

Development and Application of a Multiplex Real-Time PCR Assay as an Indicator of Potential Allergenicity in Citrus Fruits

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ABSTRACT: The effects of tissue type, harvest maturity, and genetic factors on the expression of genes that related to citrus fruit allergies remain poorly understood. In the present study, a multiplex real-time PCR assay was developed to monitor the expression of citrus allergen genes individually with the advantages of much fewer sample requirements and simultaneously multiple target genes detection. Gene specific primer pairs and Taqman probes of three citrus allergen genes *Cit s 1.01*, *Cit s 2.01*, and *Cit s 3.01* and the house-keeping gene β -actin were designed based on gene sequence differences. The PCR results showed that differential expression patterns were found during the ripening process. The expression levels of *Cit s 3.01* were much higher than those of *Cit s 1.01* and *Cit s 2.01* in both peel and pulp tissues among 10 citrus cultivars. Data suggested that Kao Phuang Pummelo could be safely consumed with a potential low risk in allergenicity. Considering that assessing allergenicity is one of the tests in food safety, this assay might also facilitate the breeding and production of “allergy-friendly” citrus fruits.

KEYWORDS: citrus allergen, tissues, harvest maturity, genetic factors, multiplex real-time PCR

INTRODUCTION

Citrus fruit, one of the most important fruits, can be consumed as fresh fruit, jam, and juice or used in cosmetic products.¹ The various bioactive compounds such as vitamin C, carotenoids, flavonoids, and limonoids in citrus play an important role in improving human health and preventing diseases, such as cardiovascular disease, asthma, diabetes, and cancer, with their antioxidant, anti-inflammatory, and signaling properties.^{2–5} However, there is growing evidence that citrus fruits are one of the important allergic plant foods in the public perception surveys of food allergy.^{6–8} Three main citrus species, including sweet oranges (*Citrus sinensis*), tangerines (*Citrus reticulata*), and lemons (*Citrus lemon*) are recognized as sources of allergenic reactions for human beings as documented by the World Health Organization and the International Union of Immunological Societies (WHO-IUIS). These allergic reactions caused by the consumption of citrus fruits vary from a mild oral allergy syndrome to severe anaphylaxis cases.⁹ Currently, there is no approved therapeutics for the treatment of food allergy other than avoidance.^{10,11} Considering the serious negative effects of allergens from citrus fruits on the life quality of allergic patients, developing a sensitive and efficient analytical technique as an indicator of potential allergenicity in citrus fruits is beneficial for the choice of citrus fruits with no or low allergen content.

Cit s 1, *Cit s 2*, and *Cit s 3* are the three major orange allergens recently identified. *Cit s 1* is a 24 kDa germin-like protein (GLP) recognized by patients' sera IgE.¹² Orange profilin *Cit s 2* is another highly prevalent allergen based on its unexpectedly high reactivity in vitro and in vivo.¹³ *Cit s 3*, a member of the lipid transfer protein (LTP) pan-allergen family, behaves as a minor allergen (approximately 35% prevalence).^{12,14,15} *Cit l 3* and *Cit r 3* have been recognized as allergens in lemon and tangerine, respectively.^{16,17} Actually, recent research suggests that *Cit s 1*, *Cit s 2*, and *Cit s 3* from sweet orange may have different individual isoforms, leading to great difficulties in specifically quantifying

citrus allergens. A study of *Cit s 1* in the flavedo of lemon indicated that *Cit s 1* could be present in different isoforms ranging from 20 to 120 kDa.¹⁸ Eight putative allergen genes from mandarin have been identified, including three *Cit c 2* and five *Cit c 3*.¹⁹ The in silico analysis revealed that 15 new sequences belong to GLPs (*Cit cl 1*), two more belong to nsLTPs (*Cit cl 3*), and other new sequences belong to profilins (*Cit cl 2*) by searching the citrus genome database (<http://www.citrusgenomedb.org/species/clementina>).²⁰ These studies indicated that *Cit s 1* may be a complex gene family composed of 15 different isoforms with high similarity of sequences, each of which codes for a different isoallergen. Meanwhile, *Cit s 2* and *Cit s 3* have three and seven different isoforms, respectively. It has been reported that isoallergens from other fruits might differ greatly in their allergenic properties.^{21,22} Thus, distinguishing targeted allergen genes from their isoallergen genes warrants the development of a quantification method for the expression of citrus allergen genes.

Currently, enzyme-linked immunosorbent assay (ELISA), quantitative real-time polymerase chain reaction (real-time PCR), and liquid chromatography–tandem mass spectrometry (LC–MS/MS) are precise and sensitive methods for the detection of allergen in food products.²³ Proteomics has primarily been applied to quantify the amounts of clementine allergen but failed to distinguish isoallergens or variants.²⁰ Few recombinant allergens and commercial antibodies for fruits and vegetables have been obtained,²⁴ especially for citrus fruits. Based on the polyclonal antibody and monoclonal antibody with a strong immunoreactivity against *Cit s 2*, a sandwich ELISA has been developed for the assessment of profilin concentration in 12 citrus fruits, without distinguishing isoallergens of *Cit s 2*.²⁵ In addition, the

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Table 1. Species of 10 Citrus Cultivars^a

cultivars	abbreviation	Citrus species	commercial harvest
Red Tangerine	RT	<i>Citrus tangerine</i> Tanaka	November to December
Bendizao Tangerine	BT	<i>Citrus succosa</i> Hort. ex Tanaka	first–third week of November
Guoqin No. 1 Satsuma	GNS	<i>Citrus unshiu</i> Marc.	first–third week of October
Newhall Navel Orange	NNO	<i>Citrus 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>C. 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
Cocktail Grapefruit	CG	<i>Citrus paradisi</i> Macf.	second–fourth week of December
Star Ruby Grapefruit	SRG	<i>C. paradisi</i> Macf.	second–fourth week of December

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

production of antibodies against *Cit s 1* or *Cit s 3* has not been reported so far. For these reasons, it is difficult to identify isoallergens or variants in citrus fruits specifically through immunological detection at the protein level.

