

ORIGINAL ARTICLE

High prevalence of sensitization to gibberellin-regulated protein (peamaclein) in fruit allergies with negative immunoglobulin E reactivity to Bet v 1 homologs and profilin: Clinical pattern, causative fruits and cofactor effect of gibberellin-regulated protein allergy

Naoko INOMATA, Mami MIYAKAWA, Michiko AIHARA

Department of Environmental Immuno-Dermatology, Yokohama City University Graduate School of Medicine, Yokohama, Japan

ABSTRACT

Gibberellin-regulated protein (GRP) is a new allergen in peach allergy, with an amino acid sequence very well conserved through several botanical species. We investigated the allergenicity of GRP in fruit allergies other than peaches and identified the clinical characteristics of fruit allergy patients with GRP sensitization. One hundred consecutive Japanese patients with fruit allergies were enrolled in the present study. To identify the features of GRP sensitization, we selected patients with negative ImmunoCAP results for Bet v 1 homologs and profilin, which are marker allergens for pollen-food allergy syndrome (PFAS), or lipid transfer protein. These patients underwent specific immunoglobulin E measurements by enzyme-linked immunosorbent assay (ELISA) and skin prick tests (SPT) using purified nPru p 7. Twenty of 100 consecutive patients with fruit allergies had negative ImmunoCAP results for Bet v 1 homologs and profilin. Thirteen (65.0%) of the 20 patients had positive ELISA and/or SPT results using nPru p 7, whereas one of the 20 patients had positive ImmunoCAP results for Pru p 3. In 13 nPru p 7-sensitized patients, the causative foods were peaches (92.3%), apricots (61.5%), oranges (46.2%) and apples (30.8%). Ten patients (76.9%) had multiple causative fruits. Frequent symptoms included facial edema (92.3%) and laryngeal tightness (66.7%). In eight patients (61.5%), exercise or aspirin intake enhanced the allergic reaction onset as cofactors. The prevalence of GRP sensitization was high in Japanese fruit allergy patients except for PFAS patients. In conclusion, GRP-sensitized patients may have allergies to multiple fruits and may show peculiar characteristics such as facial swelling and cofactor dependence.

Key words: food allergy, gibberellin-regulated protein, lipid transfer protein, peamaclein, Pru p 7.

INTRODUCTION

Gibberellin-regulated protein (GRP, also known as peamaclein) is a member of the GASA gibberellin-regulated cysteine-rich protein family and an antimicrobial peptide according to classification based on biological function. GRP was registered as new peach allergen (allergen name approved by official the World Health Organization and International Union of Immunological Societies, Pru p 7) in 2013.¹ We recently identified Pru p 7 as a marker related to systemic reactions to peaches.²

By searching the Basic Local Alignment Search Tool (BLAST) algorithm, the amino acid sequence of Pru p 7, which is the only GRP homolog identified as an allergen, did not show any similarity to other well-known allergens, such as Bet

v 1 homologs, profilin and lipid transfer proteins (LTP); however, its amino acid sequence is well conserved through several botanical species. GRP may therefore be a causative allergen in allergies due to fruits regardless of the plant kingdom classification. However, the clinical relevance of GRP in allergies to fruits other than peaches is not clear. Here, we investigated the allergenicity of GRP in fruit allergy patients and identified clinical characteristics related to GRP sensitization. To identify the clinical characteristics related to GRP sensitization, we selected patients with negative immunoglobulin (Ig)E reactivity to Bet v 1 homologs and profilin (marker allergens for pollen-food allergy syndrome [PFAS]) and LTP (a marker allergen associated with systemic reactions in fruit allergies) from those fruit allergy patients enrolled in the present study.^{3,4}

Correspondence: Naoko Inomata, M.D., Ph.D., Department of Environmental, Immuno-Dermatology, Yokohama City University Graduate School of Medicine, 3-9 Fukuura, Kanazawa-ku, Kanagawa 236-0004, Japan. Email: ninomata@yokohama-cu.ac.jp
Received 7 November 2016; accepted 11 January 2017.

METHODS

Patients

One hundred consecutive patients (male : female ratio, 27:73; mean age, 31.5 years [range, 3–74]) diagnosed with fruit allergies at the Dermatology Department of Yokohama City University Hospital between January 2001 and December 2015 were enrolled in the present study (Fig. 1). Patients were diagnosed with fruit allergy if they had a convincing history of allergic reactions within 30 min of ingesting the relevant fruit and had positive results on skin prick tests (SPT) with fruits according to prick-prick method, specific immunoglobulin (Ig) E measurements for them using ImmunoCAP (Thermo Fisher Scientific, Waltham, MA, USA) and/or challenge tests with them.

