

ORIGINAL ARTICLE

Allergens

Identification of gibberellin-regulated protein as a new allergen in orange allergy

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Abstract

Background: To date, three orange allergens have been reported. However, it is still unclear whether gibberellin-regulated proteins (GRPs), identified as new allergens in other fruit allergies, are also involved in orange allergy.

Objective: To investigate the allergenicity of orange GRP and to determine the clinical characteristics of patients with orange allergy who are sensitized to orange GRP.

Methods: We enrolled 14 patients (four men, 10 women, mean age: 29.6 years) who were diagnosed with orange allergy based on relevant clinical history, positive skin test, and/or positive challenge test. Orange GRP (molecular weight: 6941.6 Da) was purified by ion-exchange column chromatography. To test for orange GRP-specific IgE, we performed ELISA, basophil activation tests, and skin prick tests. Cross-reactivity of orange GRP with native peach allergen nPru p 7 and Japanese apricot nPru m 7 was analysed by ELISA inhibition assays. IgE specific for orange, grapefruit, and peach allergens rPru p 1, rPru p 3, and rPru p 4 was measured using ImmunoCAP.

Results: Twelve of the 14 patients (85.7%) were positive for orange GRP allergy in at least one test: 71.4% (10/14) were positive by ELISA, 50% (3/6) were positive in the basophil activation test, and 100% (4/4) were positive in the skin prick test. ELISA inhibition assays revealed cross-reactivity of orange GRP with both nPru p 7 and nPru m 7. The patients showed variable positivity for specific IgE against orange, grapefruit, rPru p 1, rPru p 3, and rPru p 4 (57.1%, 71.4%, 7.1%, 0%, and 21.4%, respectively). The most frequent symptoms of orange GRP allergy were facial swelling and oropharyngeal symptoms.

Conclusions and Clinical Relevance: Orange GRP may be involved in orange allergy and may be a cross-reactive allergen between citrus fruits and the *Rosaceae* family of fruits.

KEYWORDS

allergen, basophil activation test, Cit s 7, food allergy, food-dependent exercise-induced anaphylaxis, gibberellin-regulated protein, orange (*Citrus sinensis*), peach, peamaclein, Pru p 7

1 | INTRODUCTION

The sweet orange *Citrus sinensis* is consumed world-wide in its raw form as well as in derived products, such as juice, jam, and sweets in the European and American diets. However, studies on orange allergy are limited and mostly originate in Europe. In a European cross-sectional epidemiological study involving 15 countries, orange allergy accounted for 3% of all reports regarding food allergy.¹ According to a Japanese survey of food allergy, orange accounted for 2% of the causative foods in oral allergy syndrome (<http://www.med.shimane-u.ac.jp/dermatology/FAGuideLine2015.pdf>). Although orange allergy is uncommon, patients occasionally experience anaphylaxis and food-dependent exercise-induced anaphylaxis (FDEIA).²⁻⁴

To date, three proteins have been registered as orange allergens by the World Health Organization (WHO)-International Union of Immunology Societies (IUIS), namely Cit s 1 (23-24 kDa germin-like protein), Cit s 2 (13-14 kDa actin-binding protein, profilin), and Cit s 3 (9 kDa nonspecific lipid transfer protein; LTP).⁵⁻⁷ In Europe, most orange allergy patients are sensitized to Cit s 1 or Cit s 2.⁵⁻⁷ However, it is not known whether other orange allergens exist.

Gibberellin-regulated proteins (GRPs) are plant antimicrobial peptides of the snak/GASA family. GRPs have various roles in plant responses to biotic (bacteria, fungi, and nematode pathogens) and abiotic (salinity, drought, and reactive oxygen species) stressors and participate in crosstalk with plant hormones.⁸ Thus, given their essential roles, GRPs are conserved across a broad range of plants. Sequence similarity searches using the Basic Local Alignment Search Tool (BLAST) have identified GRP homologs in many other plants, including potato, peach, grape, castor bean, and soybean plants. GRPs are small basic proteins (approximately 7 kDa) that contain 12 conserved C-terminal cysteine residues which contribute to their unusual biochemical stability.⁹

This stability of GRPs is thought to maintain their allergic capacity after passing through the gastrointestinal tract and to induce severe allergic reactions in patients sensitized to them. Among GRPs, peach GRP was identified as the first allergen, Pru p 7, in 2014.¹⁰ We reported that Pru p 7 is a marker allergen related to systemic reactions in peach allergy patients.¹¹ Pru p 7 maintains its native 3D structure up to 90°C but becomes unfolded at temperatures of 100-120°C, resulting in a partial lack of its IgE-binding epitopes.⁹ Subsequently, several GRPs from fruits were identified including Pru m 7 from Japanese apricot and a potential GRP in pomegranate.^{12,13} However, it is not yet known whether orange contains GRPs and, if it does, whether the GRPs are allergenic.

Here, we identified GRP as a new allergen in orange allergy. Furthermore, we investigated its cross-reactivity with Pru p 7 and Pru m 7 and evaluated the clinical characteristics of patients with sensitization to orange GRP.

Note: The partial amino acid sequence of orange GRP and data characterizing its biochemical, clinical, and immunological features have been submitted to the WHO-IUIS Subcommittee for Allergen

Nomenclature, which has assigned the designation of Cit s 7 for the new orange allergen.

2 | METHODS

2.1 | Patients

We enrolled 14 consecutive patients diagnosed with orange allergy at the Dermatology Department of Yokohama City University Hospital in Yokohama between January 2002 and December 2012. A diagnosis of orange allergy was made if the patient had a history of allergic reactions such as skin reactions (including weals, flushing, and angioedema), respiratory symptoms, gastrointestinal symptoms, loss of consciousness, and hypotension, within 2 h of orange ingestion and had positive results on orange skin prick tests (SPTs) and/or challenge tests with orange. Open food challenges using fresh orange were performed as previously described, except in patients with a recent history (within 1 year) of anaphylaxis after orange ingestion and a positive SPT to orange.^{14,15}

Patient clinical histories were evaluated to determine the involvement of cofactors that contributed to FDEIA. The time period accepted for a potential relationship with a food-allergic reaction was 4 h after food ingestion, in combination with exercise.¹⁶ FDEIA was diagnosed based on a clinical history of orange allergy and/or positive results of an exercise challenge combined with orange ingestion.

This study (B1001111003) was approved by our institutional review board. Written informed consent was provided by all patients.

2.2 | Preparation of orange crude extract

Oranges (*Citrus sinensis*, Valencia cultivar) were obtained at a commercially unripe stage from a local grocery store. After peeling and removing seeds, the whole orange was homogenized in a blender at a 1:1 (w/v) ratio in extraction solution (2 mM EDTA, 10 mM sodium *N,N*-diethyldithiocarbamate, 3 mM sodium azide, and a 2% suspension of solid polyvinylpyrrolidone).¹⁷ The extract was then filtered through gauze and centrifuged at 10 000 *g* for 15 min at 4°C. The supernatants were collected and designated the orange crude extract.

2.3 | Purification of orange GRP

GRP was purified from the orange crude extract by ion-exchange chromatography and membrane filtration, as described in the Appendix S1. The protein concentration was estimated based on the molar extinction coefficient at 280 nm (6710 M/cm). Protein purity was assessed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, and *N*-terminal amino acid sequencing as described in the Appendix S1.

2.4 | Purification of Pru p 7 and Pru m 7

Native peach GRP (nPru p 7) and Japanese apricot GRP (nPru m 7) were purified from peach pulp by ion-exchange chromatography as previously described (Appendix S1).^{12,18}

2.5 | Synthesis of polyclonal antibodies specific to Pru p 7 and Pru p 3 peptides

On the basis of the amino acid sequences of Pru p 7 and Pru p 3 (UniProt Knowledgebase accession nos. P86888 and P81402), individual peptides were synthesized by solid-phase peptide synthesis using Fmoc chemistry to show the basic synthesis steps as described previously.¹⁹ The purity of peptides was <99.23% as determined by high-performance liquid chromatography. To ensure that peptides induced an immune response and produced antibodies, ECP-CYRDLKNSKGN originating from GRP and CCNGCRNVNNLART originating from LTP were conjugated to keyhole limpet haemocyanin as a carrier protein. Three rabbits (Nippon SLC Co., Shizuoka, Japan) were immunized by four subcutaneous injections of 25 µg of individual bovine serum albumin-conjugated peptides emulsified in Freund's complete adjuvant, 20 µg of Freund's incomplete adjuvant, and 10 µg of 10 mM phosphate-buffered saline (PBS) (pH 7.4) containing 0.15 M NaCl at 2-week intervals. On the 14th day after the final injection, antiserum was collected from rabbits.