Real-time PCR is a fast, highly sensitive, and reproducible technique to study gene expression. The applications of real-time PCR have been reported for the detection of allergen DNA fragments or coding sequences in Brazil nut,²⁶ almonds,²⁷ hazelnut, and soy.²⁸ Moreover, allergy genes of several fruits such as apple²⁹ and peach³⁰ have the features of tissue- and cultivar-specific expression and revealed differential effects under certain environmental conditions.^{31,32} However, the previous studies on citrus allergy were rarely involved in the expression of citrus allergy genes. Semiquantitative reverse transcription PCR and real-time PCR indicated that *Cit s 1*, *Cit s 2*, and *Cit s 3* present different patterns in the expression of clementine allergens in ungerminated and germinated pollens. *Cit s 1* and *Cit s 3* are two common allergens expressed in citrus fruit and pollen, while *Cit s 2* is present in citrus fruit and not detectable in pollen.³³ However, previous studies have neither covered the full set of orange

allergens nor developed the PCR primer pairs for *Cit s 1*, *Cit s 2*, and *Cit s 3* which were identified and submitted to WHO-IUIS.

Knowledge of regularities of allergen gene expressions in citrus fruits would provide useful information to citrus fruit breeders, growers, and consumers for the selection of hypoallergenic genotypes and the consumption of fruits with low amounts of allergens. The application of real-time PCR methodology to develop an indicator of potential allergenicity in citrus fruits is ideal. Therefore, the aim of this study is to (i) develop a highly sensitive and reproducible real-time PCR assay for the simultaneous detection of citrus allergen genes and (ii) evaluate the expression of allergen genes (*Cit s 1.01*, *Cit s 2.01*, and *Cit s 3.01*) in the pulp and peel of 10 citrus cultivars during harvest maturity.

MATERIALS AND METHODS

Plant Material. Ten citrus cultivars used in this paper are shown in Table 1. Unripe (2 weeks before predicted optimal harvest date), ripe, and overripe (2 weeks after harvest of ripe fruits) citrus fruits were obtained from citrus trees grown at the National Center for Citrus Breeding, Huazhong Agricultural University. Peels and pulps of the fruits were separated with scalpels, frozen immediately in liquid nitrogen, and stored at -80°C for further analysis.

RNA Extraction and cDNA Synthesis. Total RNA was isolated from plant materials (peels and pulps) using the Trizol reagent (Invitrogen, Carlsbad, CA). Contaminating DNA was digested with DNase I (Invitrogen, Carlsbad, CA). Total RNA was reverse transcribed with random hexamers using a Prime-Script first-strand cDNA synthesis Kit (Takara, Dalian, China). The concentration of cDNA samples was quantified using a Nano-Drop 1000 spectrophotometer (Thermo Scientific, Wilmington, U.S.A.).

Primer and Probe Design. The information on sweet orange allergens was obtained from the WHO-IUIS (<http://www.allergen.org>). Coding DNA sequences were retrieved from the sweet orange genome (<http://citrus.hzau.edu.cn/orange/>) and a BLAST search in the NCBI database using GenBank Protein Accessions of sweet orange allergens obtained from the WHO-IUIS. To identify new members of citrus allergen gene families in silico analysis of the genome information, two steps were required. First, nucleotide and protein sequences of citrus allergens were used to blast the sweet orange genome based on the calculating statistical significance of matches. According to the level of similarity, new homologous citrus allergen genes were likely to be identified. Second, protein domains were also an

Table 2. Sequence of Primers and Probes

gene		primer and probe sequences (5'-3')	length (bp)	product size (bp)	T _m (°C) ^a
<i>Cit s 1.01</i>	F	ACCCTTGGCATATCAGCATT	20	97	54.5
	R	CCAGGACTAAGAAGATTTCCG	21		53.1
	P	(FAM)-ACATTCACCCCCGTGCCAGCGAAATCT-(Eclipse)	27		66
<i>Cit s 2.01</i>	F	CGGTTCTCTTGCCCCAACTGGT	22	130	61.9
	R	GGCTTGGCCCGTTTCTTGACG	22		61.5
	P	(Cy3)-ACTGGTTTGCACCTTGGAGGCACCAAGTACA-(BHQ2)	31		66.1
<i>Cit s 3.01</i>	F	GAGGGCGTTTCTCCTCACCGC	21	124	63.1
	R	CCGCGGATTGTTCCGTAAGCTC	22		61.4
	P	(Cy5)-ATGCTGTAGCGCGTCAGGTCTCTCAA-(BHQ2)	27		65.5
β -actin	F	CCAAGCAGCATGAAGATCAA	20	100	53.3
	R	TCTGCTGGAAGGTGCTGAG	19		57.1
	P	(JOE)-TTTGATTGGAGGATCAATCCTTGCATCCCT-(BHQ1)	30		62.9

^aT_m: melting temperature.

Cit s 1

Cs5g25680 ACCCTGGCATATCAGCATTCCGTATTGACTATGCACCATATGGCCAAAGGCCACCTC**ACATTACCCCGTGGCAGCGAAATCTTCTTAGTCCTGG** 97
 Cs5g25740 C G C T T C. GC. G 97
 Cs5g25760 G G T T C. G. G 97
 Cs5g25660 G GA G C G T C. GC. G 97
 Cs5g25700 C G C G A T C. GC. G 97
 Cs5g25790 .. T G C G C T AT C CT C. GC. G 97
 Cs5g25770 .. T C G C G C AT C T CT C. GC. G 97
 Cs5g25810 G C AC CG T G CT G C. GC. G 97
 Cs5g25800 G C AC CG T G CT G C. GC. G 97
 Cs5g14290 .. T. C. A G A C AC T C T CT C. GACG T 97
 Cs5g14210 .. T. C. A G A C AC T C T CT C. GACG T 97
 Cs5g14180 .. T. C. A G A C AC T C T CT C. GACG T 97
 Cs5g14330 .. T. C. A G A C AC T C T CT C. GACG T 97
 Cs5g14230 .. T. C. A G A C AC T C T CT C. GACG T 97
 Cs5g14300 .. T. C. A G A C AC T C T CT C. GACG T 97
 Cs5g25820 .. T. C. TG. C. T. TG. T. C. T T AC C T CT C. GC. G 97

Cit s 2

Cs1g15890 CGGTCTCTTGGCCCA**ACTGGTTTGCACCTTGAGGCACCAAGTACAT**GGTAATCCAGGTTGAGCCTGGAGCTGTGATTGGTGAAGGTTCTGGT 100
 Cs4g12970 T... CA T. C. C. C A. A T A. G C. C G A. C. A 100
 Cs4g12960 T... AA. A. A. A AC. T. T T A. A. T G A. T C. C. A. G. G CC 100