In addition, we evaluated each patient's clinical history to determine the involvement of cofactors, such as non-steroidal anti-inflammatory drugs and exercise, which are associated with food-dependent, exercise-induced anaphylaxis. The time period accepted for considering a potential relationship with food-allergic reaction was 2 h before food ingestion, combined with the simultaneous intake of non-steroidal anti-inflammatory drugs and the food.⁵ Regarding exercise, a 4-h period after a food ingestion was established.⁵ Food-dependent exercise-induced anaphylaxis (FDEIA) was diagnosed based on a convincing history of fruit allergy, and/or positive results of challenge tests combined with exercise and/or aspirin.

This study was approved by our institutional review board. Written informed consent was provided by all patients.

Purification of GRP from peach (Pru p 7)

Native GRP (nPru p 7) was purified from peach pulp by ionic exchange chromatography following the protocol described in Supporting Information.^{2,6}

Measurement of nPru p 7-specific IgE by ELISA

We measured an enzyme-linked immunosorbent assay (ELISA) to measure specific IgE (sIgE) antibody to purified nPru p 7 as a representative GRP, as described in Supporting Information.⁶

Measurement of specific IgE measurements by ImmunoCAP

Serum sIgE levels (ImmunoCAP) for the relevant fruits and for recombinant allergens of Bet v 1 homologs (Bet v 1 or rPru p 1), profilin (rBet v 2 or rPru p 4) and LTP (rPru p 3) were measured. According to the information provided by the manufacturer, the cut-off value of the assay was 0.35 kUA/L as class 1.

Skin testing with nPru p 7

Eight patients gave informed consent for additional SPT with purified nPru p 7. SPT with nPru p 7 (2 µg/mL in phosphate-buffered saline) were performed and read according to a standard procedure.⁷ The skin was pricked through the allergen drop of nPru p 7 with the tip of a Prick-Lancetter (EWO CARE, Gislaved, Sweden) and the responses were read after 15 min. Histamine chloride at 10 mg/mL and vehicle (Torii Pharmaceutical, Tokyo, Japan) served as positive and negative controls, respectively. The elicited response of SPT was considered positive when the largest diameter of the wheal induced by the allergen was 3 mm or more.⁸ SPT with nPru p 7 was also carried out on five control subjects without fruit allergy.

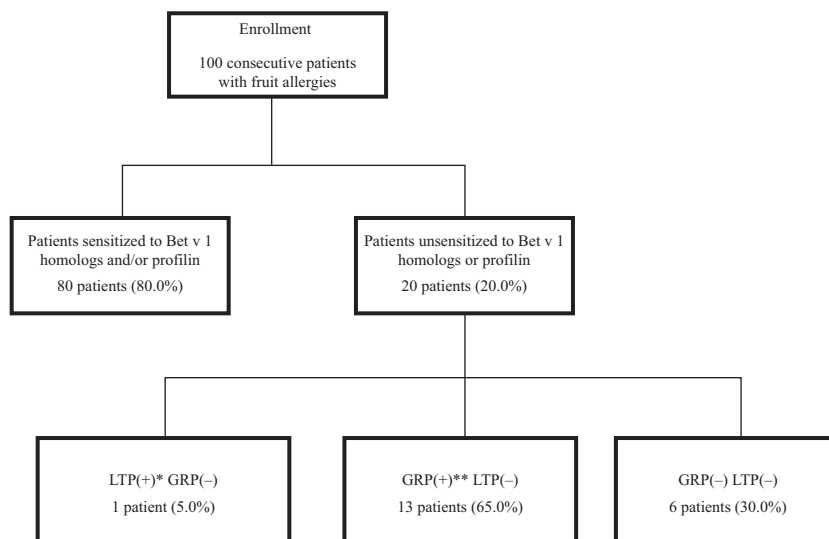


Figure 1. Patient selection flowchart. From 100 consecutive patients with fruit allergies, 20 patients had negative ImmunoCAP results for Bet v 1 homologs and profilin. Of these 20 patients, 13 patients had positive specific immunoglobulin E results by enzyme-linked immunoassay and/or skin prick test (SPT) examinations using purified nPru p 7 as a representative gibberellin-regulated protein (GRP). One patient was ImmunoCAP positive for Pru p 3 as a representative lipid transfer protein (LTP). *LTP-sensitized patients indicate patients with positive ImmunoCAP results for Pru p 3. **GRP-sensitized patients indicate patients with positive results for nPru p 7 in at least one allergic test.

