

Immunoblotting Quantification Approach for Identifying Potential Hypoallergenic Citrus Cultivars

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Supporting Information

ABSTRACT: The inherent allergens of citrus fruits, such as Cit s 1, Cit s 2, Cit s 3 can cause allergic reactions. A better understanding of the genetic factors (cultivar to cultivar) affecting the allergenic potential of citrus fruits would be beneficial for further identification of hypoallergenic genotypes. In the present study, an immunoblotting quantification approach was adopted to assess the potential allergenicity of 21 citrus cultivars, including nine subgroups (tangerine, satsuma, orange, pummelo, grapefruit, lemon, kumquat, tangor, and tangelo). To prepare highly sensitive and specific rabbit polyclonal antibodies, antigenicity of purified rCit s 1.01, rCit s 2.01, and rCit s 3.01 peptides were enhanced with high epitope density in a single protein molecule. The data integration of three citrus allergen quantifications demonstrated that the four pummelo cultivars (Kao Phuang Pummelo, Wanbai Pummelo, Shatian Pummelo, and Guanxi Pummelo) were potential hypoallergenic, compared with other 8 subgroups. Moreover, the immunological analyses with sera of allergic subjects revealed that Shatian Pummelo and Guanxi Pummelo showed the lowest immunoreactivity in 8 representative citrus cultivars. These potential hypoallergenic genotypes are of great significance to not only allergic consumers but also citrus breeders in the genetic improvement of hypoallergenic citrus as breeding resources.

KEYWORDS: citrus allergen, recombinant antigen, immunoblotting, genetic factors, hypoallergenic fruit

■ INTRODUCTION

Comparing with the main common fruit traits, for instance, appearance, fragrance, and taste, nutritional and safety properties of fruits are becoming new characteristics that consumers are concerned with in recent years.¹ The allergenic potential of foods is an important issue regarding the toxicological safety assessment.² In recent decades, fruit allergenicity has received wide attention from scientists in diverse fields, such as allergology, immunology, biochemical and molecular biology, agriculture, and food industry.³ Genus *Citrus* of Rutaceae is one of the most important fruit crops all over the world and is consumed mostly as fresh fruit, jam, and juice owing to its rich nutritional values and abundant flavor.^{4,5} Various bioactive compounds in citrus, such as vitamin C, carotenoids, flavonoids, and limonoids, play vital roles in human health promotion and disease prevention.^{6–8} However, there are some cases reported about allergic reactions that vary from a mild oral allergy syndrome to a severe anaphylaxis occurrence.^{9–12}

Avoidance of the allergenic food is strongly recommended to those who suffer from food allergies. Administration of emergency medications has been provided when accidentally exposed.¹³ Given the negative effects of the citrus allergy on allergic patients, developing hypoallergenic citrus cultivars with low amounts of allergens as an “allergy-friendly” food would be a new opportunity for citrus fruit breeders and growers.

Major citrus allergens and the corresponding genes were identified in the past.^{10,14–17} Six representative allergens from sweet oranges (*Citrus sinensis*), tangerines (*Citrus reticulata*), and lemons (*Citrus limon*) have been included in the official allergen database established by the Allergen Nomenclature Subcommittee of the World Health Organization and Interna-

tional Union of Immunological Societies (WHO/IUIS; <http://www.allergen.org/search.php>). The genes encoding those three known allergens (Cit s 1, Cs5g25680; Cit s 2, Cs1g15890; and Cit s 3, Cs6g09940) are mapped onto the orange genome.¹⁸ Moreover, Cit s 1, Cit s 2, and Cit s 3 are complex gene families composed of 16, 3, and 7 different isoforms, respectively, with high conserved sequences, where each of them codes a different individual isoallergen. However, little information is currently available on Cit s 7. Since a low dose of allergens could induce adverse reactions,¹⁹ the development of appropriate analytical methods is urgent for the detection and control of citrus allergens in food products and industry.²⁰ However, only a few methods are available for the quantification of the allergen levels in citrus fruits. Previously, sandwich enzyme-linked immunosorbent assays (ELISA) were developed for quantification of Cit s 2, a major allergen in 12 different citrus varieties.^{21,22} In our previous work, the multiplex real-time PCR assay was developed and applied to detect the expression of citrus allergen genes for the assessment of potential allergenicity in citrus fruits.¹⁸ However, due to the absence of specific antibodies against Cit s 1, Cit s 2, and Cit s 3 simultaneously, there is no method to directly quantify the allergen levels through immunological detection for the overall assessment of potential allergenicity.

The genotype (genetic factor) is considered to be a most significant factor affecting the allergenic potential of citrus

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Table 1. Species of 21 Citrus Cultivars

cultivars	abbreviation	citrus species	commercial harvest ^a
Red Tangerine	RT	<i>C. tangerine</i> Tanaka	November to December
Bendizao Tangerine	BT	<i>C. succosa</i> Hort. ex Tanaka	first–third week of November
E-gan No.1 Ponkan Tangerine	ET	<i>C. reticulata</i> Blanco cv. Ponkan	second and third weeks of November
Clementine Tangerine	CT	<i>C. clementina</i> hort.ex Tanaka	second and third weeks of November
Guoqin No. 1 Satsuma	GNS	<i>C. unshiu</i> Marc.	first–third week of October
Newhall Navel Orange	NNO	<i>C. sinensis</i> Osbeck	second week of November, second week of December
Washington Navel Orange	WNO	<i>C. sinensis</i> Osbeck	third week of November, first week of December
Caracara Navel Orange	CNO	<i>C. sinensis</i> Osbeck	first–third week of December
Rohde Red Valencia Orange	RRVO	<i>Citrus sinensis</i> Osbeck	third week of April, first week of May
Kao Phuang Pummelo	KPP	<i>C. grandis</i> (L.) Osbeck	second week of November, first week of December
Wanbai pummelo	WBP	<i>C. grandis</i> (L.) Osbeck	third week of December, first week of January
Shatian pummelo	STP	<i>C. grandis</i> (L.) Osbeck	third week of November, first week of December
Guanxi pummelo	GXP	<i>C. grandis</i> (L.) Osbeck	second week of October, first week of November
Cocktail Grapefruit	CG	<i>C. paradisi</i> Macf.	second–fourth week of December
Star Ruby Grapefruit	SRG	<i>C. paradisi</i> Macf.	second–fourth week of December
Eureka Lemon	EL	<i>C. limon</i> (L.) Osbeck	second and third weeks of November
Mexican lime	ML	<i>C. aurantiifolia</i>	first week of August, first week of November
Meiwa kumquat	MK	<i>Fortunella crassifolia</i> Swingle.	first and second weeks of December
Kiyomi Tangor	KT	<i>C. unshiu</i> Marc. cv. Miyagawa × <i>C. sinensis</i> Osbeck cv. Trovita	first–third week of January
Shiranuhi Tangor	ST	(<i>C. reticulata</i> × <i>C. sinensis</i>) × <i>C. reticulata</i>	third and fourth weeks of January
Fallglo Tangelo	FT	<i>C. reticulata</i> × <i>C. maxima</i> or <i>C. paradise</i>	first–third week of November

^aThe optimal harvest date for each cultivar was determined according to the recommendation of the National Citrus Germplasm Information System (<http://xt.cric.cn/>).

