in luciferase expression upon stimulation with the commercially available timothy pollen allergen product (P2, P3, P4, P5, and P6) and birch pollen extract (P1, P2, P3, P4, and P5) (Supplementary Fig. 1). Based on the EXiLE method, stimulation with commercially available tomato allergen product only induced slight responses in two of the four sera obtained from patients (P3 and P5) with tomato allergy (Fig. 1A). The freshly prepared tomato juice exhibited strong IgE responses (Fig. 1B), and the responses to preserved tomato extract were moderate (Fig. 1C) in these two patients. In contrast, the commercially available apple allergen product (Fig. 1D) did not induce any response up to a concentration of 100 µg/mL, whereas the freshly prepared apple skin juice (Fig. 1E) and preserved apple skin extract (Fig. 1F) caused a dose-dependent increase in luciferase expression. The difference in the order of the amplitudes of the cellular responses in some patients as shown in Figure 1E, F can be explained by the difference in the components of whole apple and apple skin. These results indicated that allergens with intact structure that can induce IgE crosslinking was retained in the freshly prepared juice and preserved fruit and vegetable extracts and have been lost or decreased by commercially available fruit and vegetable allergen products.

Next, we investigated whether the EXiLE method can be used to assess the cross-reactivity of pollen and food allergens. Briefly, specific or cross-reactive IgE in the sera were removed using antigen-coated beads, and the EXiLE activity of the supernatant was assessed. Bead treatment of the timothy pollen allergen product and homemade tomato extract significantly inhibited the tomato extract-induced EXiLE responses by approximately 75% (Fig. 2A). However, the tomato extract inhibited the timothy-induced EXiLE response by only 40%, which is nearly half of that of the timothy pollen allergen product (Fig. 2B). Similar results were observed in the case of anti-human IgE stimulation (Fig. 2C). These results indicated that only a portion of the timothy-specific IgE cross-reacts with the tomato antigen, indicating that the primary sensitizer in these patients was grass pol-

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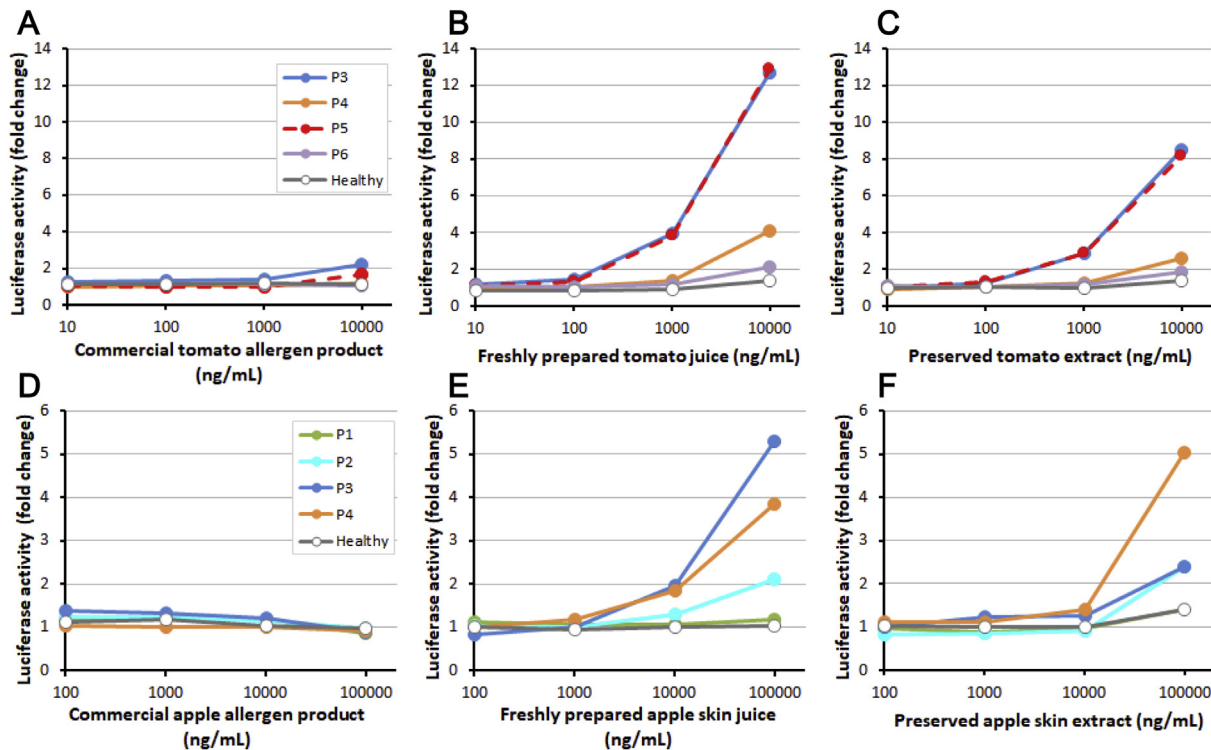