Cs1g15890 GGTGTACCGTCAAGAAACGGGCCAAGCC 130
 Cs4g12970 .. A A. A G. C. T. G. T 130
 Cs4g12960 T... A G. CA. TGGC... 130

Cit s 3

Cs6g09940 GAGGGCGTTTTCCTCCACCGCCATGCTGTAGCGGCGTCAGGTCTCTCAACGGCGCCGCCAGAACCCCTGACCGCCAAGCTGCATGCAACTGCTTGA 100
 Cs6g09950 .. C. C. C. A. A. . GT C. A CT A A 97
 Cs6g09960 CGC. G. G. G. ---AG. C GA. T. A. AG. . T. TC. A. . CC. . A. C. GAGC. TT 97
 Cs6g09970 .. C. GCCG. GA. GA. ---. AAC. . T. C. A. A. G. A. A. T. AAT. TC. GT. AG. T. GA. C. TG. T. CG. 97
 Cs7g13570 .. AAT. CAGCGG. . G. ----C. GAT. . GT. G. A. AA. G. C. ATAA. TCCC. TG. A. A. GT. . A. G. TG. T. 94
 Cs7g12190 .. AAC. C. GACG. . G. ----. CGAT. . T. G. A. AA. GA. . ATTA. TCC. G. T. TG. G. G. GG. . G. G. A. . G. 94
 Cs7g01050 .. GAAA. CACCAAG. . G. ----. T. C. T. A. C. TCAG. TGCATCTA. T. A. C. . G. TG. TAAGA. A. C. T. . TGA. . A. T. 94

Cs6g09940 ACGAGCTTACGGAACAATCCGCGG 124
 Cs6g09950 G. A. CGC. T. CTAA 121
 Cs6g09960 .. ACC. GCT. . CT. C. A. AAA 121
 Cs6g09970 .. A. GT. A. TGG. TCGTA 121
 Cs7g13570 GGC. GCT. CTCGC. A. . C. AA 118
 Cs7g12190 GGC. GCT. CTCGCTA. . CTAA 118
 Cs7g01050 GAC. AGC. ---CAG. AAATGAA 115

Actin

Cs1g05000 CCAAGCAGCATGAAGATCAAGGTCGTGGCTCCACCAGAGAGAAAATACAGTGT**TTGGATTGGAGGATCAATCCTTGCATCCCTCAGCACCTTCCAGCAGA** 100
 1. It02236 T G. A. A G. G C A C. G. . AT. G. T. 100
 Cs8g18400 A. T. A. G. C. T. G. T. C. C. . T. A. T. 100
 Cs6g06250 T. G. T. T. C. G. T. C. A. C. TT. G. T. 100
 1. It02699 .. T. T. A. T. T. C. A. G. C. T. T. T. A. T. A. 100
 Cs7g29220 T A A T G. G. T C A C C T. G. T. T. T. A. 100

Figure 1. Sequence alignment and location of the designed primers and probes. Cs5g25680, Cs1g15890, Cs6g09940, and Cs1g05000 marked in bold are the accession numbers of *Cit s 1.01*, *Cit s 2.01*, *Cit s 3.01*, and *CitACT7*, respectively. Partial SNPs specific to the target genes are also marked in bold. The primers are shown in forward arrow and reverse arrow, and the probes underlined.

important determinant for new citrus allergens since they contained Cupin_1 domain, Profilin domain, and Tryp_alpha_amyl domain, respectively. The utilization of Web site tool (<http://pfam.xfam.org/search>) facilitated selection and screening. Additionally, β -actin (*CitACT7*)³⁴ was chosen as a housekeeping gene to be used as an internal loading control. In order to quantify β -actin (*CitACT7*) accurately, similarity sequences of β -actin were also analyzed in silico. The coding sequences of sweet orange allergen genes and β -actin were aligned using the BioEdit v7.2.5 and manually adjusted when necessary. Single nucleotide polymorphisms (SNP) among the genes *Cit s 1.01*, *Cit s 2.01*, and

Cit s 3.01 were identified separately as reference materials for designing the primers and probes.

Available primer pairs and gene-specific Taqman probes were designed using Oligo 7 primer analysis software.³⁵ The length of the amplicons should be 50–150 bp for optimal PCR efficiency. At least one of the targeted gene-differentiating SNPs or specific haplotype was located at the probe region to ensure the specific hybridization of the probe with the target gene. In addition, the strategy to improve the success rate of TaqMan real-time PCR was to design and verify the primer sets from the various regions of the gene sequences of sweet orange allergens. Regions of identity

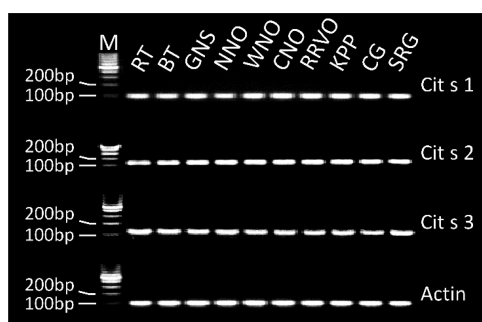


Figure 2. Agarose gel electrophoresis of PCR products obtained with the primer pairs designed on allergen genes. The expected length for *Cit s 1.01*, *Cit s 2.01*, *Cit s 3.01*, and β -actin are 97, 130, 124, and 100 bp, respectively. Lane 1: Marker, lane 2: Red Tangerine (RT), lane 3: Bendizao Tangerine (BT), lane 4: Guoqin No. 1 Satsuma (GNS), lane 5: Newhall Navel Orange (NNO), lane 6: Washington Navel Orange (WNO), lane 7: Caracara Navel Orange (CNO), lane 8: Rohde Red Valencia Orange (RRVO), lane 9: Kao Phuang Pummelo (KPP), lane 10: Cocktail Grapefruit (CG), lane 11: Star Ruby Grapefruit (SRG).

were surveyed for possible cross-hybridization with other allergen genes and confirmed by the search against the sweet orange genome database³⁶ using the BLAST program. The probes and primers were synthesized by BGI Tech Solutions Co., Ltd. (Beijing, China). Their sequences are shown in Table 2.