2.6 | SDS-PAGE

Orange crude extract (10 mg per lane) or purified orange GRP (100 ng per lane) was subjected to SDS-PAGE (12%, w/v) under reducing conditions using the electrophoresis system Bolt™ (Thermo Fisher Scientific, Waltham, MA, USA) according to the method of Laemmli, following the instructions supplied by the manufacturer.²⁰ For SDS-PAGE under reducing conditions, the samples were mixed with 0.1 M Tris (pH 6.8) containing 4% (w/v) SDS, 20% (w/v) glycerol, 10% (w/v) 2-mercaptoethanol, and 0.02% (w/v) bromophenol blue. The samples were denatured by heating at 70°C for 10 min. To ensure correct protein separation and visualization, proteins on the gel were stained with Coomassie Brilliant Blue R-350 (Wako Pure Chemical Industries, Osaka, Japan) and Silver Staining II kit (Wako) following the instructions provided by the manufacturer.

2.7 | Immunoblot analysis

To demonstrate the lack of contamination of orange LTP in the purified orange GRP, we performed immunoblotting using polyclonal antibodies specific to Pru p 7 and Pru p 3 peptides. The orange crude extract and purified orange allergen were subjected to SDS-PAGE (12% (w/v), 10 ng per lane) under reducing conditions using the electrophoresis system Bolt™ (Thermo Fisher Scientific) according to the method of Laemmli, following the instructions provided by the manufacturer.²⁰ Proteins separated by SDS-PAGE under reducing conditions were transferred onto an Immobilon-P polyvinylidene difluoride

membrane (Merck Millipore, Bedford, MA, USA) by wet transfer blotting methods. The membrane was incubated in 10 mM PBS (pH 7.5) containing 0.1% Tween 20 and 5% skim milk for blocking.

The membrane was incubated for 1 h at room temperature with allergen-specific antibodies in PBS/0.05% Tween 20. Bound primary antibodies were detected by horseradish peroxidase-conjugated goat anti-mouse IgG (Promega, Tokyo, Japan) and an ECL Western Blotting Detection Kit (GE Healthcare, Little Chalfont, UK), following the manufacturer's instructions. The obtained chemiluminescence signals were detected on X-ray films (Hyperfilm MP, GE Healthcare).

2.8 | Measurement of IgE specific for native orange GRP and nPru p 7 by ELISA

Specific IgE (sIgE) antibodies against purified orange GRP or nPru p 7 in patient sera were quantified by ELISA as described in the Appendix S1.¹⁰ Sera were considered positive if sIgE levels were >2.32 U/mL or >0.485 U/mL for orange GRP or nPru p 7, respectively.

2.9 | ELISA inhibition assay

To investigate cross-reactivity between sIgE for orange GRP and Pru p 7, we performed ELISA inhibition assays using individual sera from 10 patients (Patients 1-10) who had positive ELISA results for orange GRP. In addition, cross-reactivity of orange GRP with nPru p 7 and nPru m 7 was confirmed by ELISA inhibition assays with pooled serum from these 10 patients.

In brief, one of three GRPs was coated in microtitre wells as the solid phase at 2 µg/mL. An individual or pool of patient sera was diluted 1:10 and preincubated with 1, 0.1, and 0.01 µg/mL of the competing allergen overnight at 4°C. Results were evaluated by determining the ratio of the levels of sIgE for one GRP detected after preincubation with another GRP to that without preincubation. The remainder of the assay was performed using the ELISA method described in the Appendix S1. We also performed control experiments using bovine serum albumin as a nonhomologous protein. All assays were performed in duplicate, and the results are the means of these experiments.

2.10 | Specific IgE measurements by ImmunoCAP

Serum IgE levels specific for orange, grapefruit, peach, and the recombinant allergens rPru p 1, rPru p 3, rPru p 4, rBet v 1, and rBet v 2 were measured by ImmunoCAP assays (Thermo Fisher Scientific). According to the information provided by the manufacturer, the cut-off value of the assay was 0.35 kUA/L (class 1).

2.11 | Basophil activation test (BAT)

Six patients provided additional informed consent for BATs. A commercial kit (AllergenCity Kit; Beckman Coulter, Brea, CA, USA) was used for quantification of basophil CD203c expression by flow cytometry, as described previously.²¹ Briefly, heparinized whole

TABLE 1 Clinical characteristics of patients with orange allergy

No. of case	Age/sex	Orange allergy		Other fruit allergy	Other allergy	Serum total IgE (IU/mL)
		Symptom	Cofactor			
1	16/M	Or, Ur	None	Peach, apple ^a	PO, AR, BA	27
2	31/F	Ae, Na	None	Peach, apple, grape ^a , lemon, grapefruit, strawberry, kiwi	PO	85
3	50/F	Ae, Or, Ey, Ly, Dy, Ab	None	Peach ^a , apple, strawberry	PO	460
4	40/F	Ae	Cycling, take bathing	Kiwi ^a , melon, banana	AD, PO	82
5	18/F	Ae	None	Peach ^a , Japanese apricot, apple, strawberry lemon, grapefruit	PO, AD	621
6	8/M	Or, Na	None	Melon (orange ^a)	PO, AD	161
7	15/M	Ae, Ur, Dy	Running	Peach, Japanese apricot, apple, cherry, grapefruit (orange ^a)	PO, AR, BA	829
8	46/F	Or	None	Peach ^a , apple, grapefruit, corn	PO	157
9	27/F	Ae, Ey, Na	Jogging	Peach ^a , grape, cherry	PO	32
10	52/F	Ae	None	Peach ^a , Japanese apricot, apple, strawberry, cherry, tomato, almond	PO	243
11	14/M	Ur, Dy	Walking	Peach, Japanese apricot ^a , apple, pear	PO	281
12	24/F	Ae, Or, Ey, Na	None	Peach ^a , Japanese apricot, lemon, grape	PO	838
13	27/F	Or, Ab	None	Peach ^a , grape, strawberry, persimmon, watermelon, cherry, banana, kiwi, soybean	PO, AR	47
14	47/F	Ae, Or	None	Peach ^a , apple, cherry, watermelon	PO	93

Ab, abdominal symptoms; AD, atopic dermatitis; Ae, angioedema; AR, allergic rhinitis; BA, bronchial asthma; Dy, dyspnoea; Ey, eye symptoms; La, laryngeal tightness; Na, nasal symptom; ND, not done; PO, pollinosis; Or, oral symptoms; Ur, urticaria.

^aThe causative food of the first episode is indicated.

blood from six patients with orange allergy or four nonallergic subjects (control) was incubated for 15 min with orange GRP at the indicated concentrations (0.01, 0.1, or 1 ng/mL) together with phycoerythrin-cyanin 7-conjugated anti-CD3, fluorescein isothiocyanate-conjugated antichemoattractant receptor-homologous molecule on Th2 cells (CRTH2), and phycoerythrin-conjugated anti-CD203c antibodies. Anti-IgE antibody (4 µg/mL) or PBS was used as positive or negative controls, respectively, and was added in place of orange GRP. The cells were then analysed on a flow cytometer. Basophils were detected based on forward and side scatter characteristics, negative CD3 expression, and positive CRTH2 expression. Up-regulation of CD203c on basophils was determined using a threshold defined by the fluorescence of unstimulated cells (negative control) and was expressed as the percentage of CD203c^{high} cells in the sample. At least 500 basophils were analysed per sample. A sample was considered positive for activation if >10% basophils were CD203c^{high} and CD203c expression was induced to a level greater than that of the negative control.

2.12 | Skin testing with orange GRP

Four patients with orange allergy provided informed consent for additional SPTs with purified orange GRP (2 µg/mL in

PBS), which were performed according to a standard procedure.²² The skin was pricked with a drop of allergen from the tip of a Prick-Lancetter (EWO CARE AB, Sweden), and the responses were read after 15 min. Histamine chloride at 10 mg/mL and vehicle (Torii Pharmaceutical Co, Tokyo, Japan) served as positive and negative controls, respectively. The elicited response was considered positive if the largest allergen-induced weal diameter was ≥3 mm.²³ SPT with orange GRP was also performed on four control subjects without orange allergy.

3 | RESULTS

3.1 | Demographic data of study subjects

We enrolled 14 patients (four men, 10 women) with orange allergy in this study (Table 1). Patients had a mean age of 29.6 years (range: 8–52 years) and had allergies elicited by fresh orange and fresh orange juice. Patient histories were notable for other food allergies in 100.0% (14/14), pollinosis in 100.0% (14/14), allergic rhinitis in 21.4% (3/14), bronchial asthma in 14.3% (2/14), and atopic dermatitis in 21.4% (3/14).