Fig. 1. EXiLE responses to tomato or apple extract. RS-ATL8 cells in the sera of patients with pollinosis were sensitized with sera from patients allergic to tomato (P3, P4, P5, and P6) or a healthy control serum and were stimulated with a commercially available tomato allergen product (A), freshly prepared tomato juice (B), or preserved tomato extract (C). Each value of luciferase activity is the average of those in duplicated wells. Results of each single experiment are shown in [Supplementary Table 2](#). Similarly, the EXiLE responses to a commercially available apple allergen product (D), freshly prepared apple skin juice (E), or preserved apple skin extract (F) in patients allergic to apple (P1, P2, P3, and P4) or a healthy control serum are shown.

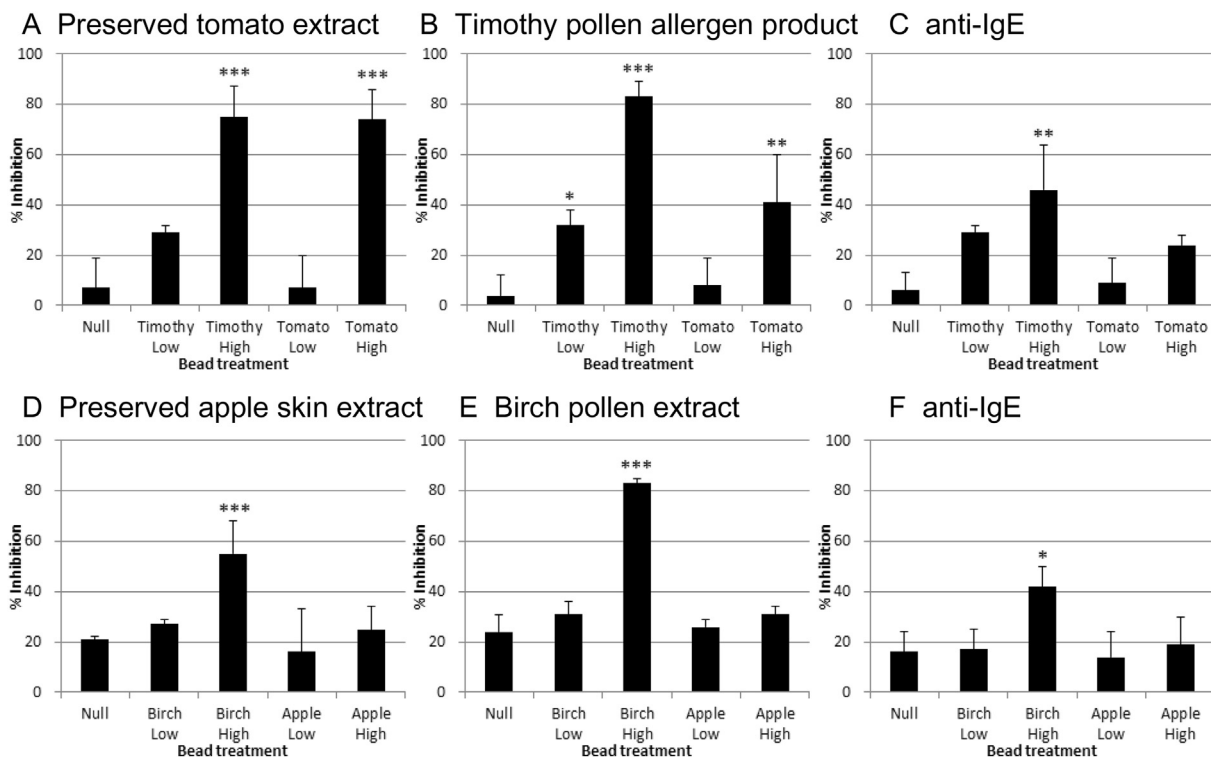


Fig. 2. Cross-reactivity between pollen and apple/tomato. Sera from patients allergic to tomato (P3, P4, and P5) were treated with a commercially available timothy pollen allergen product- or preserved tomato extract-coated beads (A–C) and were used for the sensitization of RS-ATL8 cells. Then, the EXiLE responses to preserved tomato extract (A), timothy pollen allergen product (B), and anti-IgE (C) were measured. Similarly, sera from patients allergic to apple (P2, P3, and P4) were treated with an extract from commercially available birch pollen or preserved apple skin extract (D–F); then, the EXiLE responses to preserved apple skin extract (D), birch pollen extract (E), and anti-IgE (F) were measured. The concentrations of antigens used for coating were as follows (all µg/mL): Null, 0; Timothy Low, 1; Timothy High, 1000; Birch Low, 1; Birch High, 1000; Tomato Low, 10; Tomato High, 5000; Apple Low, 30; and Apple High, 6000. Data are presented as the mean percent inhibition $\pm$ the standard deviation for three individual sera. Percent inhibition values for each patient are average of duplicated experiments. Dunnett's test was performed. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