Specificity Test. The specificity of designed primers and TaqMan probes was validated in three ways. First, the sequences of primers and probes were blasted with sweet orange genome in silico to ensure that these primers perfectly matched only with sequences corresponding to the target genes. Second, the specificity of the primer pairs for their target genes was confirmed by conventional PCR and agarose gel electrophoresis analysis. Ten citrus cultivars (Table 1) were chosen as templates for conventional PCR to check the generality of the designed primers. The PCR reactions were carried out in the 20 μ L reaction system consisting of 10 ng of cDNA, 0.25 μ M each primer, and 2 $\times$ Taq Master Mix (Vazyme Biotech, China) on a T100 Thermal Cycler (Bio-Rad, U.S.A.) under the following PCR program: an initial denaturation step at 94 $^{\circ}$ C for 5 min, followed by 35 cycles of denaturation at 94 $^{\circ}$ C for 30 s; annealing at 55 $^{\circ}$ C for 30 s; elongation at 72 $^{\circ}$ C for 20 s; last step at 72 $^{\circ}$ C for 5 min. Amplification products were electrophoresed on 2.0% agarose gels in 0.5 $\times$ TBE buffer at 100 V for approximately 30 min and stained with EtBr for visualization. The reproducibility of PCR patterns was verified by triplicate experiments. Finally, the direct sequencing of the amplicons obtained from the 10 citrus cultivars was performed to ensure that only the specific target sequences were amplified and highly conserved (Figure 3). Sequencing of the PCR products was performed by BGI Tech Solutions Co., Ltd. DNA sequences were edited using Chromas Pro 1.22 software (Technelysium Pty, Ltd., Australia). Alignments between sequences of the same amplicons obtained from the 10 citrus cultivars were performed using the BioEdit v7.2.5.

Analytical Sensitivity and Amplification Efficiency. Gene synthesis with the sequences shown in Figure 1 was applied to produce the template DNA for TaqMan real-time PCR which was done by Wuhan GeneCreate Biological Engineering Co., Ltd. (Wuhan, China). The target DNA fragments were inserted into pUC19 vector. The plasmid DNA was normalized to 20 ng/ μ L with deionized water for a consistent TaqMan real-time assay. Nucleic acid concentrations were measured using a NanoDrop 1000 spectrophotometer. The analytical sensitivity

of the assays was established with dilutions of four gene templates: pUC19-*Cit s 1* DNA, pUC19-*Cit s 2* DNA, pUC19-*Cit s 3* DNA, and pUC19-*actin* DNA. DNA template mix was composed of these gene templates with an equivalent amount for the multiplex real-time PCR assay. The templates were diluted to 20 ng/ μ L (corresponding to 20 ng of DNA per PCR tube) followed by 10-fold dilutions down to 0.2 fg/ μ L with 20 ng/ μ L pUC19 DNA solution, which kept the total DNA amount constant. These tests were performed for both the singleplex and multiplex assays. The limit of detection (LOD) was determined as the lowest concentration at which amplification occurred for all three replicates. Slope, PCR efficiency (E), and correlation coefficient (R^2) were calculated according to the plotted points from the establishment of LOD. The corresponding real-time PCR amplification efficiencies were calculated according to the equation.³⁷

Real-Time PCR Condition. TaqMan real-time PCR reactions were performed with an Applied Biosystems 7500 Real Time PCR System using AceQ U+ Probe Master Mix (Vazyme Biotech, China). This Mix contains heat-labile uracil–DNA glycosylase (UDG). Treatment with heat-labile UDG is useful for preventing the reamplification of carryover PCR products.³⁸ All reactions were performed in biological and technical triplicate. On each plate, at least two nontemplate controls were used to check the PCR performance. An average of technical triplicate was performed for each sample, while the biological triplicate was analyzed independently. The 20 μ L reaction volume contained 10 μ L of AceQ U+ Probe Master Mix, 1 μ L of template DNA, 0.4 μ L of each primer (10 μ M), 0.2 μ L of Taqman probe (10 μ M), and 8 μ L of ddH₂O. The reactions were cycled as follows: 37 $^{\circ}$ C for 2 min, 95 $^{\circ}$ C for 5 min, 95 $^{\circ}$ C for 10 s, and 60 $^{\circ}$ C for 40 s (40 cycles). All reactions were performed according to the standard manufacturers' protocols. Results were analyzed using 7500 System SDS software. Raw data were analyzed using the default settings of the software.

Optimization of Singleplex and Multiplex Real-Time PCR Assays. Differences in C_t between single and multiplex reactions can often be mitigated by adjusting primer and probe concentrations. For each singleplex assay, optimized conditions were the 20 μ L reaction volume consisting of 10 μ L of AceQ U+ Probe Master Mix, 1 μ L of template DNA, 0.4 μ L of each primer (10, 20, or 30 μ M), 0.2 μ L of Taqman probe (10, 20, or 30 μ M), 0.2 μ L of ROX Reference Dye II, 7 μ L of ddH₂O, and an additional 0.8 μ L of MgCl₂ (25 mM). The same primer sequences for the singleplex reactions were used for the multiplex assay, but all oligonucleotides were pooled in a single tube with optimal primer concentrations from singleplex reactions. A similar real-time PCR procedure was followed for the Taqman probe assay. The primer mix and probe mix obtained were applied to the final multiplex reaction. These reactions were run in the ABI 7500 Real-Time PCR system.

Statistical Analysis. The relative expression level in this study was calculated using the $2^{-\Delta\Delta C_t}$ method.³⁹ 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, NY). Origin Pro 8.0 SR4 (Origin Lab, Northampton, MA) was used to analyze data and make graphs.