RESULTS

Selection of patients without PFAS

Eighteen (80.0%) of 100 consecutive patients with fruit allergies had positive ImmunoCAP results for Bet v 1 homologs and/or profilin, indicating that they were diagnosed with PFAS (Fig. 1).

Prevalence of sensitization to LTP

In the 20 patients with negative ImmunoCAP results for Bet v 1 homologs or profilin, only one patient (5%) had positive ImmunoCAP results for rPru p 7.

Prevalence of sensitization to nPru p 7

Among the 20 fruit allergy patients with negative ImmunoCAP results for Bet v 1 homologs and profilin, 11 patients (55.0%) had positive results (range, 0.50–3.36 unit/mL; mean, 1.18 U/mL) in ELISA with nPru p 7. In addition, eight patients gave informed consent for SPT with purified nPru p 7. Seven of the eight patients including two patients (patients 3 and 6) with negative ELISA results for nPru p 7 showed positive SPT results for nPru p 7 (Table 1). Therefore, 13 of 20 patients with fruit allergies and negative IgE reactivity to Bet v 1 homologs and profilin (five males and eight females; mean age, 31.8 years) were sensitized to nPru p 7 (Fig. 1).

Causative fruits in fruit allergies with GRP sensitization

In these patients, the most frequent causative fruits belonging to the Rosaceae family were peaches (12 patients, 92.3%), followed by apricots including Japanese apricot (eight patients, 61.5%), apples (four patients, 30.8%), strawberries (four patients, 30.8%), cherries (two patients, 15.4%) and pear (one patient, 7.7%) (Table 2).

Regarding fruits in the Rutaceae family, the prevalence of sIgE positivity to oranges (six patients, 46.2%) was higher than that to lemons and grapefruits (two patients each, 15.4%). Additionally, in families other than Rosaceae and Rutaceae, causative foods were grapes (two patients, 16.7%), figs (one patient, 8.3%), melons (one patient, 8.3%), tomatoes (one patient, 8.3%) and pineapples (one patient, 8.3%). Ten patients (76.9%) had multiple causative fruits. One patient reacted to two foods, three patients to four foods, two patients to five foods, and two patients to six foods. Three patients had single causative foods: two patients developed allergic reactions after the ingestion of peaches and one patient after grapes.

Clinical symptoms in fruit allergies with GRP sensitization

In the most severe episodes experienced by the 13 patients sensitized to nPru p 7, the most frequent symptom was facial swelling ($n = 12$, 92.3%), especially the eyelids, followed by generalized urticaria ($n = 10$, 76.9%), laryngeal tightness ($n = 9$, 69.2%), nasal symptoms ($n = 8$, 61.5%), eye symptoms ($n = 7$, 53.8%), dyspnea ($n = 6$, 46.2%), oral symptoms ($n = 6$, 46.2%), loss of consciousness ($n = 4$, 30.8%), abdominal symptoms ($n = 3$, 23.1%) and hypotension ($n = 1$, 7.7%) (Table 1). Thus, 12 patients (92.3%) developed anaphylactic

reactions, and four patients (30.8%) experienced anaphylactic shock, accompanied by hypotension and loss of consciousness. Foods that caused the most severe episodes were peaches ($n = 6$, 46.2%), apricots ($n = 4$, 30.8%), strawberries ($n = 2$, 46.2%) and grapes ($n = 1$, 7.7%).

Cofactors in fruit allergies with GRP sensitization

Interestingly, exercise and the intake of aspirin were cofactors and enhanced the onset of allergic reactions in 10 patients (76.9%) and one patient (7.7%), respectively (Table 1). In two patients (patients 1 and 7), food challenge tests combined with exercise and/or aspirin showed positive results. The other eight patients did not give informed consent for combined challenge tests because during the onset of their episodes they experienced severe systemic reactions after the ingestion of causative fruits and the risks of challenge tests include anaphylaxis. The causative foods involved with episodes associated with cofactors were peaches, apricots, apples, pears, cherries, strawberries and grapes.

Other allergic diseases in fruit allergies with GRP sensitization

In terms of the patients' histories of other allergies, the prevalence of pollinosis was 92.3% (12/13), bronchial asthma 15.8% (2/13) and atopic dermatitis 15.8% (2/12).