Source	Accession No.	Amino acid sequence			
		1	10	20	30 40
Orange	Genpept *XP_006472264	<u>G</u> SDFCD <u>S</u> KCAVR <u>C</u> SKAGREDRLKYCGICCDKCHCVPST			
Potato	Uniprot KB Q948Z4	-- S -----	KL -----	LA -----	EE - K -----
Peach	Uniprot KB P86888	-- S -----	G -----	YQE -----	E -----
Japanese apricot	Genpept *XP_016648029.1	-- S -----	G -----	YQE -----	E -----
Grape	Uniprot KB E0CP56	-- S -----	A -----	MKD -----	EE - K -----
Soybean	Uniprot KB C6SY10	-- P -----	Q - A - -	YVED - -	RF - - E - - N - -
		41	50	60	Identity (%)
		YGHKDECPCYRDLKNSKGKPKCP			-
		-- N - H - - - - -	K - - - - -	S - - -	90
		-- N - - - - -	- - - - -	N - - - -	87
		-- N - - - - -	- - - - -	N - - - -	87
		-- N - H - - - - -	K - K - - - -	Q - - - -	81
		-- N - - - - -	- - - - -	M - - - -	81

FIGURE 1 Alignment of the amino acid sequences of orange gibberellin-regulated protein (GRP) and its homologs from other plants. The 18 amino acid N-terminal sequence (underlined) was confirmed by sequencing of the isolated allergen. The 12 cysteine residues are highlighted in grey, conserved residues are indicated by dashes, and the percentage sequence identity to orange GRP is indicated on the right

3.2 | Identification of GRP as a new orange allergen

To determine whether orange contains a GRP homologous to those described for other fruits, we separated the orange crude extract by SDS-PAGE under reducing conditions and sequenced an 8-kDa protein band. An N-terminal sequence, GSDFCDKCAVRCSKAGR, corresponded to a predicted orange GRP peptide (accession number: XP_006472264.1) registered in the GenPept database. Similarity analysis revealed that orange GRP XP_006472264.1 shared 87.3% amino acid identity with Pru p 7 (accession number: P86888) registered in UniProt KB (Figure 1). On the basis of homology searches, orange GRP was most similar to potato GRP (also known as Snakin-1) (90% identity), followed by Pru p 7 (87%), Pru m 7 (87%), grape GRP (81%), and soybean GRP (81%).

3.3 | IgE-immunoblotting with orange GRP

SDS-PAGE of the orange crude extract under reducing conditions showed multiple protein bands with molecular weights ranging from approximately 5 to 90 kDa. SDS-PAGE of purified orange GRP revealed a single band with a molecular weight of 8 kDa (Figure 2A).

3.4 | Analysis of the purity of orange GRP by immunoblotting using anti-Pru p 3 and Pru p 7 peptide antibodies

Rabbit anti-Pru p 7 peptide antibodies bound to a protein with a molecular weight of 8 kDa in the orange crude extract and the purified orange GRP, but not to a protein with a molecular weight of

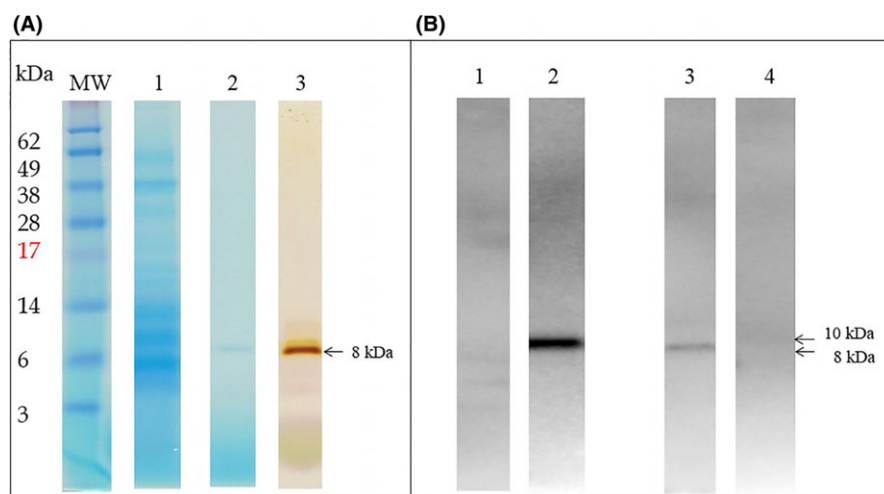


FIGURE 2 Purity of orange gibberellin-regulated protein (GRP) and reactivity of polyclonal antibodies to Pru p 7 and Pru p 3 peptides. (A) Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of orange extract, 10 µg/lane (lane 1) and the purified orange GRP, 10 ng/lane, stained by the Coomassie blue staining method (lane 2) and silver staining method (lane 3). (B) Separation of proteins in orange extract, 10 µg/lane (lanes 1 and 2) and the purified orange GRP, 10 ng/lane (lanes 3 and 4). Proteins were electrotransferred to polyvinylidene difluoride membranes and incubated with anti-Pru p 7 peptide antibodies (lanes 1 and 3) or anti-Pru p 3 peptide Ab (lanes 2 and 4)

10 kDa in any of the extracts (Figure 2B). Rabbit anti-Pru p 3 peptide antibodies bound to the protein with a molecular weight of 10 kDa in the orange crude extract, but not to the protein with a molecular weight of 8 kDa in the purified orange GRP. These results indicated no contamination of orange LTP in the purified orange GRP.

3.5 | Detection of IgE specific for orange GRP and Pru p 7 by ELISA

We found that sera from 10 of the 14 patients (71.4%) with orange allergy were positive for orange GRP-specific IgE (mean: 10.6 U/mL, range: 2.33–27.2 U/mL) (Table 2). Of these 14 patients, seven were also positive for nPru p 7-sIgE (0.84 U/mL, range: 0.50–1.27 U/mL). Thus, levels of orange GRP-sIgE were higher than nPru p 7-sIgE levels in these patients.

3.6 | Basophil activation tests

Orange GRP induced CD203c up-regulation in a concentration-dependent manner in three of the six patients (50%) (Patient 10 (white square), Patient 11 (black square), and Patient 12 (white triangle)) and none of the nonallergic subjects (Figure 2, Table 2). The rate of CD203c up-regulation induced by orange GRP was increased by 80% in Patient 11 (white square) compared with Patient 10 (white square) and Patient 12 (white triangle). Regarding the mean rate of CD203c up-regulation after incubation with 1 ng/mL orange GRP, 17.9% (2.2%–80.0%) of basophils from the patients were CD203c^{high} compared with 2.7% (1.4%–4.0%) of basophils from the control subjects (Figure 3).

3.7 | Skin testing

In the SPT, all four patients tested were positive for orange GRP. Of these, two patients were negative for orange GRP-sIgE by ELISA (Table 2). The mean weal diameter induced by orange GRP was 8.3 mm, with a range of 5.2 to 14.2 mm (Table 2). The four control subjects had negative SPTs with orange GRP.

3.8 | ELISA inhibition assays

In individual-serum inhibition experiments, each allergen completely or partially cross-inhibited specific IgE binding in a concentration-dependent manner (Figure 4). The inhibition rate of 1 µg/mL orange GRP to nPru p 7-sIgE binding (white dots) ranged from 10% to 100%, whereas the inhibition rate of 1 µg/mL nPru p 7 against orange GRP-IgE binding (black dots) ranged from 11.4% to 100%. IgE binding to nPru p 7 was completely inhibited by orange GRP in Patients 2, 4, 6, and 10. For orange GRP on the solid phase, IgE binding was completely inhibited by nPru p 7 in Patient 10. In the sera of the other 9 patients, the inhibition was <72.1%. Comparisons between the inhibition rates of individual sera showed that in Patients 2, 7, 8, 9, and 10, each allergen cross-inhibited specific IgE

binding to a similar extent. In Patients 4, 5, and 6, the inhibition rate of orange GRP to nPru p 7-sIgE binding was higher than that of nPru p 7 to orange GRP-sIgE binding. In contrast, in Patients 1 and 3, Pru p 7 was a stronger inhibitor than orange GRP.

Next, ELISA inhibition experiments using three GRPs were carried out with pooled serum. IgE binding to orange GRP was inhibited by nPru p 7 and nPru m 7 with a maximum inhibition rate of 40.2% and 44.7%, respectively (Figure 5). In the case of nPru p 7 on the solid phase, IgE binding was inhibited by orange GRP, reaching a maximum of 36.7%, which was less than that for Pru m 7 on the solid phase (55.7%). These results indicated that orange GRP may be more likely be cross-reactive to Pru m 7 than Pru p 7.

3.9 | Clinical features of patients with orange allergy with GRP sensitization

Twelve of the 14 patients (85.7%) were allergic to orange GRP based on positive results in at least one orange GRP assays (Table 2).