RESULTS AND DISCUSSION

Development and Optimization: Method of Establishing a Specificity and Sensitivity Multiplex Real-Time PCR Assay. Primer and Probe Design. The development of a

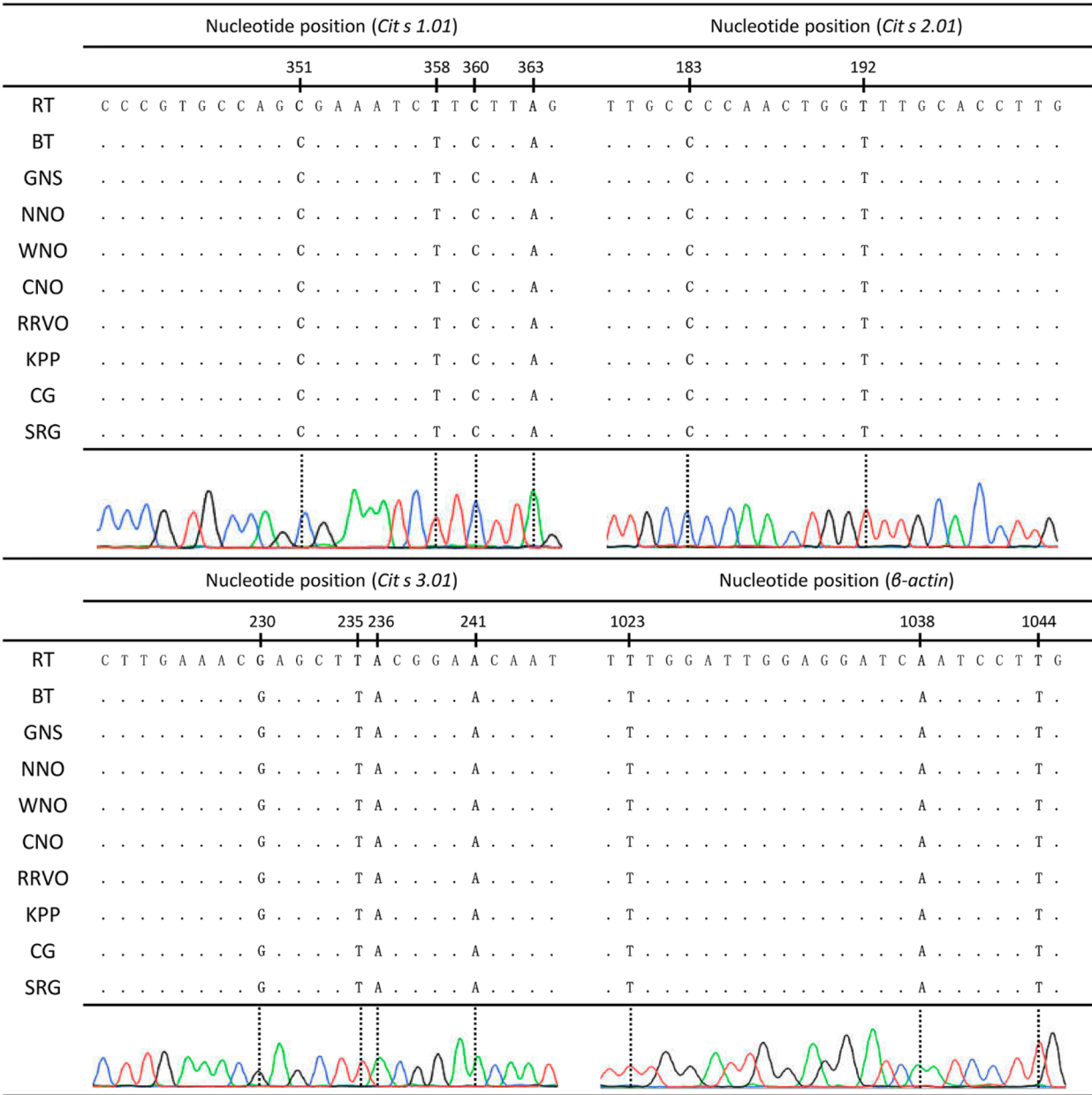


Figure 3. Diagnostic nucleotide analysis. Consensus sequences from the 10 citrus samples are aligned with the base calls and trace file peaks obtained from sequenced *Cit s 1.01*, *Cit s 2.01*, *Cit s 3.01*, and β -actin with specific primers. Sequence fragments shown do not include the primer binding regions. The nucleotide position is relative to the gene-differentiating SNP position of allergen coding genes, and the shaded regions indicate diagnostic nucleotide sites. A, adenine, green; T, thymine, red; C, cytosine, blue; G, guanine, black.

multiplex real-time PCR assay for citrus allergen genes began with the design of specific primers and probes. The accession numbers of *Cit s 1.01*, *Cit s 2.01*, *Cit s 3.01*, and *CitACT7* were Cs5g25680, Cs1g15890, Cs6g09940, and Cs1g05000, respectively. In addition to the original *Cit s 1.01*, *Cit s 2.01*, *Cit s 3.01*, and β -actin, 15 *Cit s 1* genes, 2 *Cit s 2* genes, 6 *Cit s 2* genes, and 5 β -actin genes were discovered (Figure 1). Data of multiple sequence alignments were used to design the specific real-time PCR primers and probes from each of the target sequences. For example, the reverse primer and the probe of Cs5g25680 (*Cit s 1.01*) are located in a partial coding region, with four SNPs and one SNP differentiating the *Cit s 1.01* gene respectively from all other genes (Figure 1). The probe selection is based on estimated the melting temperature (T_m), which was identified to be within 65 °C (± 3 °C). To develop a multiplex real-time PCR

assay, four probes used in the reactions were labeled with different reporter dyes (FAM, Cy3, Cy5, and JOE) and nonfluorescent quenchers (Eclipse, BHQ1, and BHQ2). The primers and probes for each of the genes are listed in Table 2.

Specificity. To set up the specific real-time PCR systems, the fragments of citrus allergen genes and β -actin were amplified from the cDNA of 10 citrus cultivars. Only one band shown in Figure 2 confirmed that the designed primers amplified a single fragment with the expected length (*Cit s 1.01*, 97 bp; *Cit s 2.01*, 130 bp; *Cit s 3.01*, 124 bp; β -actin, 100 bp). Next, the direct sequencing of the amplicons obtained from the 10 citrus cultivars was performed. Many nucleotide positions that were relative to the gene-differentiating SNP positions of allergen and β -actin coding genes were marked in Figure 3: four nucleotide positions for *Cit s 1.01* (351, 358, 360, and 363), two nucleotide positions