In terms of peach allergen components, ImmunoCAP to rPru p 1, rPru p 3 and rPru p 4 was negative in all 13 patients. Additionally, almost all patients (12/13, 92.3%) were sensitized to Japanese cedar pollens. Four patients (patients 1, 10, 11 and 12) were sensitized to Betulaceae family pollens, such as alder and birch pollen, even though they had negative ImmunoCAP for Pru p 1 or Pru p 4. The prevalence of the positive rate of ImmunoCAP was 33.3% (4/12) for grass and 40% (4/10) for ragweed.

DISCUSSION

Our findings showed a high prevalence (65.0%) of sensitization to nPru p 7 as a representative GRP in Japanese fruit allergy patients without sensitization to Bet v 1 homologs and profilin. Importantly, to clarify which clinical features are related to GRP sensitization, patients without PFAS and LTP allergy were selected. However, a limitation of the present study was the lack of screening sensitization to all fruit allergen components, including thaumatin-like protein.

Gibberellin-regulated protein is the first allergen among antimicrobial peptides according to classification by biological function. GRP protects plants from pathogens and has an essential role in plant development.⁹ In addition, Pru p 7 has heat-stability and digestion-resistance because of an unusually high cysteine content (19% of total residues), similar to LTP. GRP is thus presumed to be a true food allergen that sensitizes by itself via the gastrointestinal tract and to induce severe reactions after ingestion.^{10–12}

In our patients who were sensitized to Pru p 7, the causative foods were from a wide range of plant kingdom classifications. The Rosaceae family was the most frequently involved,

Table 1. Clinical characteristics of fruit allergy patients sensitized to gibberellin-regulated protein

No. of cases	Age/sex	Allergic histories	Most severe episode	Cofactor	sIgE against Pru p 7		ImmunoCAP (U/mL)*				Pollen
					ELISA [†] U/ml	SPT [‡] mm	Pru p 1	Pru p 3	Pru p 4	Plant-derived food	
1	58/F	PO	Apricot La, Na, Ae	Ex	0.55	11.5	<0.35	<0.35	<0.35	Pe <0.35, Or <0.35	Ce 16.4 , Al <0.35, Bi 0.35 , Gr <0.35, Ra <0.35
2	27/M	PO	Peach Ae, Ur, Dy	None	0.84	nd	<0.35	<0.35	<0.35	Pe, Ap, St, Or <0.35	Ce <0.35, Al <0.35, Bi <0.35, Gr <0.35, Ra <0.35
3	52/F	PO	Apricot La, Ae, Ur	Ex	0.27	7.4	<0.35	<0.35	<0.35	Pe 1.42 , Ap <0.35, St <0.35, Or <0.35	Ce 162.0 , Al <0.35, Bi <0.35, Gr <0.35, Ra <0.35
4	41/F	PO	Peach Ey, Na, Ae, U, Co, Hy	None	1.01	nd	<0.35	<0.35	<0.35	Pe 0.57 , Ap <0.35	Ce 33.3 , Al <0.35, Gr <0.35, Ra nd
5	15/F	PO, BA, AD	Peach Ae, Ur, Dy, Co	Ex	1.95	nd	<0.35	<0.35	<0.35	Pe 3.4 , Ap <0.35, St <0.35, Or 0.6	Ce >100 , Al <0.35, Bi nd, Gr nd, Ra nd
6	24/F	PO	Peach Or, Ey, La, Na, Ae, Ur, Co	Ex	0.40	12.1	<0.35	<0.35	<0.35	Pe 0.67 , Ap <0.35, Or 1.24 , Grf 1.58 , To 0.57	Ce 95.8 , Al <0.35, Bi <0.35, Gr <0.35, Ra <0.35
7	39/M	PO	Apricot Ey, La, Na, Ae, Ur	Ex or NSAIDs	0.66	20.4	<0.35	<0.35	<0.35	Pe 0.95 , Ap <0.35, St <0.35 Or <0.35	Ce 14.5 , Al <0.35, Bi <0.35, Gr <0.35, Ra <0.35
8	32/F	None	Peach Or, Ey, Na, Ae, Ur, Dy, Ab	None	0.91	4.8	<0.35	<0.35	<0.35	Pe 0.59 , Ap <0.35, Or 0.59 , To 0.44	Ce 135.0 , Al <0.35, Bi <0.35, Gr 38.6 , Ra 3.54
9	14/M	PO	Apricot La, Ae, Ur, Dy, Ab	Ex	0.60	11.9	<0.35	<0.35	<0.35	Pe 4.5 , Ap <0.35, St <0.35, Or 2.52	Ce 77.5 , Al <0.35, Bi <0.35, Gr <0.35, Ra 0.66
10	16/M	PO, AR, BA	Peach Or, La, Ur	Ex	0.50	nd	<0.35	<0.35	<0.35	Pe <0.35, Ap <0.35, Me <0.35, To 0.44	Ce 11.3 , Al 0.44 , Bi 0.64 , Gr <0.34, Ra 1.03
11	18/F	PO, AD	Strawberry Or, Ey, La, Na, Ae, Ur	Ex	0.97	nd	<0.35	<0.35	<0.35	Pe 1.61 , Ap 1.08 , St 1.17 , Or 2.02	Ce 152.0 , Al 2.26 , Bi 2.43 , Gr 10.6 , Ra 8.10
12	50/F	PO	Strawberry La, Na, Ae, Dy, Co	Ex	3.36	nd	<0.35	<0.35	<0.35	Pe 0.87 , Ap nd, St 0.59 , Or 1.10	Ce 60.6 , Al 0.40 , Bi 0.74 , Gr 0.94 , Ra nd
13	27/F	PO	Grape Or, Ey, Na, Ae, Dy	Ex	1.62	6.8	<0.35	<0.35	<0.35	Pe 0.96 , Ap <0.35, St 0.72 , Or 0.43	Ce 14.6 , Al <0.35, Bi <0.35, Gr 0.51 , Ra 0.14