Table 2. Sequence of Primers^a

primer name	primer sequences (5′–3′)
Cit s 1.01-P1	CCCCTGGGATCCCCGGAATTCAAAGCGATCCAGTTCCTGAT
Cit s 1.01-P2	TTTACCAGAAACCACCAGAACCGTTACCGTTGATGAATTTGT
Cit s 1.01-P3	GGTTCCTGGTGGTTCCTGGTAAAGCGATCCAGTTCCTGATCGG
Cit s 1.01-P4	GTCACGATGCGGCCGCTCGAGTTAGTTACCGTTGATGAATT
Cit s 2.01-P1	CCCCTGGGATCCCCGGAATTCTCTTGGCAGACCTACGTTGA
Cit s 2.01-P2	AGAACCAGAAACCACCAGAACCCAGACCCTGGTCGATCAGGT
Cit s 2.01-P3	GGTTCCTGGTGGTTCCTGGTTCCTGGCAGACCTACGTTGACGA
Cit s 2.01-P4	GTCACGATGCGGCCGCTCGAGTTACAGACCCTGGTCGATCA
Cit s 3.01-P1	GCTGATATCGGATCCGAATTCGCGGCGCTGAACTGGTTTG
Cit s 3.01-P2	AGAACCAGAAACCACCAGAACCCAGACCCTGGTCGATCAGGT
Cit s 3.01-P3	GGTTCCTGGTGGTTCCTGGTTCCTGGCAGACCTACGTTGACGA
Cit s 3.01-P4	GTGGTGGTGGTGGTGCTCGAGTTAACGAACACGAGAGCAGT

^aThe 15 bp extensions required for In-Fusion cloning are indicated in bold text. The linker sequence of fusion protein is underlined. *Eco*RI Sites in the primers Cit s 1.01-P1, Cit s 2.01-P1, and Cit s 3.01-P1 and *Xho*I Sites in the primers Cit s 1.01-P4, Cit s 2.01-P4, and Cit s 3.01-P4 are indicated in italics.

fruits.¹⁸ Moreover, in the long term, identification of hypoallergenic citrus cultivars is beneficial to both the allergic consumers and the citrus breeders. Thus, we wished to establish an immunoblotting quantification approach for citrus allergens, e.g., Cit s 1, Cit s 2, and Cit s 3, and to further identify potential hypoallergenic citrus fruits and to provide relevant guidelines for inspiring the future improvement of hypoallergenic citrus cultivars.

MATERIALS AND METHODS

Plant Materials. The characters of 21 citrus cultivars used in this study are shown in Table 1. These citrus fruits were obtained from the National Center for Citrus Breeding, Huazhong Agricultural University. Peels (both flavedo and albedo) and pulps of these fruits were separated with scalpels, frozen immediately in liquid nitrogen, and stored at −80 °C for further analysis.

Citrus Fruits Protein Extraction. Citrus fruit proteins were extracted as previously described with some modification.³ Citrus fruits samples (peel or pulp) were manually ground with a mortar and pestle in liquid nitrogen. Five grams of powders were mixed with the extraction buffer containing 25 mM Tris, 192 mM glycine (pH 9.0), 6 M urea, 10% (w/v) glycerol, 2.5% SDS (w/v), and 2.5% β-mercaptoethanol (v/v) in a ratio of 1:2 (w/v), at 4 °C for 1 h with gentle stirring. During homogenization, 33 mg of polyvinyl-pyrrolidone (insoluble) per gram of tissue was added. After centrifugation twice at 8000 rpm, in 4 °C for 20 min, the supernatant of the extraction solution was collected, passed through the muslin cloth, and dialyzed with dialysis membranes (MW: 3500D, MD44, Biosharp, U.S.A.) overnight against 20 mM sodium phosphate buffer (pH 7.0). The protein concentration of the extracted sample was measured using the Bio-Rad protein assay, BSA as a standard. All chemicals were purchased from Sangon Biotech Co., Ltd. (Shanghai, China).

Plasmid Construction. First, amino acid sequences of published Cit s 1.01, Cit s 2.01, and Cit s 3.01¹⁸ were analyzed for putative signal peptides using SignalP (www.cbs.dtu.dk/services/SignalP/).²³ The entire coding sequence minus the N-terminal signal peptide of these genes were acquired. To facilitate the expression in *E. coli* BL21 (DE3; TransGen Biotech, Beijing, China), the coding sequence of Cit s 1.01, Cit s 2.01, and Cit s 3.01 was optimized based on the JCat (JAVA Codon Adaptation Tool, <http://www.jcat.de/>),²⁴ and the genes were synthesized by Wuhan GeneCreate Biological Engineering Co., Ltd. (Wuhan, China). Subsequently, the synthesized genes were used as templates to amplify the corresponding regions using primers P1 and P2, primers P3 and P4, and primers P3 and P2 (Table 2), respectively. All primers were synthesized by Tsingke Biological Technology (Wuhan, China). In parallel, the plasmid PGEX-6p-1 and pET-32a (+) were digested with *Eco*RI and *Xho*I (Invitrogen, Carlsbad, CA), respectively. The three PCR fragments were mixed with corresponding digested plasmid and ligated by using ClonExpress MultiS One Step Cloning Kit (Vazyme, China) according to the manufacturer's protocol. The mixture was kept at 37 °C for 30 min, and then placed on ice for 5 min. Then, the resulting products were transformed into *E. coli* DH5 α (TransGen Biotech, Beijing, China) and transformants were selected on LB agar plates containing 50 μ g/mL ampicillin. PCRs were performed on selected colonies to confirm the presence of inserts. The recombinant plasmids were designated as PGEX-6p-1/GST-(Cit s 1.01) $\times$ 2, PGEX-6p-1/GST-(Cit s 2.01) $\times$ 3, and pET-32a(+)/Trx-His-(Cit s 3.01) $\times$ 2 and verified by DNA sequencing.

Expression, Purification, and Identification of Recombinant Citrus Allergen Proteins. To induce the expression of rCit s 1.01, rCit s 2.01, and rCit s 3.01, *E. coli* BL21 (DE3) transformed with the plasmid of PGEX-6p-1/GST-(Cit s 1.01) $\times$ 2, PGEX-6p-1/GST-(Cit s 2.01) $\times$ 3, or pET-32a(+)/Trx-His-(Cit s 3.01) $\times$ 2 was cultured in LB medium that contained 50 μ g/mL ampicillin at 37 °C for 12 h with shaking (200 rpm). Subsequently, the culture was added to another fresh LB medium (1:1000) at 37 °C with shaking (200 rpm). When the OD₆₀₀ of the culture reached 0.5 AU, the heterologous expressions of three recombinant citrus allergen proteins were initiated by IPTG induction at 0.1 mM at 16 °C for 20 h.

After that, *E. coli* BL21 (DE3) was collected by centrifugation at 4 °C for 10 min at 8000 rpm. The cell pellets were resuspended in lysis buffer (50 mM Tris, 100 mM NaCl, 5% glycerol, and 1 mM PMSF). Sonication was performed on ice using Sonics VCX750 (Sonics & Material, Inc.) at 30% output for 20 cycles of 20 s on and 20 s off. The supernatant of the cell lysates was achieved by centrifugation at 4 °C for 20 min at 16 000g and stored at 4 °C. rCit s 1.01 and rCit s 2.01 fusion proteins were purified by an affinity column that had been packed with Glutathione Sepharose 4B beads (GE Healthcare) and equilibrated with five column volumes (CVs) of lysis buffer. The supernatants were incubated with glutathione beads with rotation for 2 h at 4 °C. The beads were washed with nine CVs of washing buffer (50 mM Tris, 100 mM NaCl and 5% glycerol) to remove unwanted cellular constituents or contaminants. Bound GST fusion proteins were eluted twice with elution buffer (50 mM Tris, 100 mM NaCl and 5% glycerol and 10 mM glutathione), 20 min each, at 4 °C. rCit s 3.01 fusion protein was purified by an affinity column that was packed with Ni-NTA agarose (Qiagen) and equilibrated with five CVs of the lysis buffer. The supernatant was loaded onto the column and then washed with nine CVs of washing buffer (lysis buffer, containing 20 mM imidazole, pH 8.0). The bound rCit s 3.01 fusion proteins were eluted with six CVs of 100–300 mM (with gradient) imidazole in lysis buffer (pH 8.0). Then, the collected solution was ultrafiltered in a centrifuge (5 °C, 15 min, 5000 rpm) using a Millipore ultrafiltration unit with a cutoff of 10 kDa. The protein concentration of rCit s 1.01, rCit s 2.01, and rCit s 3.01 was measured using the Bio-Rad protein assay with BSA as a standard. The purity of these recombinant proteins was assessed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

These three recombinant citrus allergen proteins were identified by MALDI-TOF MS analysis. The protein sample was first separated out by SDS-PAGE analysis and stained with Coomassie brilliant blue (CBB). The band that contained rCit s 1.01, rCit s 2.01, and rCit s

3.01 was cut out and digested by trypsin in the gels of SDS-PAGE. The peptide mapping of the trypsinized sample was carried out by AB SCIEX MALDI TOF-TOF 5800 analyzer (AB SCIEX, U.S.A.).