Three of these patients had developed anaphylactic reactions, but none had experienced anaphylactic shock. Interestingly, light exercise, such as jogging, walking, and cycling, was cofactors for four patients (three self-reported and one by challenge testing). In three patients, challenge with fresh orange induced nasal symptoms, oropharyngeal symptoms, facial angioedema, and generalized urticaria.

Patients with orange GRP allergy had histories of facial angioedema, especially the eyelids (8/12, 66.7%), oropharyngeal symptoms (5/12, 41.7%), nasal obstruction (4/12, 33.3%), urticaria (3/12, 25.0%), eye symptoms (3/12, 25.0%), dyspnoea (3/12, 25.0%), laryngeal tightness (1/12, 8.3%), and abdominal symptoms (1/12, 8.3%).

Seven (58.3%), eight (66.7%), and nine (75%) of the 12 patients had positive ImmunoCAP results for orange, grapefruit, and peach allergens, respectively. Of the patients with orange GRP allergy, two had positive ImmunoCAP results to rPru p 1, three to rPru p 4, and none to rPru p 3 (Table 2). In terms of pollen sensitization, ImmunoCAP results showed that all patients tested positive for Japanese cedar pollen allergy, and six were allergic to grass pollen (50.0%). Only one patient showed positive ImmunoCAP results to the birch pollen allergens rBet v 1 and rBet v 2. The mean total IgE concentration for the 14 patients was 318 IU/mL (range: 27–838 IU/mL; reference range for our hospital laboratory: 1–300 IU/mL) (Table 1).

4 | DISCUSSION

In the present study, we identified GRP as a new orange allergen. Twelve of 14 patients (85.7%) with confirmed orange allergy were sensitized to orange GRP based on positive results in at least one of three assays (ELISA, BAT, or SPT). In addition, ELISA inhibition assays using sera from orange allergy patients showed cross-reactivity of orange GRP with Pru p 7 and Pru m 7. These results indicated

TABLE 2 Diagnostic profiles of patients with orange allergy

No. of case	Serum-specific IgE (kUA/mL, class)										BAT (%) ^c	SPT (mm) ^d		Challenge	
	Orange					Peach						Orange GRP	Orange		
	Orange	Orange GRP ^a	Grapefruit	Peach	Pru p 1	Pru p 3	Pru p 4	Pru p 7 ^b	Grass pollen	Japanese cedar pollen					Birch pollen
											Bet v 1	Bet v 2	Orange GRP	Orange	Orange
1	0.10 (0)	4.93	<0.35 (0)	<0.34 (0)	<0.35 (0)	<0.35 (0)	0.53 (1)	0.50	<0.35 (0)	11.3 (3)	<0.35 (0)	0.59 (1)	ND	6.0	ND
2	0.19 (0)	2.33	<0.35 (0)	1.04 (2)	<0.35 (0)	<0.35 (0)	<0.35 (0)	0.40	14.6 (3)	23.7 (6)	<0.35 (0)	<0.35 (0)	ND	5.8	Ae, Na
3	1.10 (2)	3.37	0.71 (2)	0.87 (2)	<0.35 (0)	<0.35 (0)	<0.35 (0)	0.40	0.94 (2)	606 (6)	<0.35 (0)	<0.35 (0)	ND	3.0	Or
4	<0.10 (0)	27.2	0.446 (1)	<0.1 (0)	<0.35 (0)	<0.35 (0)	<0.35 (0)	1.08	<0.35 (0)	11.5 (3)	<0.35 (0)	<0.35 (0)	ND	4.6	ND
5	2.02 (2)	23.1	3.65 (3)	2.09 (2)	<0.35 (0)	<0.35 (0)	<0.35 (0)	0.96	10.6 (3)	238 (6)	<0.35 (0)	<0.35 (0)	ND	9.7	ND
6	<0.35 (0)	11.9	0.743 (2)	<0.34 (0)	<0.35 (0)	<0.35 (0)	0.904 (2)	0.94	0.53 (1)	63.5 (5)	ND	ND	ND	3.7	ND
7	0.51 (1)	2.95	<0.35 (0)	1.42 (2)	32.9 (3)	<0.35 (0)	<0.35 (0)	0.33	<0.35 (0)	158 (6)	66.2 (5)	<0.35 (0)	ND	12.4	Ur
8	1.95 (2)	5.49	2.56 (2)	2.17 (2)	9.45 (3)	<0.35 (0)	0.949 (2)	0.39	17.7 (3)	45.3 (4)	ND	ND	2.5	10.6	ND
9	0.72 (1)	2.78	0.54 (1)	0.96 (2)	<0.35 (0)	<0.35 (0)	<0.35 (0)	1.27	0.51 (1)	20.5 (4)	<0.35 (0)	<0.35 (0)	5.9	5.1	7.4
10	<0.35 (0)	21.8	<0.35 (0)	1.42 (2)	<0.35 (0)	<0.35 (0)	<0.35 (0)	0.27	<0.35 (0)	162 (6)	ND	ND	11.1	6.3	ND
11	4.50 (3)	1.63	2.06 (2)	4.50 (3)	<0.35 (0)	<0.35 (0)	<0.35 (0)	0.60	<0.35 (0)	77.5 (6)	<0.35 (0)	<0.35 (0)	80.1	7.2	14.2
12	1.96 (2)	0.72	0.71 (1)	1.36 (2)	<0.35 (0)	<0.35 (0)	<0.35 (0)	0.40	<0.35 (0)	14.5 (3)	<0.35 (0)	<0.35 (0)	13.2	3.9	5.2
13	<0.35 (0)	2.29	3.65 (3)	<0.10 (0)	<0.35 (0)	<0.35 (0)	<0.35 (0)	0.54	5.93 (3)	12.8 (6)	<0.35 (0)	<0.35 (0)	6.3	8.2	ND
14	0.743 (2)	1.24	0.714 (2)	1.98 (2)	<0.35 (0)	<0.35 (0)	<0.35 (0)	0.20	<0.35 (0)	28.2 (5)	<0.35 (0)	<0.35 (0)	ND	11.6	ND

BAT, basophil activation test; GRP, gibberellin-regulated protein; ND, not done; SPT, skin prick test.
^aSpecific IgE levels> 2.32 unit/mL (U/mL) were considered positive for orange GRP. Positivity was indicated in bold.
^bSpecific IgE levels> 0.49 unit/mL (U/mL) were considered positive for nPru p 7. Positivity was indicated in bold.
^cPercentage of basophil CD203c expression by orange in a basophil activation test.
^dThe largest diameter of the weal induced by the allergen.

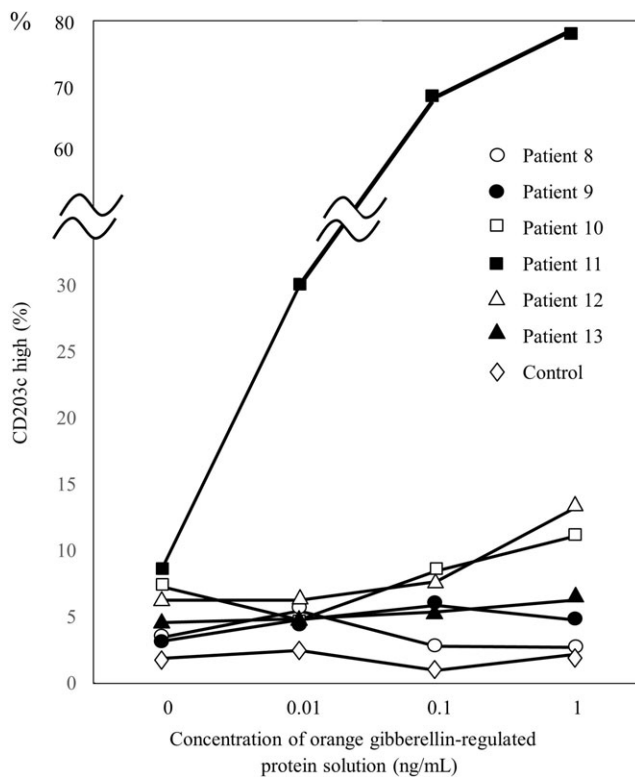


FIGURE 3 Basophil activation test with orange gibberellin-regulated protein (GRP). Flow cytometry of CD203c expression on basophils (CD3-negative, CRTH2-positive) from six patients with orange allergy and four nonallergic subjects (shown as the mean) after incubation with the indicated concentrations of purified orange GRP. The percentage of CD203c^{high} cells after incubation with anti-IgE as a positive control was 68.1%, 36.6%, 47.2%, 83.6%, 68.6%, 12.9%, and 73.6% for patients 8–13 and control subjects, respectively

that orange GRP induces allergic reactions that are cross-reactive to GRPs from the *Rosaceae* family of fruits.