len. Moreover, neither timothy nor tomato allergens lost their intact structure on the beads.

Next, we investigated the cross-reactivity of birch pollen and apple antigens (Fig. 2D–F). As shown in Figure 2D, birch pollen-coated beads significantly inhibited the preserved apple skin extract-induced EXiLE responses. However, the apple extract-coated beads did not significantly inhibit the responses. The same was true for the cases of birch pollen extract (Fig. 2E) and anti-IgE (Fig. 2F) stimulation. These results indicate cross-reactivity between birch and apple antigens; however, apple antigens are highly unstable on the polystyrene beads. The inhibition rate of birch pollen-coated beads was only partial for preserved apple skin extract stimulation (55%) compared with that for birch pollen extract stimulation (80%), indicating that the primary sensitization of these patients occurred with birch pollen.

Using preserved sera, preserved extracts, and a cultured mast cell line, the EXiLE method can detect cross-reactive IgE and allergen-specific IgE from several microliters of serum. The use of freshly prepared tomato or apple skin juice, which is a stimulating antigen, was also effective. Therefore, the EXiLE method can be used for the screening of potentially cross-reactive fruit and vegetable allergens in patients allergic to pollen. In addition to pollen allergen-specific IgE titers, this novel *in vitro* method may help clinicians in diagnosing PFS before performing the *in vivo* prick-prick test, which is burdensome.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.alit.2019.12.007>.

Conflict of interest

The authors have no conflict of interest to declare.

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Supplementary Methods

Pollen-associated food allergy syndrome (PFS) patient sera

Patients diagnosed with PFS at Sagamihara National Hospital were enrolled in the study. PFS was diagnosed in patients with sensitization to pollen, a convincing clinical history of oral allergy syndrome (OAS) symptoms to fresh tomato or apple, and a positive prick–prick test using fresh tomato or apple compared to 10 mg/mL histamine dihydrochloride as a positive control.¹ They were tolerant to cooked tomato or apple. Patients' written informed consent for the investigation of their serum samples was obtained. The study was approved by the institutional review board of Kanagawa Prefectural Institute of Public Health (No. 5 in 2014) and the ethics committee of Sagamihara National Hospital (No. 5 in 2014) and was performed in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki). The serum of a healthy control was purchased from Cosmo Bio Co., Ltd. (Tokyo, Japan).

Commercial allergen products

The birch pollen was purchased from Biostir, Inc. (Hyogo, Japan). The allergen products for the skin-prick test (timothy grass pollen, tomato, and apple) were purchased from Torii Pharmaceutical Co. (Tokyo, Japan).

Freshly prepared tomato juice and apple juice

A whole tomato (Momotaro) was cut into four pieces and the hull was removed.

Similarly, an apple (Fuji) was cut into eight pieces and the skin, flesh, and core were separated. The tomato fruit and apple skin each were weighed and blended separately in a chilled food processor, and the supernatants were clarified by centrifuging for 30 minutes at 15,000 revolutions per minute (rpm). The protein concentrations of the juices were determined immediately by the bicinchoninic acid (BCA) method. The juices then were used in the IgE crosslinking-induced luciferase expression (EXiLE) test.

Homemade preserved soluble extracts from whole tomato and apple skin

A tomato was quartered and stored at -80°C for more than one day. Extraction buffer was prepared by dissolving one tablet of protease inhibitor cocktail (Complete; Roche Diagnostics K.K., Tokyo, Japan) in 50 mL 20 mM phosphate buffer (pH 6.8).² A piece of the frozen tomato was weighed, an equivalent amount of extraction buffer was added, and they were homogenized in a chilled food processor. The crude extract was centrifuged at 7,500 rpm at 4°C for 60 minutes and the supernatant was clarified further by centrifuging for 30 minutes at 15,000 rpm. The purified tomato extract was separated into aliquots and stored at -80°C until use.