*The cut-off value of the ImmunoCAP assay was 0.35 kU/L as class 1. †Specific IgE levels >0.49 U/mL (the mean unit $\pm$ 10 SD of the negative control value) was considered positive for nPru p 7. Positivity is indicated in bold. ‡Positivity is indicated in bold. Five control subjects without fruit allergy had negative SPT results for nPru p 7. Ab, abdominal symptoms; AD, atopic dermatitis; Ae, angioedema; Al, Alder pollen; Ap, apple; AR, allergic rhinitis; BA, bronchial asthma; Bi, birch; Ce, Japanese cedar pollen; Co, loss of consciousness; Dy, dyspnea; ELISA, enzyme-linked immunosorbent assay; Ex, exercise; Ey, eye symptom; F, female; Gr, grass; Grf, grapefruit; Me, melon; Hy, hypotension; La, laryngeal tightness; M, male; Na, nasal symptom; nd, not done; NSAIDs, non-steroidal anti-inflammatory drugs; Or, orange; Pe, peach; PO, pollinosis; Ra, ragweed; SD, standard deviation; SPT, skin prick test; St, strawberry; To, tomato; Ur, urticaria.

Table 2. Distributions of causative fruits in plant kingdoms, clinical severity and involvement of cofactors in gibberellin-regulated protein allergy patients

Plant classification of the causative food															
Rosids															
Rosales order															
Rosaceae family															
Case no.	Causative food no.	Peach	Apricot	Apple	Cherry	Pear	Strawberry	Fig	Moraceae family	Vitales order		Sapindales order		Commelinids	Asterids Solanales order
										Vitaceae family	Grape	Orange	Lemon		
1	4	An [†]	An*	—	An*	—	—	Na	—	—	—	—	—	—	—
2	1	An [†]	—	—	—	—	—	—	—	—	—	—	—	—	—
3	5	Ae	An [†]	Ur	—	—	Ur	—	—	—	—	Ae	—	—	—
4	1	An-S [†]	—	—	—	—	—	—	—	—	—	—	—	—	—
5	4	An-S [†]	An*	—	An*	—	An*	—	—	—	—	—	—	—	—
6	6	An [†]	An*	—	—	—	—	—	—	—	—	—	—	—	—
7	2	An [†]	An [†]	—	—	—	—	—	—	—	—	—	—	—	—
8	6	An [†]	An	—	—	—	—	Ae/Ur	Ae/Ur	—	—	—	—	—	—
9	5	An	An [†]	An	—	An*	—	—	—	—	—	An	—	Ab	—
10	5	An [†]	An*	An*	—	—	—	—	—	—	—	Or	—	—	—
11	6	Ae	An*	An*	—	—	An [†]	—	—	—	—	Or	Or	—	Or
12	4	An	—	—	—	—	An-S [†]	—	—	—	—	—	—	—	—
13	1	—	—	—	—	—	—	—	—	Ae [†]	—	An	—	—	—
Prevalence	Multiple	12/13	8/13	4/13	2/13	1/13	4/13	2/13	15.4%	23.1%	6/13	2/13	2/13	1/13	1/13
	causative foods	92.3%	61.5%	30.8%	15.4%	7.7%	30.8%	15.4%			46.2%	15.4%	15.4%	7.7%	7.7%
	10/13														
	76.9%														