The inhibition assays demonstrated that inhibition rates might be related to the clinical severity of allergy. In individual-serum inhibition experiments, five patients who had similar inhibition rates with orange GRP and nPru p 7 (Patients 2, 7, 8, 9, and 10) had similar severities of allergic reactions after ingesting either orange or peach. However, of 3 patients (Patients 4, 5, and 6) demonstrating a lower inhibition rate with an inhibitor of nPru p 7, two patients (Patients 4 and 6) had never experienced allergic symptoms after eating peach. In addition, ELISA inhibition is a widely accepted method for differentiating whether an allergen is a primary sensitizer.^{24,25} In the current study, for patients with positive ELISA results for orange GRP, the causative food of the first allergic episode was peach in five patients, orange in two patients, and apple, grape, and kiwi in one patient each. Indeed, in four (Patients 3, 8, 9, and 10) of five patients (Patients 3, 5, 8, 9, and 10) where the first episode was caused by peach, nPru p 7 more strongly inhibited IgE-binding to orange GRP compared with orange GRP in the case of nPru p7 on the solid phase. One of three patients in whom orange GRP was a stronger

inhibitor had a first episode caused by orange. Thus, the inhibition rates might correlate with the primary sensitizer.

Our ELISA inhibition experiments showed that orange GRP cross-reacted with Pru p 7 and Pru m 7. Cross-reactivity of GRP-specific IgE antibodies was first shown between the *Rosaceae* family-derived GRPs, Pru p 7, and Pru m7 GRPs.¹² The present study provides the first evidence of cross-reactivity between GRPs from fruits of different families, namely the *Rosaceae* and the *Rutaceae* family. Interestingly, in the case of orange GRP on the solid phase, IgE binding was more strongly inhibited by Pru m 7 than Pru p 7, even though Pru m 7 has identical amino acid sequences with Pru p 7. We found a similar phenomenon in our previous study of Japanese apricot allergy.¹² In the ELISA inhibition experiments using individual sera from 4 patients, Pru m 7 cross-reacted to Pru p 7 with higher inhibition rates compared with Pru p 7. We did not determine the reasons for the different inhibition rates of IgE-binding to orange GRP by Pru p 7 and Pru m 7. However, these might include a conformational change in GRPs during the preparation process and the glycosylation of proteins. This might explain why differences in allergen conformation, especially the epitopes, possibly caused by modifications such as protein glycosylation, could occur even when the amino acid sequences are similar between different allergens.²⁶ Glycosylation is a common event in protein production. The conformation or proper exposure of peptidic epitopes in glycoproteins is also frequently modulated by glycosylation mediated by intramolecular carbohydrate-protein interactions. In addition, a recent French study showed that a protein with a molecular weight of 14 kDa from cypress pollen was allergenic and cross-reactive with fruit- and vegetable-originated GRPs, as determined with an immunoblotting inhibition assay.²⁷

To date, searches of all databases available in the U.S. National Library of Medicine have returned little information regarding GRPs originating from pollens, including birch, alder, and grass. However, in a previous report, patients with orange allergy frequently had pollinosis caused by grass, olive, or birch pollen (Bet v 2), suggesting these pollens might be primary sensitizers and might cross-react with orange.^{7,28,29} The main allergic symptoms of orange allergy occur in the oral cavity. In addition, allergies caused by the *Rosaceae* family of fruits, which includes fruits such as apple and peach, are frequently associated with birch pollinosis in Central and Northern Europe and with grass pollen allergy in Central Spain.³⁰ The main cross-reactive allergens involved in birch pollinosis are Bet v 1 and profilin, and in grass pollinosis, it is profilin. In the current study, patients suffered from frequent pollinosis and other fruit allergies, especially those caused by the *Rosaceae* family of fruits. Therefore, we investigated whether fruit allergies in the patients of this study were influenced by Bet v 1 homologs and/or profilins. Although ImmunoCAP results showed that six patients (50.0%) were allergic to grass pollen, only one patient had positive ImmunoCAP results to rBet v 1 and rBet v 2, representative Bet v 1 homologs and/or profilins. These results indicated that orange allergy in our patients is not caused by sensitization to Bet v 1 homologs and/or profilins.

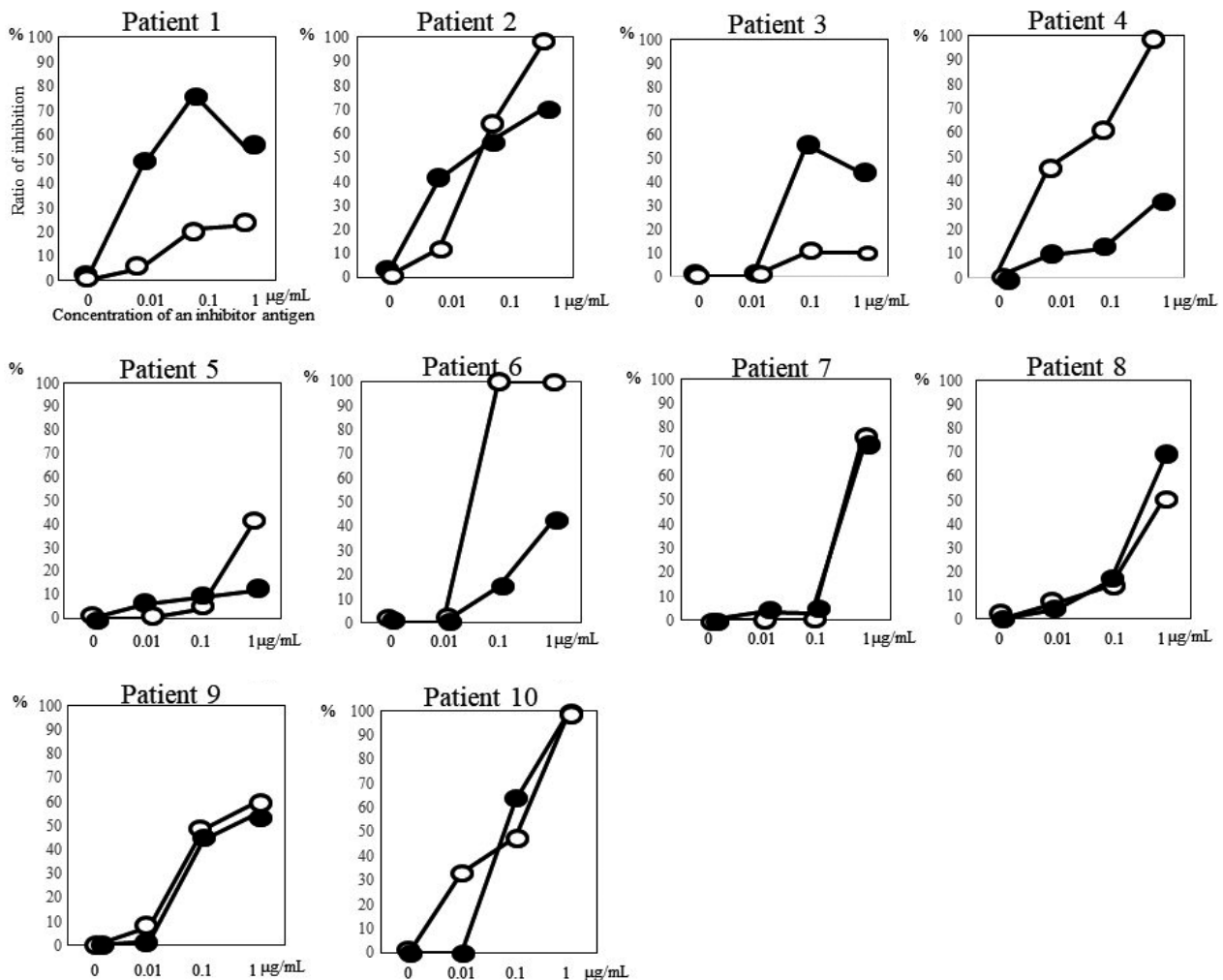
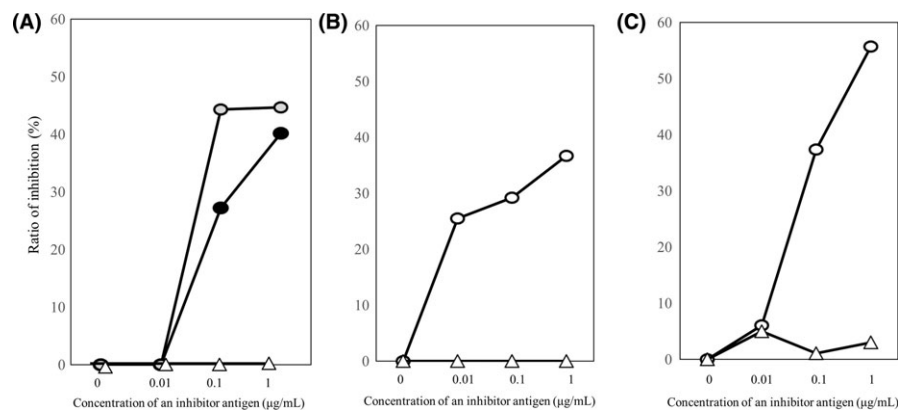