An apple was cut into eight pieces and the core was removed. The skin was frozen immediately in liquid nitrogen and stored at -80°C for more than one day. A piece of the frozen skin was weighed, an equivalent amount of extraction buffer was added. They were then homogenized immediately in a chilled food processor, followed by centrifugation in the same manner as described for the tomato extract. The apple skin extract was stored at -80°C until use.

Both extracts could be used for EXiLE tests as long as 10 months after preparation.

The EXiLE method

EXiLE tests were performed as described previously.^{3,4} Briefly, RS-ATL8 cells were sensitized with 100-fold diluted patient sera overnight in duplicated wells. The cells were washed three times with phosphate-buffered saline and stimulated with antigen-containing medium at 37°C for 3 hours in a CO_2 incubator. Luciferase activity was measured with ONE-GloTM reagent (Promega, Inc., Madison, WI, USA) and a Mithras LB940 luminometer (Berthold Japan K.K., Tokyo, Japan). The results are expressed as the fold increase of light units compared to the background expression after subtraction of a blank control (without cells).

Removal of antigen-specific antibodies from serum

Dynabeads M-280 Tosylactivated (Invitrogen, Rockville, MD, USA) was used. Coating with an antigen and removal of a specific antibody from patient sera were performed according to the manufacturer's protocol. Commercially available timothy pollen allergen product, preserved tomato extract, birch pollen extract, and preserved apple skin extract were used as coating antigens. The concentrations of antigens used for coating were as follows (all $\mu\text{g/mL}$): Timothy Low, 1; Timothy High, 1000; Birch Low, 1; Birch High, 1000; Tomato Low, 10; Tomato High, 5000; Apple Low, 30; and Apple High, 6000. A 1 mg amount of prepared beads or null-beads was incubated with 5 μL serum to remove specific antibodies.

Cross-reactivity assessment

RS-ATL8 cells were sensitized with 100-fold diluted bead-treated serum and stimulated with preserved tomato extract (10 $\mu\text{g/mL}$), timothy pollen allergen product (1 $\mu\text{g/mL}$), preserved apple skin extract (100 $\mu\text{g/mL}$), birch pollen extract (1 $\mu\text{g/mL}$), or goat anti-human IgE (100 ng/mL; Bethyl Laboratories, Montgomery, TX, USA). Percent inhibition was calculated as follows:

$$\% \text{ Inhibition} = (L_{\text{UT}} - L_{\text{T}}) / L_{\text{UT}} \times 100$$

where L_{UT} and L_T are luciferase activity with untreated serum and with treated serum, respectively.

Statistical analysis

For assessment of cross-reactivity, Dunnett's test was performed with the values from samples treated with null-beads as the control group.

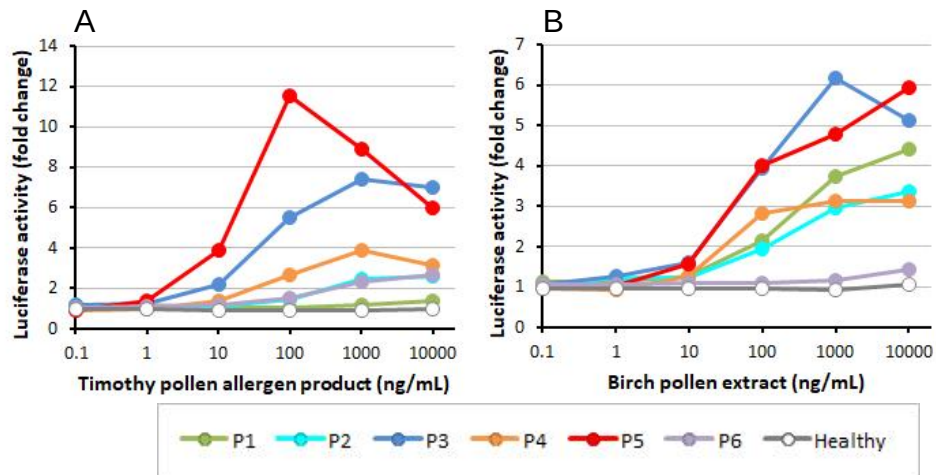
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Supplementary Fig. 1



Supplementary Fig. 1. RS-ATL8 cells were sensitized with sera from patients with pollen-associated food allergy syndrome (PFS) who were allergic to tomato or apple. The cells were then stimulated with commercially available timothy pollen allergen product (A) or birch pollen extract (B). In our preliminary experiments, the IgE crosslinking-induced luciferase expression (EXiLE) responses to these allergens with sera from healthy volunteers did not exceed 1.5-fold (data not shown). Green, Patient(P)1; cyan, P2; blue, P3; orange, P4; red, P5; purple, P6; gray, purchased healthy control.