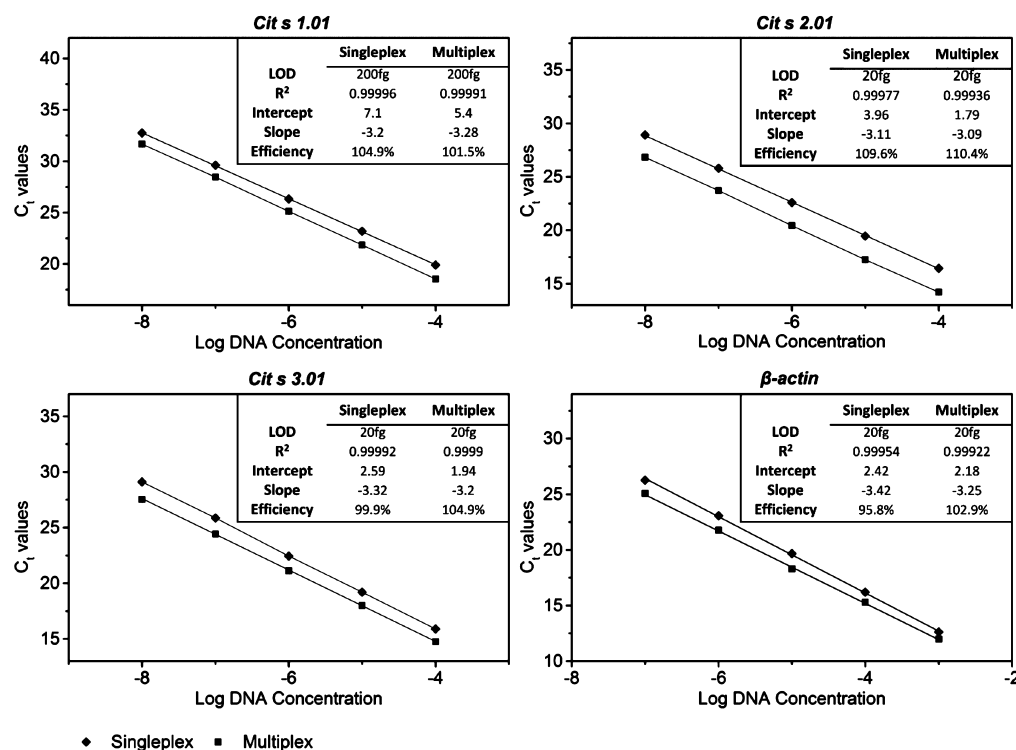


Figure 4. Comparison of singleplex and multiplex performance. ◆, standard curves of singleplex; ■, standard curves of multiplex. C_t value = mean $\pm$ SD, $n = 3$.

for *Cit s 2.01* (183 and 192), four nucleotide positions for *Cit s 3.01* (230, 235, 236, and 241), and three nucleotide positions for β -actin (1023, 1038, and 1044). Thus, the primer pairs that passed all the aforementioned validation tests had high specificity and good universal applicability for further quantitative analysis of 10 citrus cultivars.

Final Protocol of the Multiplex Real-Time PCR Assay. Multiplex real-time PCR is a variant of real-time PCR which enables amplification and quantification of multiple targets in one reaction using more than one pair of primers/probes. Compared to singleplex real-time PCR systems, multiplex real-time PCR assays offered multiple target detection in a single assay platform, reducing both cost and time.⁴⁰ In this study, a multiplex real-time PCR assay was established. First, four optimal singleplex systems were established by selecting the primer and probe concentrations with a suitable amplification efficiency ($90\% < E < 110\%$). The final concentration calculated for the pair of primers in the singleplex system was 20 nM (*Cit s 1.01*), 10 nM (*Cit s 2.01*), 10 nM (*Cit s 3.01*), and 10 nM (β -actin), respectively. The optimal probe concentration was 20 nM for four genes because high fluorescence led to high feasibility with a low number of gene copies. After verification of singleplex reactions, the multiplex reaction containing up to eight primers and four probes was performed to produce four amplicons simultaneously.