FIGURE 4 ELISA inhibition with orange gibberellin-regulated protein (GRP) and nPru p 7 using individual sera from all patients with positive IgE results for orange GRP. Individual serum was preincubated with the corresponding inhibitor (orange GRP [white dots] and Pru p 7 [black dots]) at different concentrations (0.01, 0.1, and 1 $\mu\text{g/mL}$). Specific IgE binding to orange GRP and nPru p 7 was inhibited by 10.0%-100.0% and 11.4%-100.0%, respectively. The horizontal axis represents the concentration of an inhibitor antigen, and the vertical axis represents the ratio of inhibition

FIGURE 5 ELISA inhibition with orange gibberellin-regulated proteins (GRP) (A), Pru p 7 (B), and Pru m 7 (C) in the solid phase. Pooled serum was preincubated with the corresponding inhibitor (orange GRP [white dots], Pru p 7 [black dots], Pru m 7 [grey dots] or bovine serum albumin [white triangles]) at different concentrations (0.01, 0.1, and 1 $\mu\text{g/mL}$). The horizontal axis represents the concentration of an inhibitor antigen, and the vertical axis represents the ratio of inhibition



The results of BAT, SPT, and ELISA were not always consistent in the current study. Two of three patients with negative ELISA results for orange GRP had positive BAT results, which were

consistent with the SPT results. Furthermore, in one of two patients with positive ELISA results for orange GRP, the BAT was negative. Previous reports also indicated a lack of correlation between IgE

reactivity for purified protein and patient symptoms, possibly caused by differences between the methodologies used. Ramazzina et al reported a study of pear allergy where IgE ELISA demonstrated considerable immunoreactivity of allergens to patient sera, although allergic reactions after the ingestion of pears were not reported in the clinical history of the patients.³¹ In addition, Ebo et al reported the enhanced sensitivity and specificity of BAT for the diagnosis of oral allergy syndrome caused by apple.³² Comparison between subjects with oral allergy syndrome caused by apple and healthy controls showed sensitivities and specificities of 96% and 100% for apple IgE and 88% and 100% for the apple SPT, respectively. Regarding the BAT, its sensitivity and specificity were 100%. Furthermore, recent reports have demonstrated that BATs were superior to other diagnostic tests when discriminating between peanut allergy and tolerance, particularly in difficult cases, thereby reducing the need for oral food challenge.³³ Further investigation will be required to identify the optimal diagnostic tests for orange GRP allergy.

Using the BAT, one patient (Patient 11) showed very high responses compared with other patients, possibly because the BAT might reflect higher clinical severity compared with other tests. Santos et al reported a peanut allergy study where the ratio of the percentage of CD63 basophils after stimulation with peanut and after stimulation with anti-IgE (CD63 peanut/anti-IgE) was independently associated with severity.³⁴ In our study, Patient 11 experienced the most severe reactions after ingesting orange, including generalized urticaria and dyspnoea that required emergency care.

GRP sensitization may be a good predictor for cross-reactivity to other fruits, which are likely to cause severe reactions in patients with orange allergy. In our previous study, we investigated the prevalence of sensitization to Pru p 7 as a representative GRP in fruit allergies.³⁵ We enrolled 100 consecutive Japanese patients with fruit allergies in this study. Of 20 patients with negative ImmunoCAP results for Bet v 1 homologs and profilin that were related to cross-reactivity to pollen, 13 patients (65.0%) had positive ELISA and/or SPT results for nPru p 7. Of the 13 patients, 12 patients (92.3%) developed anaphylactic reactions, and 10 patients (76.9%) had multiple causative fruits. Orange (46.2%) was the third most common cause of fruit GRP allergies, after peach (92.3%) and apricot (61.5%). Orange allergy with GRP sensitization is likely to be accompanied with other fruit allergies, such as peach, which can frequently cause systemic reactions. Therefore, GRP may be an important allergen for screening cross-reactivity to other fruits that cause severe reactions in patients with orange allergy.

Tuppo et al¹⁰ suggested that GRP might be also an allergen related to severe reactions in peach allergy with and without LTP sensitization in southern Europe. Their subjects were selected on the basis of severe clinical reactivity and discrepancies in the rPru p 3 results of several diagnostic tools using allergen preparations, including the ISAC103 microarray and ImmunoCAP. In their study, 14 (42.4%) of 33 peach allergy patients had positive reactions to purified nPru p 7. Therefore, further investigation is needed to confirm the percentage of people sensitized to orange GRP in other areas.

GRP sensitization is clinically associated with eyelid oedema, systemic reactions, and FDEIA.³⁵ These features were also observed in our patients with orange GRP allergy. Facial swelling was the most frequent symptom (66.7%) of orange GRP allergy, consistent with studies of Pru p 7 (46.2%) and Pru m 7 (71.4%) allergies.^{12,18} Eyelid oedema is most significantly associated with Pru p 7 sensitization in patients with peach allergy.¹⁸ However, the orange GRP allergy was less severe than the Pru p 7 and Pru m 7 allergies, as none of our patients with orange GRP sensitization developed anaphylactic shock after orange ingestion in this study. Additionally, oropharyngeal symptoms were observed in 41.7% of orange GRP-sensitized patients in this study. In an Italian study, 39% of 72 children and young adults with pollinosis were found to be sensitized to citrus fruits.³⁶ However, in the present study, sensitization to Bet v 1 homologs and profilin, which is related to pollen food-associated syndrome, was observed in two and three of our patients, respectively. Furthermore, exercise was a cofactor in the induction of allergic reactions in our patients, but it was less common than in patients with Pru m 7 allergy (>70%).¹² To date, three cases of FDEIA due to orange or mandarin have been reported in Japan.²⁻⁴ In one case, an 18-year-old woman developed facial redness and swelling after orange ingestion followed by exercise.⁴ An additional two paediatric patients developed allergic reactions after ingestion of fresh orange or mandarin combined with aspirin and/or exercise, although no symptoms were noted after the ingestion of citrus or aspirin alone. In one of these patients, a 10-year-old girl, IgE immunoblotting identified two proteins with molecular weights of 39 kDa and 53 kDa as possible causative allergens.³ However, the causative allergens in these three cases were not identified.

This study had some limitations, including the small number of subjects enrolled owing to the hospital-based nature of the study. In 2015, a survey regarding the prevalence of food allergies was performed by allergists at tertiary-level hospitals in Japan using a Health Labour Sciences Research Grant. According to this previous survey, orange allergy accounted for 2% of 179 patients with oral allergy syndrome in Japan (<http://www.med.shimane-u.ac.jp/dermatology/FAguideline2015.pdf>). Approximately 50 new patients per year with fruit allergies visit our hospital. On the basis of the results of the previous survey, we estimate that approximately 5 patients with orange allergy might visit our hospital every 10 years. To date, there have been few well-documented cases of allergy to sweet orange, despite its wide consumption, and little work has been done on orange allergens outside of the Mediterranean region. Therefore, further investigation of a large number of subjects is required to determine whether GRP sensitization is involved in orange allergy and/or cross-reactivity to other fruit and pollen allergens.

In conclusion, our findings indicate that GRP is a new orange allergen and is cross-reactive with the *Rosaceae* family GRP nPru p 7. Patients with suspected orange allergy should be considered as candidates for further testing for GRP sensitization and cross-reactivity to *Rosaceae* family fruits.

ACKNOWLEDGEMENTS

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CONFLICT OF INTEREST

All authors have no financial support or relationships that may pose a conflict of interest.