followed by the Rutaceae family and the Vitaceae family. Moreover, 10 of the 13 nPru p 7-sensitized patients (76.9%) were allergic to multiple fruits. Sequence searches using BLAST have demonstrated that proteins homologous to Pru p 7 are present in many other plants such as apples, oranges, grapes, castor beans, potatoes and soybeans. For example, Pru p 7 shares 82% amino acid sequence homology with potato GRP (snakin 1).¹ GRP is conserved in a broad range of plants because it has essential roles in growth and defense against plant pathogens. The wide distribution of GRP may cause this broad diversity of allergenic foods and cross-reactivity between them. Three of the patients had only one causative food; two for peaches and one for grapes. To date, no GRP other than Pru p 7 has been reported to act as a primary sensitizer. However, our results indicated that fruits other than peaches may also be initial sensitizers in GRP reactions.

Clinical manifestations of GRP sensitization can vary in severity, from anaphylaxis to symptoms restricted to the oropharyngeal area, skin or gastrointestinal tract. However, 12 of the 13 Pru p 7-sensitized patients (92.3%) experienced anaphylactic reactions after the ingestion of causative foods, and four patients developed anaphylactic shock, indicating that GRP may be related to systemic reactions in allergies not only to peaches but also to other fruits. Among our patients, the ingestion of Rosaceae family fruits, such as peaches and apricots, tended to induce more severe reactions than fruits from other families. The most frequent symptom was facial edema (12/13, 92.3%), especially eyelid edema, suggesting that facial edema may be a clinical predictor of allergy caused by GRP derived from peaches and other fruits.⁶

Surprisingly, in the majority (84.6%) of our GRP-sensitized patients, physical exercise and/or medication such as aspirin were cofactors for the development of allergic reactions after the ingestion of the causative foods, suggesting that these patients had FDEIA. In addition, all episodes involving cofactors were severe allergic reactions, such as anaphylaxis or anaphylactic shock. However, a limitation of the present study was a lack of results from challenge tests in eight of 10 patients. Regarding FDEIA, it is well established that in some patients, exposure to a food allergen is insufficient to provoke allergic reactions, which only occur under the influence of physical exercise and/or medication.^{13,14} The causative allergen components of FDEIA due to fruits have rarely been reported. Pascal *et al.*⁵ noted that in 40% of their LTP allergy patients, cofactors were involved in the clinical expression of allergic symptoms. These data emphasize the need to carefully investigate the presence of more than one trigger in episodes of allergic reactions, particularly in anaphylactic reactions.⁵ Thus, a low reproduction of clinical symptoms dependent on cofactors may lead to the underestimation of the prevalence of GRP allergies.

In the present study, PFAS accounted for 80% of the enrolled patients with fruit allergy, suggesting that PFAS may be a major type of fruit allergy in the Yokohama district of Japan. In addition, only one of the other 20% of patients had LTP allergy, even though LTP are considered a severity marker for fruit allergies in Europe.⁴ Indeed, Pascal *et al.*⁵ reported that the prevalence of positive sIgE to LTP was high (it was

100% in peach allergy) in Spanish patients who had negative results to all allergens related to PFAS by ImmunoCAP ISAC. However, Tuppo *et al.*¹ recently suggested that Pru p 7 may also be an important allergen in peach allergy with and without LTP sensitization in southern Europe. In their study, 14 (42.4%) and 26 (78.8%) of 33 peach allergy patients had positive reactions to purified nPru p 7 and nPru p 3 in SPT, respectively.¹ Their subjects were selected on the basis of the severity of clinical reactivity and discrepancies in their rPru p 3 results among several diagnostic tools using allergen preparations, including the ISAC103 microarray and ImmunoCAP.¹ Tuppo *et al.* revealed that GRP can be hidden in nPru p 3 preparations because of its electrophoretic mobility and chromatographic behavior, which are similar to those of Pru p 3. Even though GRP and LTP share several features such as electrophoretic mobility and digestion resistance, they have distinct primary and secondary structures and thus they are not likely to be cross-reactive with each other.¹ Further studies with large numbers of patients from various countries may clarify the relevance of GRP to fruit allergies. In addition, almost all patients with GRP sensitization were sensitized to Japanese cedar pollens. To date, a GRP homolog derived from the Japanese cedar pollen has never been reported. The relation between GRP allergies and Japanese cedar pollen sensitization is still unknown.