Production of Rabbit Polyclonal Antibodies. In order to generate antibodies against Cit s 1.01, Cit s 2.01, and Cit s 3.01, two New Zealand white rabbits (purchased from Hubei Provincial Center for Disease Control and Prevention, China) were immunized subcutaneously with 0.1 mg of recombinant GST-(Cit s 1.01) $\times$ 2, GST-(Cit s 2.01) $\times$ 3, and Trx-His-(Cit s 3.01) $\times$ 2 proteins, respectively. Immunization and serum collection were performed by Friendbio Science & Technology (Wuhan) Co., Ltd. The protocol was approved by the Institute of Animal Care and Use Committee (no. 201703083), according to national guidelines for animal care and handling, China. Immunization was performed every 2 weeks for a period of 3 months. The sera were collected after 3 months of immunization. Antibody titers were measured by ELISA. The sera were stored at –80 °C for further use.

Patient Sera. The orange specific pool of human IgE sera was acquired from the PlasmaLab International (Everett, WA, U.S.A.). Orange specific sera were screened and collected from 4 donors. The presence of orange reactive IgE was confirmed with the ImmunoCAP assay. The clinical characteristics of these orange allergic patients are shown in Table 3. The allergic sera were stored at –70 °C for further use.

Table 3. Characteristic of Human Sera from Orange Allergic Patients^a

patient	age	gender	CAP [kUA/L]	
			total IgE	orange
A	32	female	1340.262	9.77
B	45	female	1936	8.85
C	30	female	594.82	2.239
D	54	male	3801.799	9.513

^aSubjects were selected on the basis of clinical allergy to orange and in vitro IgE evaluation. A–D, patients reporting orange allergy.

SDS-PAGE and Immunoblotting. SDS-PAGE and immunoblotting analysis were performed according to a standard protocol. Each protein sample was mixed with 5 $\times$ sample buffer (10% SDS, 50% glycerol, 0.25 M Tris-HCl pH 6.8, β -mercaptoethanol, and 0.05% bromophenol blue) before denaturation in boiling water for 5 min. Supernatants were used for the SDS-PAGE and immunoblotting analyses. The protein samples were analyzed by SDS-PAGE with a 5% (w/v) stacking gel and a 12% (w/v) resolving gel at a constant voltage of 100 V. Proteins in the gel were detected by staining with CBB and then destained using a destaining solution that contained 10% acetic acid and 25% methanol.

For immunoblotting, the total proteins were separated by 10% SDS-PAGE and further transferred onto polyvinylidene fluoride membranes (PVDF, Millipore Corporation, Billerica, MA). PVDF blots were blocked with 5% skimmed milk powder in phosphate buffer saline with 0.1% Tween 20 (PBST, pH 7.4) for 1 h at 37 °C. Then, the blots were incubated with primary antibodies overnight with shaking at 4 °C. After washing with PBST (phosphate buffered saline with Tween 20) for six times, the blots were incubated with secondary antibodies for 1 h at room temperature. After washing with PBST for six times, the blots were incubated in chemiluminescent substrate (Immun-star HRP chemiluminescent kit, Bio-Rad, Hercules, CA). Chemiluminescent signals of protein bands were visualized using a ChemiDoc imaging system (BioRad, U.S.A.). For detection of rabbit polyclonal antibodies binding to citrus allergens, the polyclonal antisera (1:5000) of Cit s 1.01, Cit s 2.01, and Cit s 3.01 were used as the primary antibodies. Antiplant actin mouse monoclonal antibody (clone: 3T3, Abbkine, China) was used as a loading control. The second antibodies were goat anti-rabbit IgG/HRP (BA1054, BosterBio, China) and goat anti-mouse IgG/HRP (BA1050, BosterBio, China). For detection of serum IgE binding to citrus allergens, the orange specific pool of

human IgE sera (1:20) was used as the primary antibody. The second antibody was mouse monoclonal anti-human IgE/HRP (ab99806, Abcam, U.K.). Quantification of immunoblotting bands was performed by densitometry using ImageJ.²⁵

Statistical Analysis. Statistical analyses were performed on SPSS 19 with a one-way analysis of variance (ANOVA) with Duncan test where $p < 0.05$ was considered significant (IBM Corporation, Armonk New York, U.S.A.). Principal component analysis (PCA) and Pearson's correlation coefficients were performed with Origin Pro 2018C (Origin Lab, Northampton, MA, U.S.A.). Normalization of data for Pearson's correlation coefficients was calculated with the min–max normalization method.

RESULTS

Plasmid Construction. Amino acid sequencing results shown in the [Supporting Information](#), Supplementary Table 1, indicated that the optimized coding sequences of Cit s 1.01, Cit s 2.01, and Cit s 3.01 were inserted into PGEX-6p-1 and pET-32a (+) by Exnase MultiS, respectively. To verify the number of tandemly repeated genes in recombinant plasmids, DNA sequencing was performed ([Figure 1a](#)). The results showed

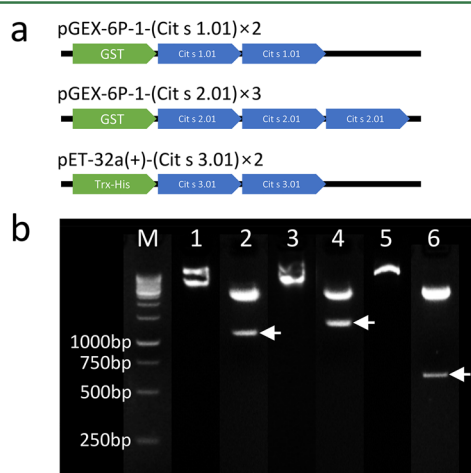


Figure 1. Plasmid construction for three citrus allergen expressions. (a) Schematic representation for plasmid construction. Two Cit s 1.01 fragments were inserted into the downstream of the GST tag region. Three Cit s 2.01 fragments were inserted into the downstream of the GST tag region. Two Cit s 3.01 fragments were inserted into the downstream of the Trx-His tag region. “pGEX-6P-1” and “pET-32a (+)” are prokaryotic Expression Vector. (b) Verification of the plasmid construction by restriction enzyme digestion analysis. The prebuilt plasmids are digested by restriction endonucleases *EcoRI* and *XhoI*. Lanes 1, 3, and 5 are nondigested empty plasmids, “pGEX-6P-1”, “pGEX-6P-1”, and “pET-32a (+)”, respectively. Lanes 2, 4, and 6 are digested plasmids, pGEX-6P-1-(Cit s 1.01)×2, pGEX-6P-1-(Cit s 2.01)×3, and pET-32a (+)-(Cit s 3.01)×2, respectively. M represents 1 kb DNA ladder from Invitrogen.

the successful constructs of recombinant prokaryotic expression plasmids PGEX-6p-1/GST-(Cit s 1.01)×2, PGEX-6p-1/GST-(Cit s 2.01)×3, and pET-32a(+)/Trx-His-(Cit s 3.01)×2, containing two tandemly repeated Cit s 1.01 genes, three tandemly repeated Cit s 2.01 genes, and two tandemly repeated Cit s 3.01 genes, respectively. To further confirm the plasmid constructs, restriction enzyme digestion by *EcoRI* and *XhoI* was conducted. The resulting digested patterns showed the proper sizes of the production bands (1365bp, 1215bp, and 711bp for Cit s 1.01, Cit s 2.01, and Cit s 3.01, respectively), matching the

predicted size of the inserted fragment within the corresponding plasmid ([Figure 1b](#)).