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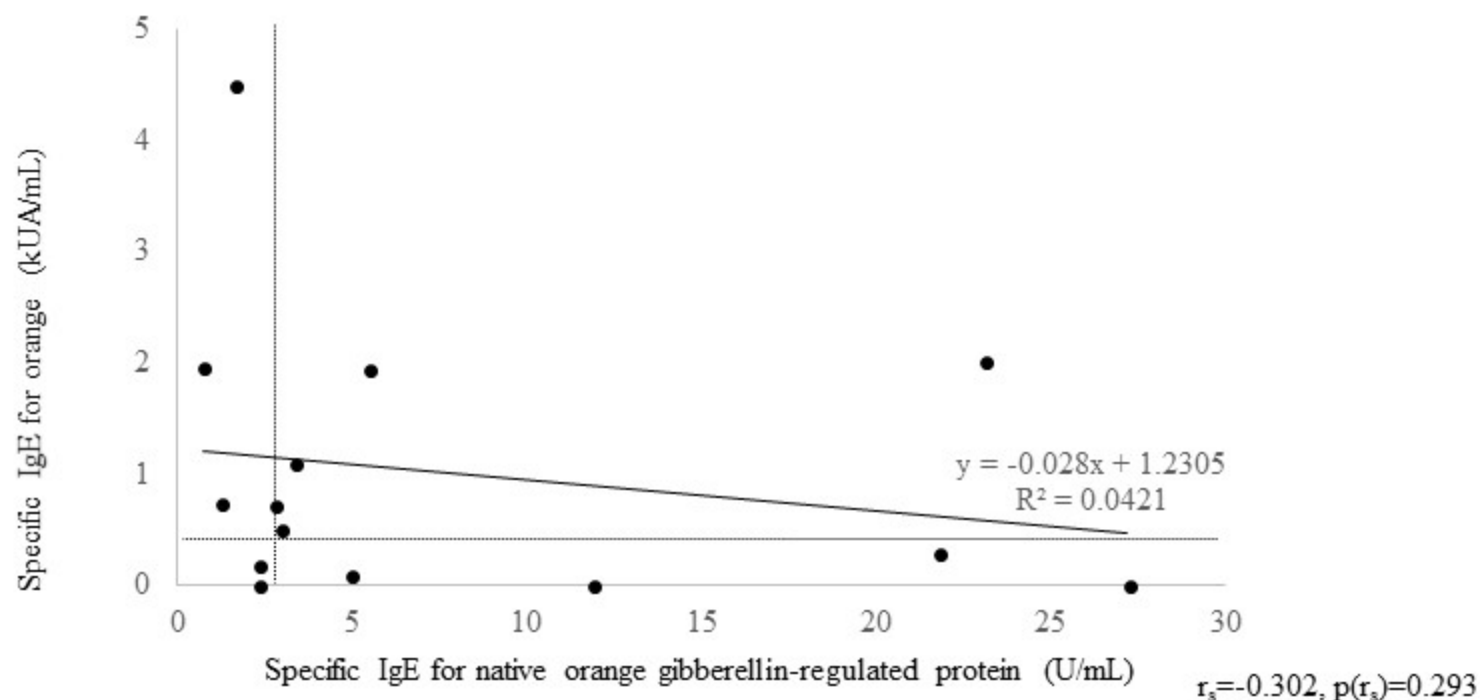
SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Fig. S1.

A.



B.

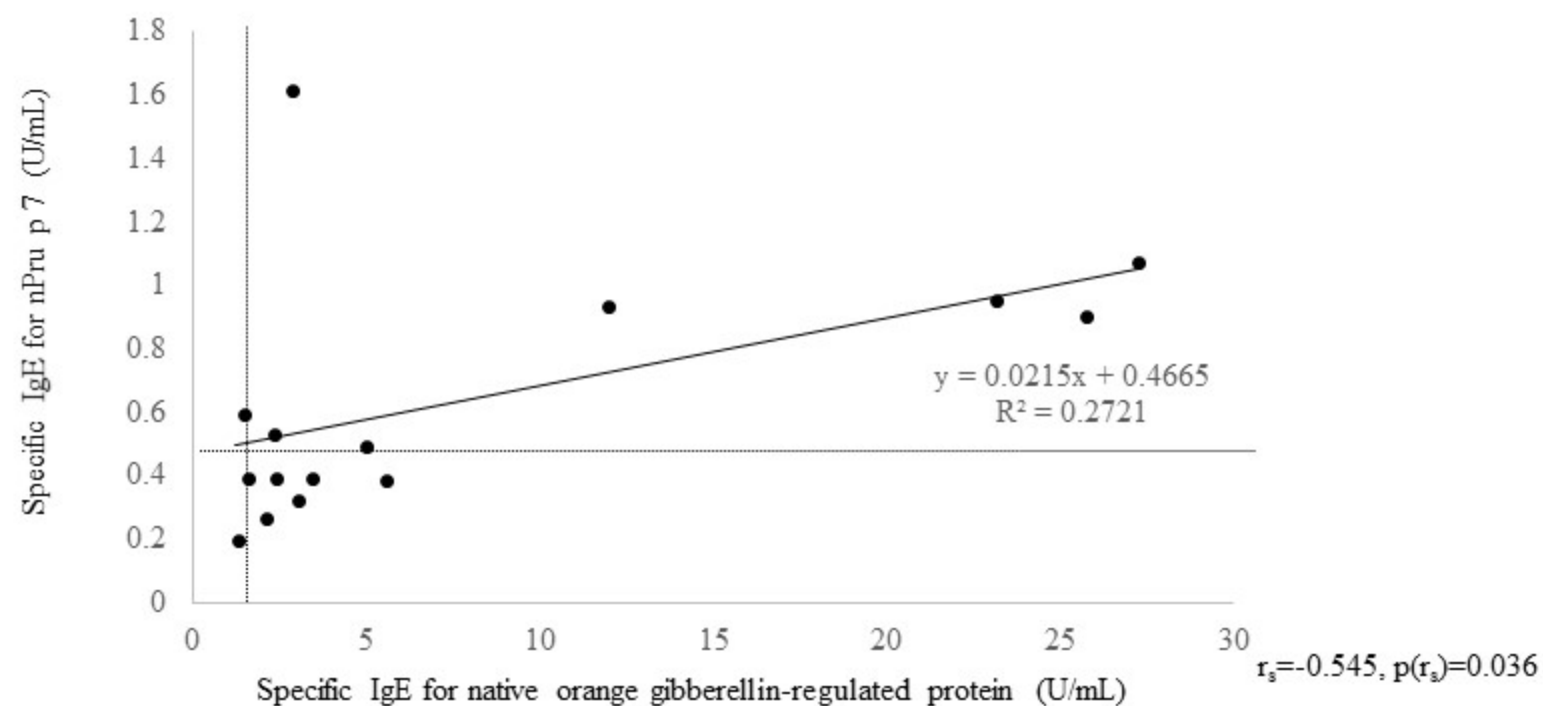
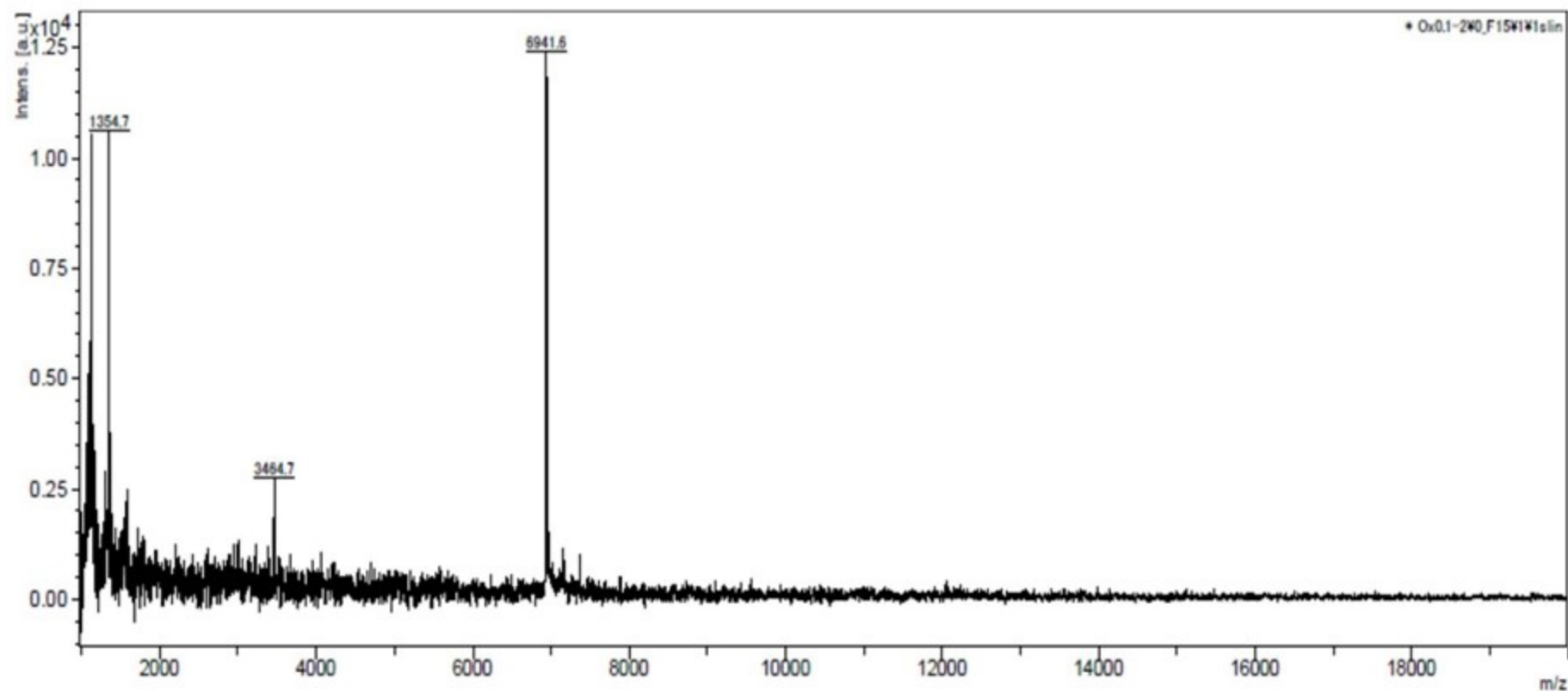


Fig. S2

Measured Average Mass = 6941.6 Da



Supplementary Information

Title: Identification of gibberellin-regulated protein as a new allergen in orange allergy

METHODS

Estimation of the protein concentration

Protein concentrations were quantified using a Qubit 2.0 fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) according to the information provided by the manufacturer.