In conclusion, the prevalence of sensitization to GRP was high in fruit allergy patients with negative IgE reactivity to Bet v 1 homologs and profilin. Our results indicate that GRP-sensitized patients may have allergies to multiple taxonomically unrelated fruits, suggesting that GRP could be a cross-reactive allergen. These patients showed peculiar clinical characteristics such as facial swelling and cofactor dependence.

ACKNOWLEDGMENTS: We thank Tsutomu Honjou of Morinaga Biological and Chemical institute for guiding allergen purification. We also thank Thermo Fisher Scientific for performing the measurement of ImmunoCAP for allergen components. This work was supported by JSPS KAKENHI (grant no. 26461663).

CONFLICT OF INTEREST: None declared.

REFERENCES

- 1 Tuppo L, Alessandri C, Pomponi D *et al.* Peamaclein - a new peach allergenic protein: similarities, differences and misleading features compared to Pru p 3. *Clin Exp Allergy* 2013; **43**: 128–140.
- 2 Inomata N, Okazaki F, Moriyama T *et al.* Identification of peamaclein as a marker allergen related to systemic reactions in peach allergy. *Ann Allergy Asthma Immunol* 2014; **112**: 175–177.
- 3 Fernandez-Rivas M. Fruit and vegetable allergy. *Chem Immunol Allergy* 2015; **101**: 162–170.
- 4 Van Zuidmeer L, Ree R. Lipid transfer protein allergy: primary food allergy or pollen/food syndrome in some cases. *Curr Opin Allergy Clin Immunol* 2007; **7**: 269–273.
- 5 Pascal M, Muñoz-Cano R, Reina Z *et al.* Lipid transfer protein syndrome: clinical pattern, cofactor effect and profile of molecular sensitization to plant-foods and pollens. *Clin Exp Allergy* 2012; **42**: 1529–1539.

- 6 Inomata N, Miyakawa M, Aihara M. Eyelid edema as a predictive factor for sensitization to Pru p 7 in peach allergy. *J Dermatol* 2016; **43**: 900–905.
- 7 Dreborg S. Skin tests used in type I allergy testing. Position paper. *Allergy* 1989; **44**: 22–30.
- 8 Konstantinou GN, Bousquet PJ, Zuberbier T, Papadopoulos NG. The longest wheal diameter is the optimal measurement for the evaluation of skin prick tests. *Int Arch Allergy Immunol* 2010; **151**: 343–345.
- 9 Berrocal-Lobo M, Segura A, Moreno M, Lopez G, Garcia-Olmedo F, Molina A. Snakin-2, an antimicrobial peptide from potato whose gene is locally induced by wounding and responds to pathogen infection. *Plant Physiol* 2002; **128**: 951–961.
- 10 Asero R, Mistrello G, Roncarolo D, Amato S, Falagiani P. Analysis of the heat stability of lipid transfer protein from apple. *J Allergy Clin Immunol* 2003; **112**: 1009–1011.
- 11 Asero R, Mistrello G, Roncarolo D, Amato S. PT with heat-processed apple peel extract to detect LTP hypersensitivity. *Eur Ann Allergy Clin Immunol* 2006; **38**: 351–354.
- 12 Ferná'ndez-Rivas M, González-Mancebo E, Rodríguez-Pé'rez R *et al.* Clinically relevant peach allergy is related to peach lipid transfer protein, Pru p 3, in the Spanish population. *J Allergy Clin Immunol* 2003; **112**: 789–795.
- 13 Lambert GP, Boylan M, Laventure JP, Bull A, Lanspa S. Effect of aspirin and ibuprofen on GI permeability during exercise. *Int J Sports Med* 2007; **28**: 722–726.
- 14 Aihara M, Miyazawa M, Osuna H *et al.* Food-dependent exercise-induced anaphylaxis: influence of concurrent aspirin administration on skin testing and provocation. *Br J Dermatol* 2002; **146**: 466–472.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Data S1. Methods.