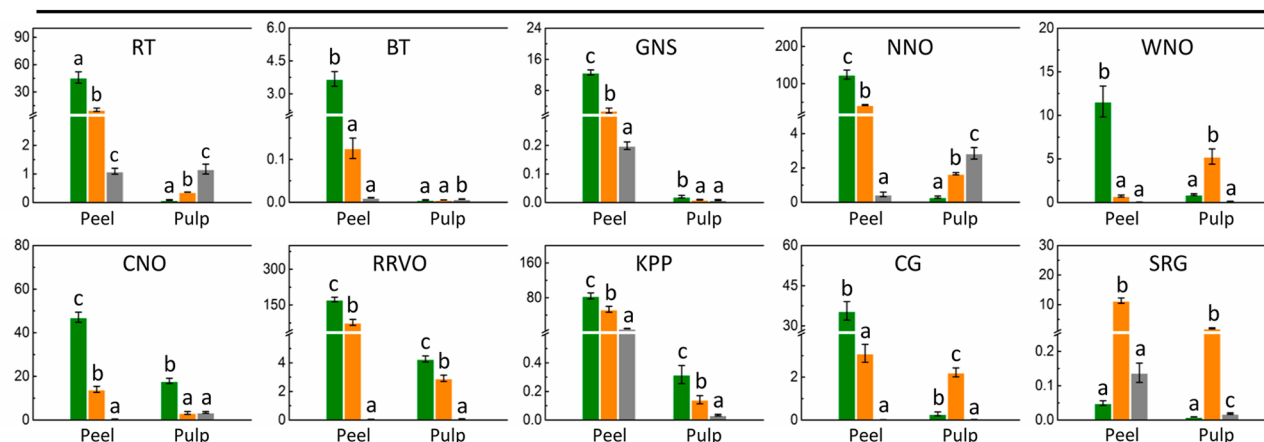
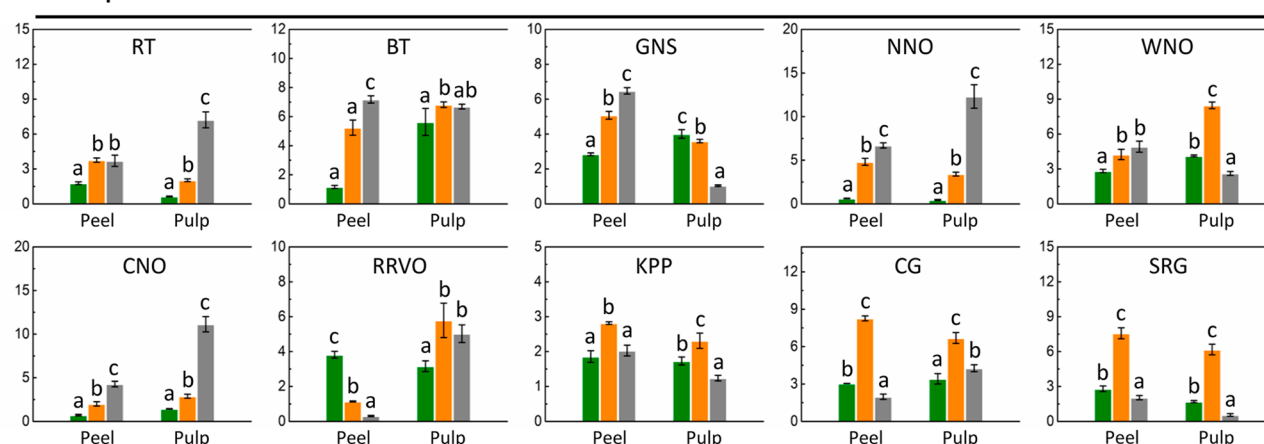
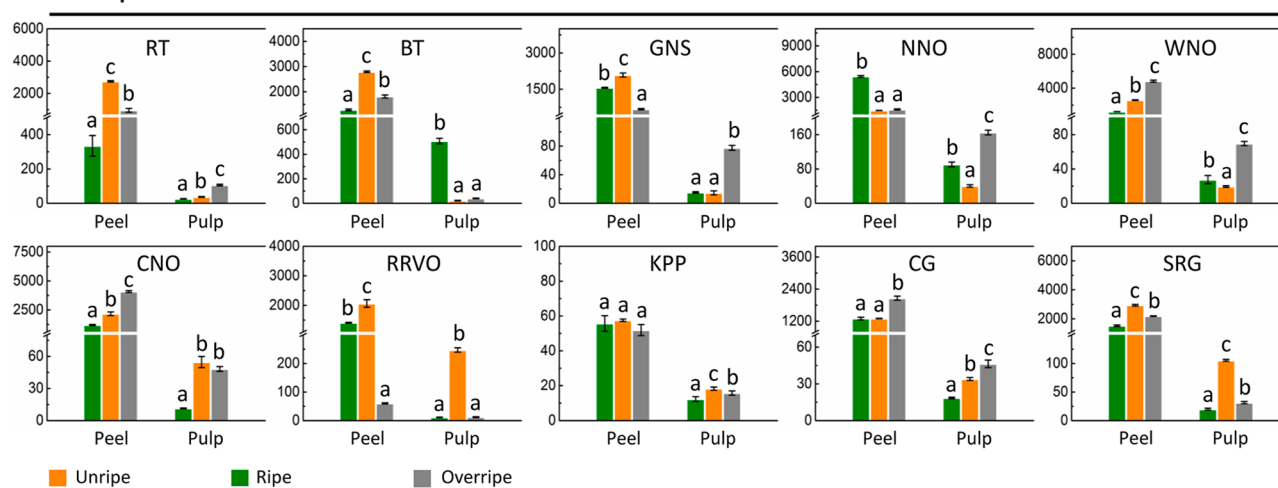
Amplification Efficiency and Analytical Sensitivity. The limit of detection (LOD), correlation values (R^2), slopes, and efficiency (E) of singleplex and multiplex assays are shown in Figure 4. The standard curves were of high quality, as indicated by correlation relationships ($R^2 > 0.999$) between the input DNA amounts and the C_t values across the standard samples (serial dilutions). The PCR efficiencies of singleplex and multiplex assays were in the range from 95.8 to 109.6% and 101.5 to 110.4%. Acceptance criteria were as follows: PCR efficiencies between 90 and 110% (corresponding to a slope of regression between -3.1 and -3.6). For the multiplex assay, the slope of the

regression curve should be between -3.9 and -2.9 corresponding to the PCR efficiency ranging from 80 to 120%,⁴¹ and the PCR efficiency of the different PCR assays within one multiplex real-time PCR was assumed not to differ more than 15%.⁴² All the real-time PCR assays presented high values of correlation and efficiency. Consequently, the intra-assay competition within the multiplex reaction did not have negative impact on the detection of these genes.

To evaluate sensitivity, 10-fold serial dilutions of allergen gene and β -actin standards (from 0.2 fg/ μ L to 20 ng/ μ L) were used to estimate the detection limits of citrus allergen gene load for the developed multiplex real-time PCR assay. The LODs achieved with the singleplex and multiplex reactions were 200 fg/ μ L (200 fg DNA per PCR tube) for *Cit s 1.01* gene and 20 fg/ μ L (20 fg DNA per PCR tube) for *Cit s 2.01*, *Cit s 3.01*, and β -actin genes, respectively. Thus, the overall of the sensitivities of singleplex and multiplex assays was satisfactory, which made it possible detect the targeted allergen genes in the multiplex real-time PCR system. The variation in sensitivity in the multiplex real-time PCR assay was caused by the competition of multiple PCR reactions in a single assay platform.⁴¹

Application: Influence of Ripening Stage and Genetic Factors on the Expression of Allergen Genes in Different Citrus Tissues. The multiplex real-time PCR assay was first applied to evaluate the influence of different fruit tissues, ripening stage, and genetic factors (cultivar to cultivar) on the allergen-related gene expression in citrus fruits. The ripening stage was divided into unripe, ripe, and overripe according to the optimal stage of maturity. In order to determine the effect of the various genetic background, 10 commonly consumed citrus cultivars (2 tangerine, 1 satsuma, 4 orange, 1 pummelo, and 2 grapefruit) were selected. The transcript level of genes that related to allergies varied among ripening stage, tissues, and cultivars (Figure 5).

Tissue Specificity. The expression patterns of three citrus allergen genes (*Cit s 1.01*, *Cit s 2.01*, and *Cit s 3.01*) in the peel and pulp

The expression of *Cit s 1.01*The expression of *Cit s 2.01*The expression of *Cit s 3.01*

RT: Red Tangerine

BT: Bendizao Tangerine

GNS: Guoqin No. 1 Satsuma

NNO: Newhall Navel Orange

WNO: Washington Navel Orange

CNO: Caracara Navel Orange

RRVO: Rohde Red Valencia Orange

KPP: Kao Phuang Pummelo

CG: Cocktail Grapefruit

SRG: Star Ruby Grapefruit

Figure 5. Expression profiles of allergen genes in the peel and flesh of citrus fruits. Each column corresponds to the expression profiles of three allergen genes, while every two rows correspond to the expression profiles of the 10 citrus species. The green bars indicate the results for unripe, the orange bars indicate the results for ripe, and the gray bars indicate the results for overripe. The expression levels are normalized with respect to β -actin. Data are means $\pm$ SD, $n = 3$. (ANOVA + Duncan's test). Values with different letters are significantly ($p < 0.05$) different from each other.

tissues were obviously dissimilar (Figure 5). For instance, the transcriptional level of *Cit s 3.01* was 10- to 10 000-fold higher than *Cit s 1.01* and *Cit s 2.01* genes in the peel of the 10 citrus cultivars.