N-terminal amino acid sequence analysis

The single band in sodium dodecyl sulfate-polyacrylamide gel electrophoresis using the orange fraction containing the 8-kDa protein, which bound to anti-Pru p 7 peptide antibodies but not to anti-Pru p 3 peptide antibodies in IgG-immunoblotting, under reducing conditions were electroblotted onto a polyvinylidene difluoride membrane as described above. After staining with Coomassie Brilliant Blue, the protein band corresponding to 8 kDa was excised and analyzed on a Procise 491 cLC protein sequencer (Applied Biosystems, Foster City, CA, USA), following the manufacturer's recommendations. Eighteen *N*-terminal amino acids were determined for the band. The obtained sequences were analyzed by the Phytozome and BLAST programs to identify proteins in the data bases.

Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry

The purity of the proteins was evaluated and the molecular mass of the purified orange protein was estimated by MALDI-TOF mass spectrometry measurements performed on an UltrafleXtreme™ (Bruker, Billerica, US).

Purification of gibberellin-regulated protein (GRP) from orange

Orange GRP was purified from orange pulp protein extract by ion-exchange column chromatography as previously described with some modifications [1,2]. Sweet orange (*Citrus sinensis*, Valencia cultivar) at the commercial unripe stage were obtained from a local store. After peeling and removing seeds, orange pulp was homogenized with an extraction solution (2 mmol/L disodium ethylenediamine tetra acetate, 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) [3]. A method based on ion-exchange column chromatography for the enrichment of fruit-allergenic extracts, as described by Martinez et al., was used [4]. After filtration through gauze, the extract was centrifuged at 10,000 ×g for 15 min at 4°C. Cation exchange resin (Sephacel Fast Flow, GE Healthcare, Little Chalfont, UK) was added to the supernatant 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.

To eliminate proteins with a molecular weight greater than 10 kDa, the sample was filtrated using Ultracel 10K Amicon Ultra filters (Merck Millipore). The sample was concentrated by ultrafiltration through Ultracel 3K Amicon Ultra filters (Merck Millipore). Further purification was achieved by ion-exchange column chromatography on a cation exchanger Sepharose Fast Flow (GE Healthcare), eluting in a stepwise gradient from 0% to 100% of a 0.2M NaCl, 20 mM acetate buffer.

Fractions containing the protein were concentrated and buffer exchanged to PBS by ultrafiltration on an Ultracel 3K Amicon Ultra filters as above. The purified orange GRP was sterilized by filtration through a 0.22- μ m pore diameter membrane (Merck Millipore) and frozen at -80°C.

Purification of Pru p 7

Pru p 7 was purified from peach pulp protein extract by ionic exchange chromatography as described by Tuppo with some modifications [1, 2]. 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) [3]. A method based on ion-exchange column chromatography for the enrichment of fruit-allergenic extracts, as described by Martinez et al, was used [4]. 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 Pru p 7 was sterilized by filtration through a 0.22- μ m pore diameter membrane (Millipore, Bedford, MA, USA) and frozen at -80°C.

Purification of Pru m 7

Japanese apricot (JA) was purified from JA pulp protein extract by ion-exchange column chromatography as previously described with some modifications [1, 2]. JA (*Prunus mume*, cultivar Nanko strain) at the commercial unripe stage were obtained from a local store. Whole JA was homogenized with an extraction solution (2 mmol/L disodium ethylenediamine tetra acetate, 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) [3]. A method based on ion-exchange column chromatography for the enrichment of fruit-allergenic extracts, as described by Martinez et al., was used.⁵ After filtration through gauze, the extract was centrifuged at 10,000 ×g for 15 min at 4°C. Cation exchange resin (Sephacrose Fast Flow, GE Healthcare, Little Chalfont, UK) was added to the supernatant 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.

To eliminate proteins with a molecular weight greater than 10 kDa, the sample was filtrated using Ultracel 10K Amicon Ultra filters (Merck Millipore,). The sample was concentrated by ultrafiltration through Ultracel 3K Amicon Ultra filters (Merck

Millipore). Further purification was achieved by ion-exchange column chromatography on a cation exchanger Sepharose Fast Flow (GE Healthcare), eluting in a stepwise gradient from 0% to 100% of a 0.2M NaCl, 20 mM acetate buffer.

Fractions containing the protein were concentrated and buffer exchanged to PBS by ultrafiltration on an Ultracel 3K Amicon Ultra filters as above. The purified JA GRP Pru m 7 was sterilized by filtration through a 0.22- μ m pore diameter membrane (Merck Millipore) and frozen at -80°C .

Measurement of IgE specific to orange GRP and Pru p 7 by ELISA

Specific IgE (sIgE) antibody to purified native orange GRP and native Pru p 7 (nPru p 7) was detected by ELISA as previously reported [2]. The wells of a SUMILON[®] multi-well, flat bottom 96-well plate (Sumitomo Bakelite Co., Tokyo, Japan) were coated with purified orange GRP or nPru p 7 (2 $\mu\text{g/mL}$ in PBS) at 50 $\mu\text{L/well}$. The plate was sealed and left overnight at 4°C . Then it 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 patient sera diluted to 20% in 1% skim milk PBS-T was added to the wells, followed by incubation for 1 hour at room temperature. The plate was then washed with 1% skim milk PBS-T. A total of 50 μL of 0.1 $\mu\text{g/mL}$ anti-human IgE-horseradish peroxidase

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). 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 that contained IgE specific to *Der f* (1686 UA/mL) from three patients were chosen as standards. Serial dilution was performed using 1% skim milk PBS-T, starting at 20 times dilution, with subsequent doubling of the dilution factor up to 100,240-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.

sIgE levels >2.32 U/mL (mean unit ± 3 SD of the negative control value) were considered positive for native orange GRP, whereas sIgE levels > 0.485 U/mL (mean unit ± 10 SD of the negative control value) were considered positive for nPrup 7.

Preparation of orange GRP solution for skin testing

Orange GRP was solubilized in PBS and sterilized by membrane filtration through a 0.22- μ m filter (Merck Millipore), in a sterile horizontal laminar flow hood. The final protein concentration was 2 μ g/mL.

Statistical analysis

Correlations between sIgE for orange and peach allergens were analyzed using Spearman's rank correlation coefficient. Absolute coefficients >0.5 were considered positive. *P* values <0.05 were considered significant.

Results

Mass spectrometry

The average masses of purified orange GRP were 6941.6 Da by MALDI-TOF mass spectrometry (Fig. S1), which was similar to that of Pru p 7 (6910.8 Da) registered in the UniProt Knowledgebase.

Correlation of sIgE levels for orange GRP with sIgE for orange and nPru p 7

Spearman's rank correlation test identified no significant correlation between levels of orange GRP-sIgE measured by ELISA and orange-sIgE measured by ImmunoCAP (Fig. S2-A). In contrast, levels of orange GRP-sIgE and Pru p 7-sIgE in the 14 patients with orange allergy were significantly correlated ($P < 0.05$; Fig. S2-B).

Figure legends

Fig. S1. Mass spectrometry measurements. Annotated mass spectrum of orange gibberellin-regulated protein, indicating an average mass of 6941.6 Da.

Fig. S2. Correlation of serum specific IgE (sIgE) for orange gibberellin-regulated protein (GRP) with sIgE for orange (A) and nPru p 7(B).

Each point represents serum from one of the 14 patients with orange allergy. Dotted lines in Fig. S2-A indicate the positivity cut-offs in the ELISA (2.32 U/mL) and ImmunoCAP (0.35 kUA/L) assays. There was no significant correlation between serum sIgE for orange GRP, and sIgE for orange measured by ImmunoCAP. Dotted lines in Figure S3-B indicate the positivity cut-offs in the orange GRP (2.32 U/mL) and nPru p 7 (0.485 U/mL) ELISA assays. A significant correlation between serum IgE for orange GRP and nPru p 7 was detected by analysis using Spearman's rank correlation coefficient.

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