Expression, Purification, and Identification of Three Recombinant Citrus Allergen Fusion Proteins. Extracted proteins from *E. coli* cells transformed with the constructed plasmids were analyzed by SDS-PAGE ([Figure 2](#)). It is apparent that a de novo synthesized polypeptide was accumulated and to be the most abundant fraction among *E. coli* total cellular proteins. These newly expressed proteins corresponded to the expected GST-(Cit s 1.01)×2, GST-(Cit s 2.01)×3, and Trx-His-(Cit s 3.01)×2 with the total mass at approximate 66, 70, and 40 kDa in the Coomassie gel, respectively. In addition, these highly soluble expressed proteins were the most abundant protein in their respective eluted samples. The final yields of the fusion proteins in 1 L of cell culture was 10.8 mg for rCit s 1.01, 15.3 mg for rCit s 2.01, and 9.6 mg for rCit s 3.01, respectively. According to the literature, such yields could be improved simply by expressing the fusion protein in the same cell physiological state but at higher cell densities.²⁶

To further characterize rCit s 1.01, rCit s 2.01, and rCit s 3.01, MALDI-TOF MS analyses were performed to determine whether the amino acid sequences matched with which were deduced from the cDNA sequences. Supplementary Figure 1 in the [Supporting Information](#) demonstrated that 7 peptide fragments for rCit s 1.01, 5 peptide fragments for rCit s 2.01, 8 peptide fragments for rCit s 3.01 were identified by MOLDI-TOF. These identified peptides covered 58.9% (Cit s 1.01), 51.9% (Cit s 2.01), and 53.9% (Cit s 3.01) of the corresponding protein sequence, respectively. Hence, these proteins expressed in *E. coli* cells were the targeted recombinant citrus allergens.

Specificity and Validation of Anti-Cit s 1.01, Anti-Cit s 2.01, and Anti-Cit s 3.01 Polyclonal Antibodies. After the immunization process, the antibody titers of anti-Cit s 1.01, anti-Cit s 2.01, and anti-Cit s 3.01 antibodies in rabbit serum determined by ELISA were $1:1.25 \times 10^7$, $1:6.25 \times 10^6$, and $1:1.25 \times 10^7$, respectively. The specificity of serum polyclonal antibodies against Cit s 1.01, Cit s 2.01, or Cit s 3.01 was evaluated by immunoblotting using the pulp protein of Newhall Navel Orange ([Figure 3](#)). The anti-Cit s 1.01 antibody only recognized Cit s 1.01 band (~23 kDa) in the corresponding lane without nonspecific band. A single band (~10 kDa) was observed using anti-Cit s 3.01 antibody. However, there were two bands shown by anti-Cit s 2.01 antibody, one at 14 kDa as predicted, and the other at about 100 kDa, likely a nonspecific band with a high molecular weight. Consequently, these three polyclonal antibodies displayed a satisfactory performance regarding binding, sensitivity, and specificity and were appropriate to quantification of citrus allergens by immunoblotting.

Quantification of Three Citrus Allergens in Different Cultivars by Specific Antibodies. Immunoblotting quantification of three citrus allergens by specific antibodies was applied to evaluate the influence of genetic factors (cultivar to cultivar) on potential allergenicity of citrus fruits. To determine the effect of the various genetic background, 21 commonly consumed citrus cultivars were selected, including 4 tangerines, 1 satsuma, 4 oranges, 4 pummelos, 2 grapefruits, 2 lemons, 1 kumquat, 2 tangors, and 1 tangelo. Tangors is a hybrid of mandarin orange (tangerine) and sweet orange, while tangelos is a hybrid of any mandarin orange with either grapefruit or pummelo. The immunoblotting results revealed that these three antibodies displayed large differences regarding the

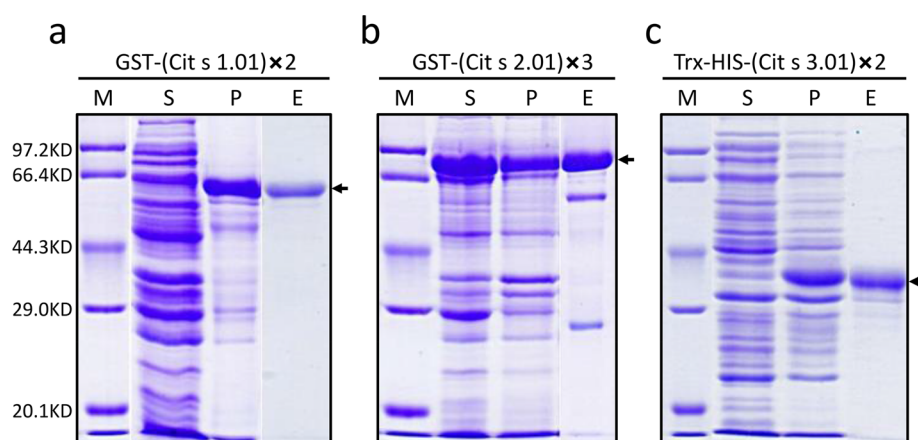


Figure 2. Expression and purification of recombinant GST-(Cit s 1)×2, GST-(Cit s 2)×3, and Trx-His-(Cit s 3)×2 fusion protein. The arrow indicates the position of the expressed protein. M, protein maker; S, soluble fractions; P, insoluble precipitate; E, protein eluted.

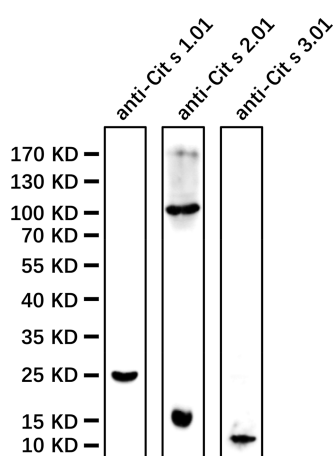


Figure 3. Specificity of polyclonal antibodies against Cit s 1.01, Cit s 2.01, and Cit s 3.01 by immunoblotting. Lane 1, Cit s 1.01 (23 kDa); lane 2, Cit s 2.01 (14 kDa); and lane 3, Cit s 3.01 (9.46 kDa).

binding intensity of allergens presented in the peels and pulps of the different cultivars, where β -actin was used as a loading control (Figure 4a).

In this study, the statistical correlation of the allergen amounts between the peel and pulp corresponding to Cit s 1.01, Cit s 2.01, and Cit s 3.01 protein was assessed by immunoblotting using those specific polyclonal antibodies generated (Supporting Information, Supplementary Table 2). It is noteworthy that there was no linear relationship regarding the general three allergen amounts in between the peel and pulp ($r = 0.09327$; $P = 0.46718$). When the three allergens were considered separately, there also was no linear relationship between the peel and pulp in Cit s 1.01 ($r = 0.00294$; $P = 0.9899$), Cit s 2.01 ($r = 0.01037$; $P = 0.96441$), and Cit s 3.01 ($r = 0.29091$; $P = 0.20077$), respectively.