Supporting information

METHODS

Purification of peach peamaclein (Pru p 7)

Peamaclein (Pru p 7) was purified from peach pulp protein extract by ionic exchange chromatography as previously described.¹ Peaches (*Prunus persica*, cultivar Asama-Hakutou strain) at the commercial ripening stage were obtained from a local store. Peach pulp was homogenized with an extraction solution (2 mmol/L disodium ethylenediamine tetraacetate, 10 mmol/L sodium N,N-diethyldithiocarbamate, 3 mmol/L sodium azide, and 2% suspended solid polyvinyl polypyrrolidone at a 1:1 [w:v] ratio).² A method based on ion-exchange column chromatography for the enrichment of fruit-allergenic extracts, as described by Martinez et al, was used.³ After filtration through gauze, the extract was centrifuged at 10,000 g for 15 min at 4°C. To the supernatant, cation exchange resin (Sephacrose Fast Flow, GE Healthcare, Little Chalfont, UK) was added and mixed overnight at 4°C. The resin was collected by centrifugation, packed and washed with 20 mmol/L phosphate buffer at pH 5.0 in a column. The proteins absorbed into the resin were eluted with 0.5 mol/L NaCl in the same buffer. The eluate was monitored at 280 nm. The separated fractions were manually collected and analyzed.

The protein concentration was quantified using Qubit 2.0 fluorometer (Thermo Fisher Scientific). The purified peamaclein was sterilized by filtration through a 0.22- μ m pore diameter membrane (Millipore, Bedford, MA, USA) and frozen at -80°C .

The protein bands in SDS-PAGE using CM extract under non-reducing conditions were electroblotted onto a PVDF membrane as described above. After staining with Coomassie Brilliant Blue, the protein band corresponding to 9 kDa was excised and analyzed on a Procise 491 cLC protein sequencer (Applied Biosystems, Foster City, CA, USA), following the manufacturer's recommendations. Ten *N*-terminal amino acids were determined for that band. The obtained sequences were analyzed by the Phytozome and BLAST programs to identify the proteins in the data bases.

Determination of specific IgE by ELISA and ImmunoCAP

Specific IgE (sIgE) antibody to purified Pru p 7 was detected using ELISA as previously described.¹ The wells of a SUMILON multi-well, flat bottom 96-well plate (Sumitomo Bakelite Co., Tokyo, Japan) were coated with the purified Pru p 7 (2 $\mu\text{g/mL}$ in PBS) at 50 μL /well. The plate was sealed and left overnight at 4°C . The plate was blocked with PBS containing 1% skim milk and 0.1% Tween 20 (PBS-T) for 1 hour at room temperature, after which 50 μL of the patients' sera diluted to 20% in 1% skim

milk PBS-T was added to the wells, followed by a further incubation for 1 hour at room temperature. The plate was then washed with 1% skim milk PBS-T. A total of 50 μ l of 0.1 μ g/mL anti-human IgE-HRP conjugate (KPL, Gaithersburg, MD, USA) in 1% skim milk PBS-T was added to the wells, and the plate was incubated for 1 hour at room temperature. The plate was washed, and the colorimetric reaction was developed by adding 1-Step Ultra TMB-ELISA (Thermo Fisher Scientific) and incubated for 15 min at room temperature. The reaction was stopped by adding 2 M H₂SO₄. Absorbance at 450 nm was read with a Multiskan™ GO Microplate Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Absorbance was converted into “unit values” based on a standard curve, which was made using house dust mite, *Dermatophagoides farinae* (*Der f*) crude extract (Life Science Laboratories, Inc., NY, US) and pooled serum containing IgE specific to *Der f* 1, whose titers were calculated by ImmunoCAP (Thermo Fisher Scientific). Serum taken from three patients, which contained IgE specific to *Der f* (1686 UA/mL) was chosen as the standard. Serial dilution was performed using 1% skim milk PBS-T, starting at 20 times dilution, with subsequent doubling of the dilution factor up to 100240-times dilution. To create a curve for the conversion of absorbance values to “unit” values, the absorbance of the 20 times-diluted serum was defined as that corresponding to 84.3 units, with the absorbance of the

40-times-diluted serum as 42.15 units, that of the 640-times-diluted serum as 2.63 units, and so forth, such that the absorbance at each dilution factor corresponded to a “unit” value. Control sera from three non-atopic subjects without food allergies were used as negative controls. All tests were performed in duplicate. Specific IgE levels > 0.485 unit/mL (U/mL) (the mean unit ± 10 SD of the negative control value) were considered positive for native Pru p 7 (nPru p 7).

REFERENCES

1. Eyelid edema as a predictive factor for sensitization to Pru p 7 in peach allergy.

Inomata N, Miyakawa M, Aihara M. *J Dermatol* 2016 [Epub ahead of print]
2. Björkstén F, Halmepuro L, Hannuksela M, Lahti A. Extraction and properties of apple allergens. *Allergy* 1980; **35**, 671-677.
3. Martínez A, Fernández-Rivas M, Martínez J, Palacios R. Improvement of fruit allergenic extracts for immunoblotting experiments. *Allergy*. 1997;**52**:155-161.