However, the transcriptional level of *Cit s 3.01* in KPP (Kao Phuang Pummelo) was equivalent to *Cit s 1.01* in six citrus cultivars (RT, NNO, CNO, RRVO, KPP, and CG). Moreover, the

transcriptional expression levels of *Cit s 1.01* and *Cit s 3.01* were higher in peel than that in pulp, while the *Cit s 2.01* showed only a slight difference between peel and pulp tissues. These results indicate that the citrus fruits which people habitually consume may contain less allergens (*Cit s 1.01* and *Cit s 3.01*) in the pulp than in the peel. The same phenomenon was also found in apple, a Rosaceae fruit, in which Mal d 1 is considered as the major allergen and shows a higher expression in peel than in flesh.²² For *Cit s 2.01*, the profilin levels in 13 citrus fruits were quantified by ELISA, and the concentration of profilin was found higher in pulp than that in peel in eight citrus cultivars (Navel, Encore, Minneola orange, Blood orange, Hyuganatsu, Shiranuhi mandarin, Kawachibankan, and Seminole tangelo).²⁵ This inconsistent phenomenon of expression between transcript and protein in peel and pulp tissues may be due to the failure in distinguishing *Cit s 2.01* from other two isoallergens (Cs4g12970 and Cs4g12960) using monoclonal and polyclonal antibodies. Regarding *Cit s 3.01*, the content was relatively low when comparing pulp to peel at the protein level,³³ which corresponded to the transcript level in the present study. Accordingly, for individuals with allergies to citrus fruits, we could further select a cultivar with less allergenicity, or they can consume only the parts of the fruit with low allergen content. In general, the pulp is the edible part of citrus fruits, except for kumquat [*Fortunella margarita* (Lour.) Swingle], an important citrus fruit, whose peel and fruit pulp can be eaten together.⁴³ Patients with anaphylactic reaction need to pay more attention to this kind of citrus fruit.

Harvest Maturity. The expression results of three citrus allergen genes in pulp demonstrated obvious differences in various harvest maturities at the transcript level (Figure 5). An obvious downward trend from unripe to overripe was observed in the expression of *Cit s 1.01* in GNS, CNO, RRVO, and KPP, *Cit s 2.01* in GNS, and *Cit s 3.01* in BT. On the contrary, an upward trend was observed in the expression of *Cit s 1.01* in RT and NNO, *Cit s 2.01* in RT, NNO, and CNO, *Cit s 3.01* in RT, GNS, and CG. The expression pattern of an increase trend from unripe to ripe and then a decrease back to the initial level in overripe was found in *Cit s 1.01* in WNO, CG, and SRG, *Cit s 2.01* in WNO, RRVO, KPP, CG, and SRG, and *Cit s 3.01* in RRVO, SRG, and KPP. With the exception of the opposite expression pattern of *Cit s 3.01* in NNO and WNO, the rest showed no significant difference in the ripening stages. A clear correlation was found between the stage of ripeness and the level of citrus allergen genes, but this downward trend was cultivar-dependent, which can be demonstrated by Mal d 1 in an apple cultivar “Golden Delicious” with an upward trend during ripeness.⁴⁴ These results illustrated that harvest maturity is one of the most important factors on the expression of allergen genes in citrus fruits, which is in agreement with the research of apple fruits.⁴⁵ Therefore, for the grower, selecting the right harvest time will be an important way to produce hypoallergenic citrus fruits. Collectively, owing to the minimum expression at the ripe stage, BT, RT, and KPP are good choices for the people who were allergic to *Cit s 1.01*, *Cit s 2.01*, and *Cit s 3.01*, respectively. There is a strong risk of allergic sensitization to consume WNO and RRVO for citrus allergic patients because of the highest expression level of *Cit s 1.01* and *Cit s 2.01* in WNO, and *Cit s 3.01* in RRVO.

Genetic Factors. The 10 cultivars genetically belong to five subgroups (tangerine, satsuma, orange, pummelo, and grapefruit). In this current study, different expression profiles for all the genes were analyzed in the 10 cultivars (Figure 5). The results revealed that the expression patterns of citrus allergen genes were different among cultivars, though there were no tissues specific

expression patterns between peel and pulp in two tangerine cultivars (RT and BT), four orange cultivars (NNO, WNO, CNO, and RRVO), and two grapefruit cultivars (CG and SRG). Among these 10 citrus cultivars, KPP could be considered as a potential low risk citrus fruit for consumers. In addition, *Cit s 2.01* exhibited a constant transcript level, and a constant gene expression pattern was also detected in Mal d 4.³¹ However, the expression patterns of *Cit s 1.01* and *Cit s 3.01* genes were different, which might be attributed to the environmental and genetic factors.⁴⁶

In conclusion, the multiplex real-time PCR assay was first developed and applied to detect the expression of citrus allergen genes. The primers and probes of these genes were designed and validated for *Cit s 1.01*, *Cit s 2.01*, *Cit s 3.01*, and β -actin. Different expression patterns of citrus allergen genes were observed in consideration of specificity of tissues, harvest maturity, and genetic factors. The results could provide new perspectives for understanding regularities of allergen gene expressions in citrus fruits and some advice on the safe consumption of citrus fruits to the public with allergenic reactions.

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Notes

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ABBREVIATIONS AND NOMENCLATURE

PCR, polymerase chain reaction; WHO-IUIS, World Health Organization and International Union of Immunological Societies; GLP, germin-like protein; LTP, lipid transfer protein; ELISA, enzyme-linked immunosorbent assay; LC–MS/MS, liquid chromatography–tandem mass spectrometry; NCBI, National Center of Biotechnology Information; SNP, single nucleotide polymorphism; TBE, tris-boric acid-EDTA; LOD, limit of detection; R^2 , correlation coefficient; E , PCR efficiency; UDG, uracil-DNA glycosylase; AVONA, a one-way analysis of variance; IBM, Applied Biosystems; FAM, 6-carboxy-fluorescein; ROX, carboxy-X-Rhodamine; BHQ, black hole quencher

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