To further compare the amounts of three citrus allergens in the citrus cultivars selected, the immunoblotting bands were quantified by densitometry, with β -actin as a loading control (Figure 4b). For Cit s 1.01 allergen, the highest amounts in the pulp were found in Caracara Navel Orange, Washington Navel Orange, Eureka Lemon, and Cocktail Grapefruit, whereas the lowest amounts were in Kao Phuang Pummelo, Wanbai pummelo, Guoqin No. 1 Satsuma, Guanxi pummelo, Bendizao Tangerine, and Shatian pummelo. Moreover, the highest values of Cit s 1.01 allergen in the peel were observed in Rohde Red

Valencia Orange, Kiyomi Tangor, Caracara Navel Orange, and Red Tangerine, and the lowest were in Eureka Lemon, Fallglo Tangelo, Washington Navel Orange, Bendizao Tangerine, Meiwa kumquat, and Mexican lime. Regarding the pulp Cit s 2.01, the highest immunoreactivities were found in Rohde Red Valencia Orange, Bendizao Tangerine, Kiyomi Tangor, and Newhall Navel Orange, and the lowest was discovered in Mexican lime, E-gan No.1 Ponkan Tangerine, Kao Phuang Pummelo, Wanbai pummelo, Shatian pummelo, and Guanxi pummelo. In the peel, the highest reactive bands of Cit s 2.01 were detected in Washington Navel Orange, Fallglo Tangelo, Star Ruby Grapefruit, and Cocktail Grapefruit, whereas the lowest were in Wanbai pummelo, Shatian pummelo, Rohde Red Valencia Orange, Guanxi pummelo, Clementine Tangerine, and E-gan No.1 Ponkan Tangerine. The highest Cit s 3.01 in the pulp were found in Star Ruby Grapefruit, Caracara Navel Orange, Red Tangerine, and Cocktail Grapefruit, while the lowest appeared in Shatian pummelo, Guanxi pummelo, Kiyomi Tangor, Wanbai pummelo, Eureka Lemon, and Shiranuhi Tangor. The highest Cit s 3.01 in the peel were observed in E-gan No.1 Ponkan Tangerine, Kiyomi Tangor, Clementine Tangerine, and Rohde Red Valencia Orange, and the lowest were detected in Fallglo Tangelo, Mexican lime, Eureka Lemon, Wanbai pummelo, Kao Phuang Pummelo, and Shatian pummelo.

Data Integration through Principal Component Analysis. To assess the difference among 21 citrus cultivars concerning all three allergens, PCA was conducted (the mean values used; $n = 3$). Min–max normalization which is one of the popular techniques applied for relevance score normalization²⁷ was performed before the PCA. PCA allows the definition of centroids of all cultivars. These citrus cultivars genetically belong to 9 subgroups (including tangerine, satsuma, orange, pummelo, grapefruit, lemon, kumquat, tangor, and tangelo). The first three components with eigen values = 3 could explain that more than 80.33% of the total quantitative variation was found for three allergen amounts of 21 citrus cultivars in the peel and the pulp. The first principal component could explain 36.79% of the total variance observed for the parameters considered in the analysis. The second principal component explained 27.69% of the total variance. Ten of 21 citrus cultivars were clustered into 3 main subgroups, as can be seen from the scatter diagram plotted according to the first two components (Figure 5). For instance, 4 tangerine cultivars

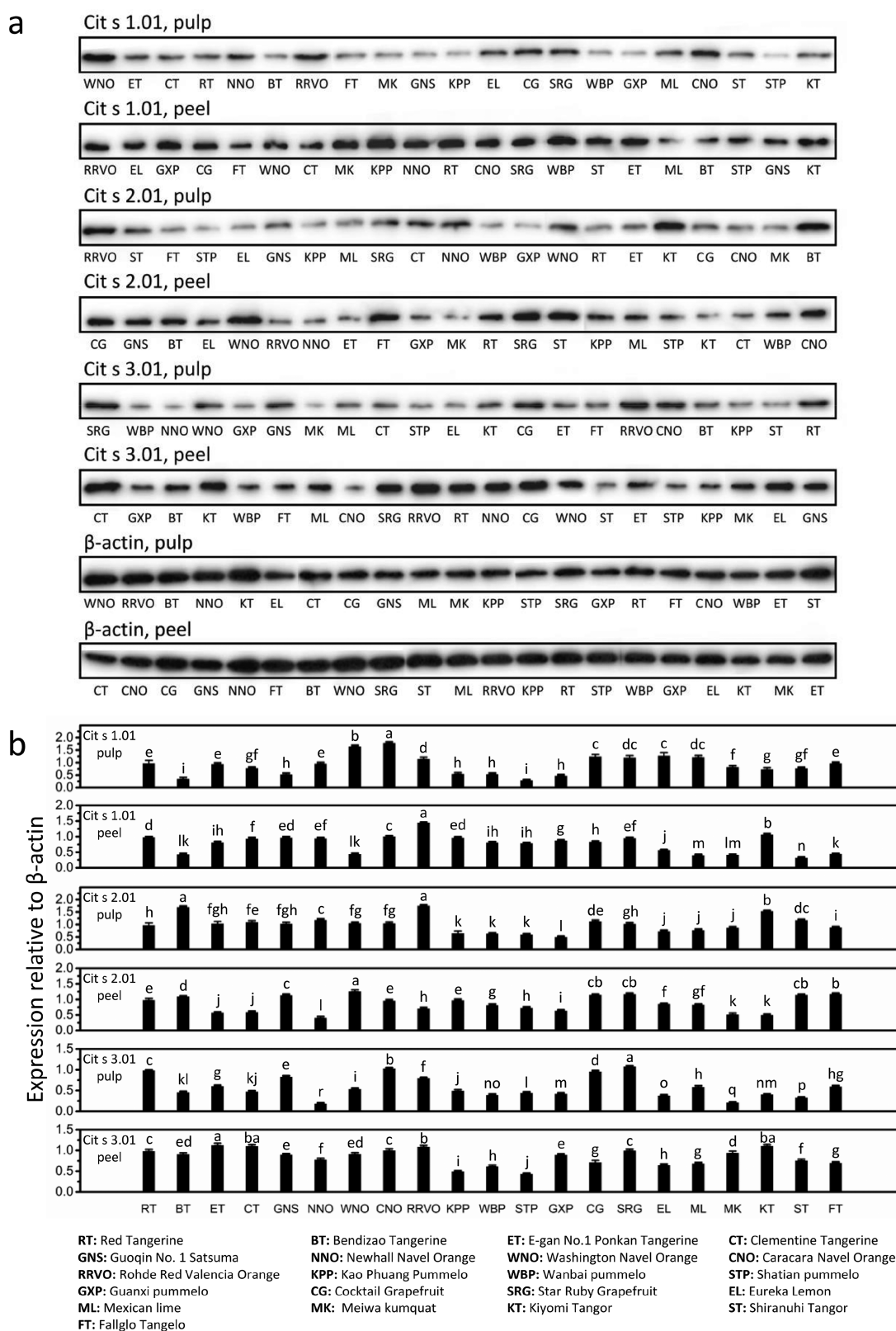


Figure 4. Immunoblotting quantification of Cit s 1.01, Cit s 2.01, and Cit s 3.01 in 21 citrus varieties listed in Table 1. (a) A representative image of the blots is shown on the chart. (b) The charts represent the intensity (as arbitrary units) of the bands measured in three sample replicates. Bars represent standard deviation. Letters indicate statistically different ($P \leq 0.05$; $n = 3$).

(Red Tangerine, Bendizao Tangerine, E-gan No.1 Ponkan Tangerine, and Clementine Tangerine), 4 pummelo cultivars (Kao Phuang Pummelo, Wanbai pummelo, Shatian pummelo, and Guanxi pummelo), and 2 grapefruit cultivars (Cocktail

Grapefruit and Star Ruby Grapefruit) were closely clustered, respectively. These three main subgroups were clearly discriminated. Accordingly, the pummelo subgroup might be potentially hypoallergenic compared with other citrus cultivars.

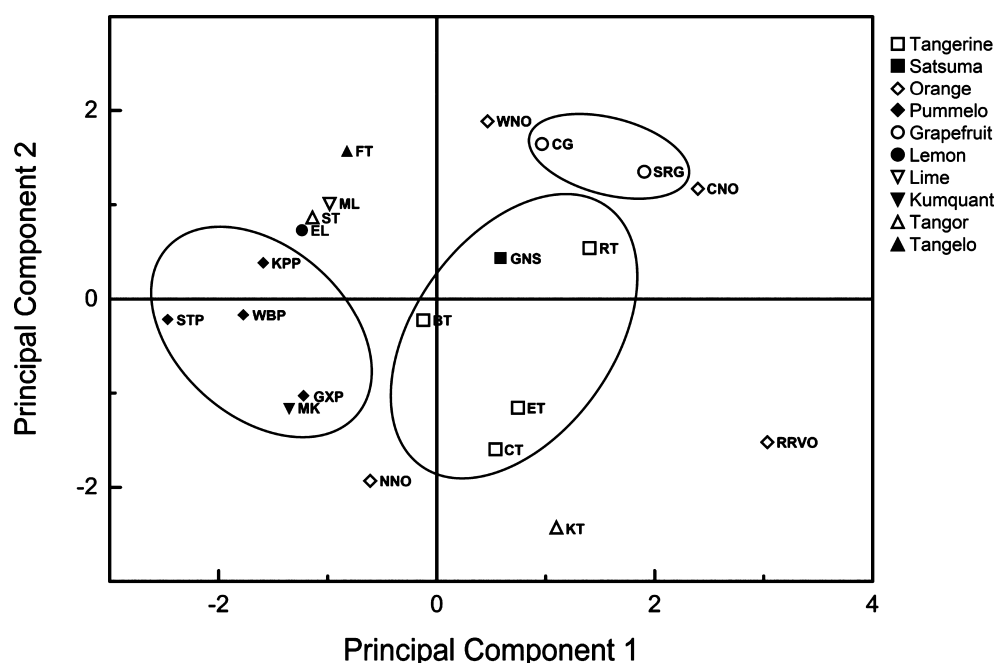


Figure 5. Principal component analysis (PCA) of all of the data concerning the quantification of three citrus allergen fractions in the 21 citrus varieties listed in Table 1. Three subgroups of varieties are identified according to the first two components.

A summary of the PCA results is also provided in the Supporting Information (Supplementary Tables 3 and 4).

IgE Immunoreactivity of 8 Citrus Cultivars. A pool of sera collected from orange allergic patients was used to evaluate the allergenicity of 8 selected citrus cultivars with these polyclonal antibodies (Figure 6). The immunoblotting of human IgE sera was evaluated by using the pulp protein of Newhall Navel Orange (Figure 6a). When the immunoblotting bands were quantified by densitometry, β -actin was also a loading control (Figure 6b,c).

For Cit s 1.01 in the pulp, the highest immunoreactivities were measured in Eureka Lemon and Clementine Tangerine, whereas the lowest were found in Cocktail Grapefruit, Guoqin No. 1 Satsuma, and Guanxi pummelo (Figure 6c). Moreover, the highest signals of Cit s 1.01 in the peel were detected in Caracara Navel Orange and Eureka Lemon, and the lowest were observed in Cocktail Grapefruit, Guanxi pummelo, and Shatian pummelo. About Cit s 2.01 in the pulp, the highest immunoreactivities were found in Clementine Tangerine and Guoqin No. 1 Satsuma, and the lowest were discovered in Cocktail Grapefruit, Guanxi pummelo, and Shatian pummelo. In the peel, the highest signals of Cit s 2.01 were detected in Caracara Navel Orange and Clementine Tangerine, whereas the lowest were examined in Guanxi pummelo, Cocktail Grapefruit, and Shatian pummelo. The highest Cit s 3.01 related immunoreactivities in the pulp were found in Clementine Tangerine and Guoqin No. 1 Satsuma, while the lowest were in Meiwa kumquat, Shatian pummelo, and Eureka Lemon. The highest levels of Cit s 3.01 in the peel were observed in Clementine Tangerine and Guoqin No. 1 Satsuma, and the lowest were detected in Shatian pummelo, Meiwa kumquat, and Eureka Lemon. The comprehensive analysis of the allergen immunoreactivity in 8 selected citrus cultivars indicated that the pummelo cultivars (e.g., Guanxi pummelo and Shatian pummelo) had great potential to be developed as hypoallergenic citrus fruits.

DISCUSSION

High epitope density in a single protein molecule significantly enhances antigenicity and immunogenicity.^{28,29} We innovatively designed and constructed three recombinant prokaryotic expression plasmids of PGEX-6p-1/GST-(Cit s 1.01) $\times$ 2, PGEX-6p-1/GST-(Cit s 2.01) $\times$ 3, and pET-32a(+)/Trx-His-(Cit s 3.01) $\times$ 2, which contained 2 tandemly repeated Cit s 1.01 genes, 3 tandemly repeated Cit s 2.01 genes, and 2 tandemly repeated Cit s 3.01 genes, respectively. Consequently, the recombinant fusion proteins, GST-(Cit s 1.01) $\times$ 2, GST-(Cit s 2.01) $\times$ 3, and Trx-His-(Cit s 3.01) $\times$ 2, contained twice, triple, and twice the epitope densities than the corresponding natural citrus allergens, respectively. Also, the single protein molecular weight of these recombinant fusion citrus allergens rose to theoretical 75097.08, 69364.25, and 41621.31 Da, which gave rise to the enhancement of their epitope densities. The sensitivity and specificity of rabbit polyclonal antibodies were tested by ELISA and immunoblotting (Figure 4). The previous studies had reported that different sizes of the immunoblotting bands of Cit s 1 in lemon had notably different isoforms or oligomers (dimers or trimers) in size,^{30,31} however, there was just one band for Cit s 1.01 in the immunoblotting (Figure 3), which may be caused by the high specificity of the anti-Cit s 1.01 polyclonal antibody. In fact, there were several weak bands with higher molecular weight than 24–25 kDa in the IgE immunoblotting (Figure 6a). The further study is warranted to confirm whether these band are isoforms or oligomers of Cit s 1.01.

Germin-like protein (Cit s 1.01),³² profilin (Cit s 2.01),³³ and nonspecific lipid transfer protein (Cit s 3.01)³⁴ are three highly conserved allergens among various plant foods based on similarities in amino acid sequences, which in turn might be associated with potential broad cross-reactivities of the anti-Cit s 1.01, anti-Cit s 2.01, and anti-Cit s 3.01 antibodies with other germin-like proteins, profilins, and nonspecific the lipid transfer proteins. Accordingly, the anti-Cit s 1.01, anti-Cit s 2.01, and

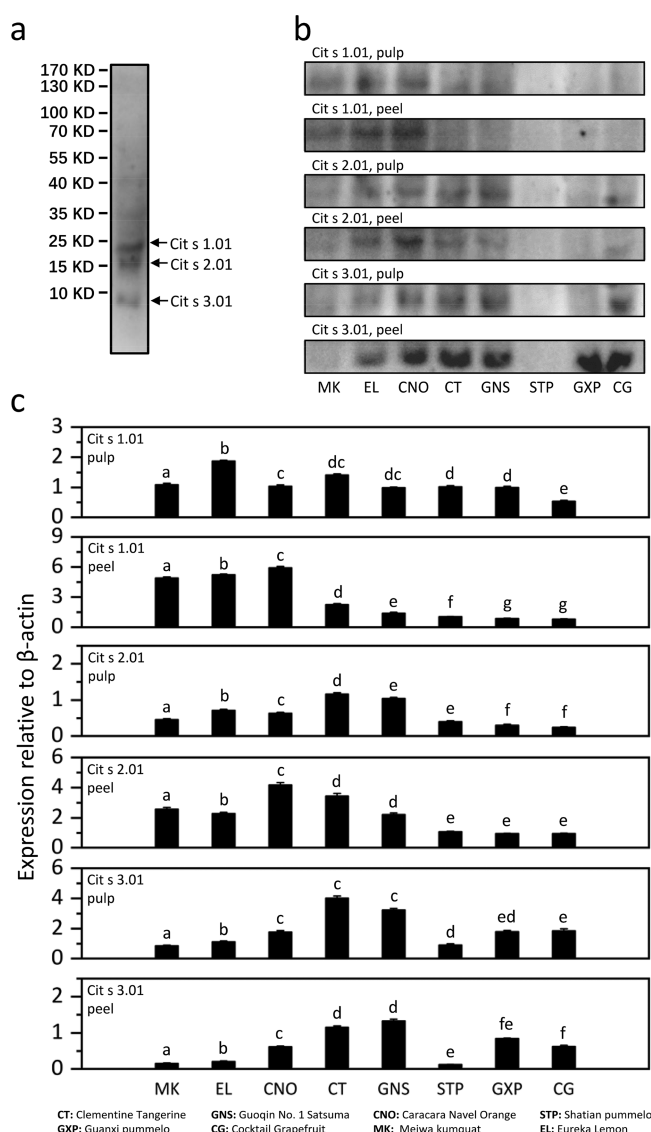


Figure 6. Immunoblotting quantification of Cit s 1.01-related, Cit s 2.01-related, and Cit s 3.01-related immunoreactivity with patients' sera. (a) The immunoblotting of the Newhall Navel Orange pulp protein with human IgE sera. (b) The image of the blots with patients' sera is shown in the chart. (c) The charts represent the intensity (as arbitrary units) of the bands measured in three sample replicates. Bars represent standard deviation. Letters indicate statistically different ($P \leq 0.05$; $n = 3$).

anti-Cit s 3.01 antibodies could be used to detect these allergens in other fruits and vegetables.

The most significant findings in the current study were the difference of the potential allergenicity among 9 subgroups (tangerine, satsuma, orange, pummelo, grapefruit, lemon, kumquat, tangor, and tangelo; Figure 4), in addition to the fact that the pummelo subgroup showed lower potential allergenicity along with a reduced variability of all of the measured parameters. The results were consistent with our previous reports as determined by a multiplex real-time PCR assay.¹⁸ Kao Phuang Pummelo could be considered as a potential low risk citrus fruit for consumers among 10 citrus cultivars (Red Tangerine, Bendizao Tangerine, Guoqin No. 1 Satsuma, Newhall Navel Orange, Washington Navel Orange, Caracara Navel Orange, Rohde Red Valencia Orange, Kao

Phuang Pummelo, Cocktail Grapefruit, and Star Ruby Grapefruit). Moreover, an immunoblotting quantification analyses with the sera pool of orange allergic patients further validate that Shatian pummelo and Guanxi pummelo were low in allergenicity among 8 citrus cultivars (Figure 6). Consequently, integrated analysis of the quantitative data from the multiplex real-time PCR assay, the immunoblotting with polyclonal antibodies, and the immunoblotting with the sera pool of allergic patients confirmed that pummelo cultivars (e.g., Shatian pummelo and Guanxi pummelo) could be considered as potential hypoallergenic citrus fruits, which are of great significance to the allergic consumers and breeders.

In succession, one unexpected result in the PCA chart is that the high variability of quantitative values of 4 orange cultivars (Newhall Navel Orange, Washington Navel Orange, Caracara Navel Orange, and Rohde Red Valencia Orange) are clustered apart, while another citrus subgroup clusters closely with one another (Figure 5). At present, several hundred sweet orange cultivars originated through mutations which change their horticultural characteristics with considerably commercial importance.^{35,36} The genetic variation of these sweet orange cultivars was higher than that of pummelo and mandarin, as sweet orange might originate from a backcross hybrid between pummelo and mandarin.³⁷ Accordingly, the diversity of agronomic traits, such as the size, color, type, and ripening season of the fruit, the number of seeds per fruit, and flowering time, among sweet orange cultivars is usually high. The diversity of morphological and genetical features may be the reason for clustering orange cultivars apart.

Notably, there is no linear relationship in the allergen amounts between the peel and the pulp, corresponding to Cit s 1.01, Cit s 2.01, and Cit s 3.01 protein measured with the polyclonal antibodies. In fact, the different expression in the peel and pulp of citrus allergens might be attributed to the environmental and genetic factors.^{18,38} Generally, the pulp is the edible part of citrus fruits, except for kumquat, whose peel and pulp can be eaten together. Although fewer amounts (Figure 4a) and weak immunoreactivity (Figure 5b) of Cit s 1.01, Cit s 2.01, and Cit s 3.01 are detected in the peel and pulp of kumquat, more attention should be paid to this potentially allergenic citrus fruit, for consumers with allergies to citrus fruits.

In conclusion, 21 citrus cultivars were collected and assessed for their potential allergenicity throughout an immunoblotting quantification approach. Three specific rabbit polyclonal antibodies produced for Cit s 1.01, Cit s 2.01, and Cit s 3.01 were facilitated to achieve the relevant and reliable quantification information of allergen in these citrus fruits. Accordingly, the data integration through PCA demonstrated that four pummelo cultivars (Kao Phuang Pummelo, Wanbai pummelo, Shatian pummelo, and Guanxi pummelo) were shown to be potentially hypoallergenic, comparing with other eight subgroups (including tangerine, satsuma, orange, grapefruit, lemon, kumquat, tangor, and tangelo). Moreover, the immunological analyses with a sera pool from orange allergic patients identified that Shatian pummelo and Guanxi pummelo were hypoallergenic among 8 citrus cultivars (Clementine Tangerine, Guoqin No. 1 Satsuma, Caracara Navel Orange, Shatian pummelo, Guanxi pummelo, Cocktail Grapefruit, Eureka Lemon, and Meiwa kumquat). As one of the healthy features for human beings, allergenicity in citrus should not be entirely "forgotten" with the accelerated genetic improvement. These findings could provide new perspectives and some advice

of citrus allergy for citrus fruit breeders, growers, merchants, and consumers.

■ ASSOCIATED CONTENT

■ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.7b05722.

Supplementary Table 1 shows the amino acid sequence and the optimized coding sequence of Cit s 1.01, Cit s 2.01, and Cit s 3.01. Supplementary Table 2 shows correlation matrix of the allergen amounts between the peel and pulp. Supplementary Tables 3 and 4 show eigenvalues of the correlation matrix and extracted eigenvectors for principal component analysis. Supplementary Figure 1 shows the identification of three recombinant citrus allergens by MALDI-TOF. (PDF)

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Notes

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■ ABBREVIATIONS USED

WHO-IUIS	World Health Organization and International Union of Immunological Societies
ELISA	enzyme-linked immunosorbent assay
PCR	polymerase chain reaction
AVONA	analysis of variance
PCA	principal component analysis
PMSF	phenylmethanesulfonyl fluoride
CVs	column volumes
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
MALDI-TOF	matrix-assisted laser desorption/ionization time of flight mass spectrometry
CBB	Coomassie brilliant blue
PBST	phosphate buffered saline with Tween 20
IPTG	isopropyl β -D-thiogalactoside

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Supporting Information belonging to

**Immunoblot Quantification Approach for Overall Assessing the Potential Allergenicity in
Citrus Fruits**

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Supplementary Table 1. The amino acid sequence and the optimized coding sequence of Cit s 1.01, Cit s 2.01 and Cit s 3.01

Name	Sequence
Cit s 1.01 [*]	KAIQFLIGFALLALASSLASASDPSPLQDICVAIEDPKDGVFVNGKFKDKPKLARAEDFFFSGLGKPGNTANRLGVDETDANVE
	QIPGLNLTGISAFRIDYAPYGQRPPHIHPRASEIFLVLEGTLYVGVFTSDQLNNTLIAKVLNKGDFVFPQGLIHFQFNIGKTNAAY AYSALNSQFPGEVTIADTVFGANPSINPDFLGKAFQLDPKIVKDLQNKFINGN
Cit s 2.01 [*]	SWQTYVDDHLMCDIDGHHLTSAIVGHDGSVWAQSSNFPQFKPEEIAAIMKDFEEPGLAPTGLHLGGTKYMVIIQEGPAV
	RGKKGSGGVTVKKTGQALIFGIYDEPLTPGQCNMIVERLGDYLIDQGL
Cit s 3.01 [*]	AALKLVCALLLCILVTAPVTNAITCGQVTASLAPCIPFLRTGGRFPPPPCCSGVRSLNGAARTTPDRQAACNCLKRAYGTIRGIK
	PNVAAGLPSQCGVRIPYKISPSTDCSRVR
Cit s 1.01 [†]	AAAGCGATCCAGTTCCTGATCGGTTTCGCGCTGCTGGCGCTGGCGTCTTCTCTGGCGTCTGCGTCTGACCCGTCTCCGCTG CAGGACATCTGCGTTGCGATCGAAGACCCGAAAGACGGTGTTTTTCGTTAACGGTAAATTCTGCAAAGACCCGAAACTGG CGCGTGCGGAAGACTTCTTCTTCTCTGGTCTGGGTAAACCGGGTAACACCGCGAACCCTGCTGGGTGTTGACGAAACCGA CGCGAACGTTGAACAGATCCCGGGTCTGAACACCCCTGGGTATCTCTGCGTTCCGTATCGACTACGCGCCGTACGGTCAGC GTCCGCCGCACATCCACCCGCGTGCGTCTGAAATCTTCTGTTCTGGAAGGTACCCTGTACGTTGGTTTCGTTACCTCTG ACCAGCTGAACAACACCCCTGATCGCGAAAGTTCTGAACAAAGGTGACGTTTTTCGTTTTCCCGCAGGGTCTGATCCACTTC CAGTTCAACATCGGTAAAACCAACGCGCGCGGCTACTCTGCGCTGAACTCTCAGTTCCCGGGTGAAGTTACCATCGCGG ACACCGTTTTTCGGTGCGAACCCTGCTATCAACCCGGACTTCCTGGGTAAAGCGTTCCAGCTGGACCCGAAAATCGTTAAA GACCTGCAGAACAAATTCATCAACGGTAACTAA
	TCTTGGCAGACCTACGTTGACGACCACCTGATGTGCGACATCGACGGTCACCACCTGACCTCTGCGGCGATCGTTGGTCA CGACGGTTCTGTTTGGGCGCAGTCTTCTAACTTCCCGCAGTTCAAACCGGAAGAAATCGCGGCGATCATGAAAGACTTC GAAGAACCGGGTTCTCTGGCGCCGACCGGTCTGCACCTGGGTGGTACCAAATACATGGTTATCCAGGGTGAACCGGGTG CGGTTATCCGTGGTAAAAAAGGTTCTGGTGGTGTTACCGTTAAAAAAACCGGTCAGGCGCTGATCTTCGGTATCTACGAC GAACCGCTGACCCCGGGTCAGTGCAACATGATCGTTGAACGTCTGGGTGACTACCTGATCGACCAGGGTCTGTAA
Cit s 2.01 [†]	GCGGCGCTGAAACTGGTTTGC GCGCTGCTGCTGTGCATCTGGTTACCGCGCCGGTTACCAACGCGATCACCTGCGGTCA GGTTACCGCGTCTCTGGCGCCGTGCATCCCGTTCTGCGTACCGGTGGTCGTTTCCCGCCGCCCGCTGCTGCTCTGGTGT
	TCGTTCTCTGAACGGTGCGGCGCGTACCACCCCGGACCGTCAGGCGGCGTGCAACTGCCTGAAACGTGCGTACGGTACC ATCCGTGGTATCAAACCGAACGTTGCGGCGGGTCTGCCGTCTCAGTGCGGTGTTTCGTATCCCGTACAAAATCTCTCCGTC TACCGACTGCTCTCGTGTTCTGTTAA
Cits3.01 [†]	

* The amino acid sequence

The optimized coding sequence

Supplementary Table 2. Correlation matrix of the allergen amounts between the peel and pulp

	Cit s 1.01 (pulp)	Cit s 1.01 (peel)	Cit s 2.01 (pulp)	Cit s 2.01 (peel)	Cit s 3.01 (pulp)	Cit s 3.01 (peel)
Cit s 1.01 (pulp)	1	0.00294	0.12789	0.24753	0.45413	0.27056
Cit s 1.01 (peel)	0.00294	1	0.25345	-0.31916	0.42708	0.35109
Cit s 2.01 (pulp)	0.12789	0.25345	1	0.01037	0.14896	0.5962
Cit s 2.01 (peel)	0.24753	-0.31916	0.01037	1	0.51626	-0.22234
Cit s 3.01 (pulp)	0.45413	0.42708	0.14896	0.51626	1	0.29091
Cit s 3.01 (peel)	0.27056	0.35109	0.5962	-0.22234	0.29091	1

Supplementary Table 3. Eigenvalues of the correlation matrix for principal component analysis

	Eigenvalue	Percentage of Variance	Cumulative
1	2.20744	36.79%	36.79%
2	1.66133	27.69%	64.48%
3	0.95108	15.85%	80.33%
4	0.73786	12.30%	92.63%
5	0.35286	5.88%	98.51%
6	0.08942	1.49%	100.00%

Supplementary Table 4. Extracted eigenvectors for principal component analysis

	Coefficients of PC1	Coefficients of PC2	Coefficients of PC3
Cit s 1.01 (pulp)	0.36767	0.35428	0.1903
Cit s 1.01 (peel)	0.39327	-0.32922	-0.66624
Cit s 2.01 (pulp)	0.43768	-0.22986	0.51388
Cit s 2.01 (peel)	0.09187	0.68933	0.10548
Cit s 3.01 (pulp)	0.49477	0.38537	-0.39545
Cit s 3.01 (peel)	0.51514	-0.29929	0.2972

Supplementary Figure 1. The identification of three recombinant citrus allergens by MALDI-TOF. These recombinant citrus allergens are Cit s 1.01 (a), Cit s 2.01 (b), Cit s 3.01 (c), respectively. Inset, effective peptides by MALDI-TOF are shown. Sequences in bold are the same as those in Cit s 1.01, Cit s 2.01, Cit s 3.01, based on MALDI-TOF result.

