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Differences in the Carotenoid Content of Ordinary Citrus and Lycopene-Accumulating Mutants

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High-performance liquid chromatography, coupled with photodiode array detection, was used to analyze the carotenoid composition of peel and juice vesicle tissues of ordinary and lycopene-accumulating mutants (referred to as red mutants in this article) of orange, pummelo, and grapefruit. Thirty-six major carotenoids, including some cis−trans isomers, were separated on a C_{30} reversed phase column, and 23 of them were identified on the basis of retention times and spectral characteristics with authentic standards. Carotenoid profiles varied with tissue types, citrus species, and mutations. β-Citraurin occurred in the peel of oranges but not in juice vesicles, whereas the reverse was found for violaxanthin, 9-*cis*-violaxanthin, and luteoxanthin. The diversity of carotenoids in peel and juice vesicle tissues and the fact that there was over 250 times higher content of total carotenoids in peels of Yuhuan pummelo than juice vesicles suggested that the biosynthesis of carotenoids in these two tissues was independent and exchange of carotenoids between the tissues was not likely. Lutein was observed in peels of pummelos and grapefruits and juice vesicles of ordinary pummelo but not in orange tissues. Accumulation of lycopene and β-carotene was observed in red mutant citrus, except for the peel of Cara Cara red orange. Additionally, phytoene accumulated in all tissues except for the peel of Chuzhou Early Red pummelo. No obvious change in the total content of xanthophylls was observed in the Cara Cara red orange. Ordinary grapefruit (Marsh) tissues and pummelo (Yuhuan) juice vesicles were almost devoid of carotenoids, and in red mutants, the content of total carotenoids increased dramatically up to 790-fold. The different changes in carotenoid content and profiles in mutant(s) of different citrus species suggest that the underlying mechanisms for the mutations might be different.

KEYWORDS: Orange; pummelo; grapefruit; carotenoids; lycopene; mutants; high-performance liquid chromatography

INTRODUCTION

Carotenoids are the principal pigments in ripe fruit peels of most citrus cultivars, contributing to the various fruit colors ranging from golden yellow to red. The occurrence of carotenoids in juice vesicles makes citrus fruits important sources of dietary carotenoids. Citrus serves as an important species for studies on plant carotenoid metabolism, because of the richness of carotenoid composition with approximately 115 carotenoids, including geometric isomers, reported by 1973 (*1*).

Carotenoids are isoprenoids generally consisting of eight isoprene units and are synthesized in plastids of higher plants. While citrus have some specific carotenoids including β-citraurin, the backbone pathways are identical to other plants (**Figure 1**). However, the carotenoid compositions vary greatly between plant species. Lycopene, which accounts for more than half of total carotenoids in ripe tomato fruits (*2*), is absent in common citrus fruits. Because of the deep red color and a strong protective effect of lycopene against carcinogenesis and cardiovascular disease (*3*), lycopene-accumulating citrus mutants, referred to as red mutants in this article, drew the attention of researchers as early as the 1930s.

Grapefruits (*Citrus paradisi* Macf.) are citrus with the greatest number of red mutants (more than 10), including famous cultivars such as Marsh Pink (also named Thompson, a mutant from Marsh grapefruit), Ruby Red (also named Redblush, a mutant from Marsh Pink), and Star Ruby (originated from Walters grapefruit after several mutations; both Marsh and Walters originated from Duncan grapefruit) (*4*, *5*). The main carotenoids of grapefruit mutants were identified as lycopene and β-carotene by early researchers from the 1930s to the 1950s (*6*−*8*), but other carotenes and xanthophylls were seldom

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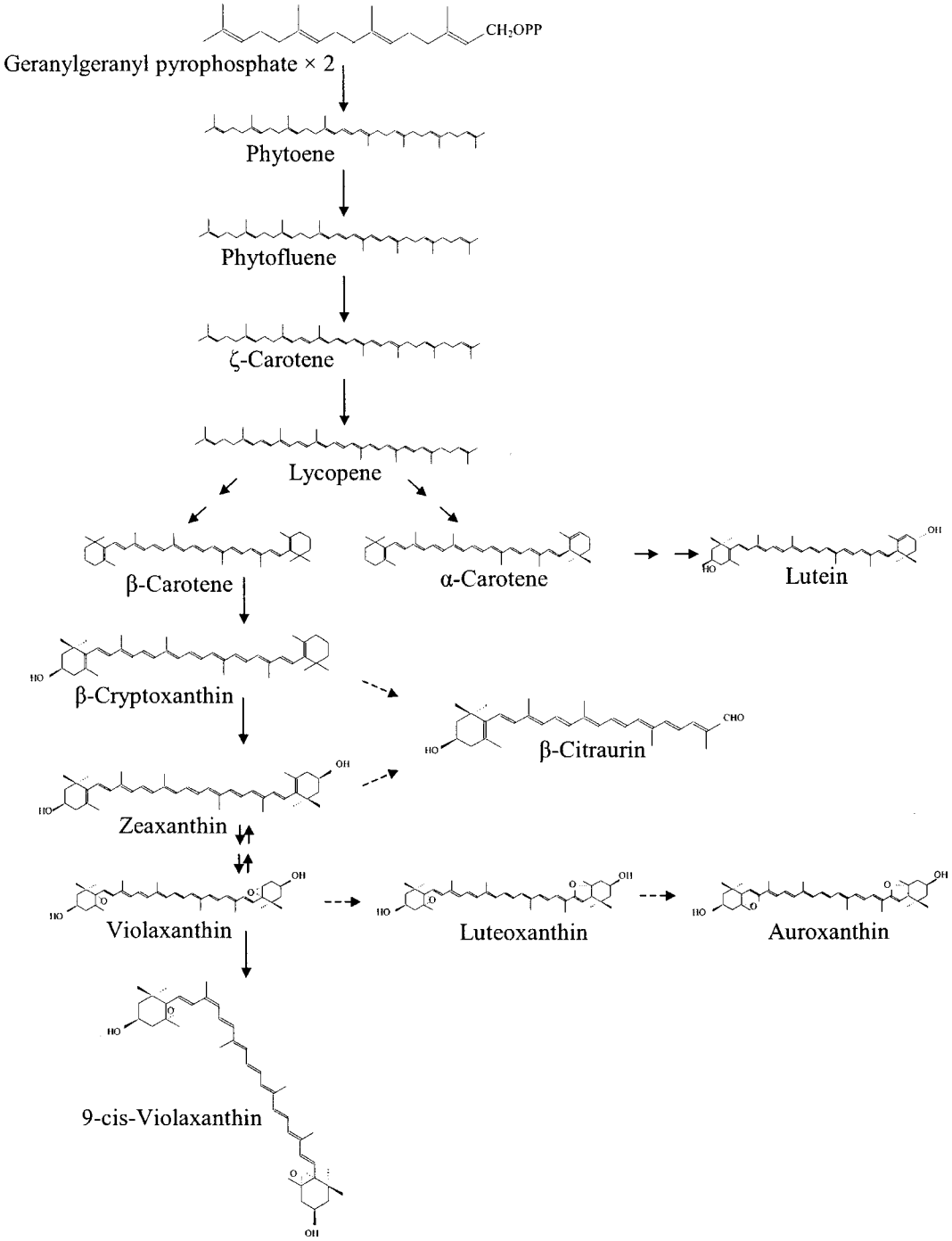

Figure 1. Citrus carotenoid biosynthetic pathway. Dashed arrows indicate proposed reactions on the basis of structural formulae.

investigated. More recently, Lee (*9*) reported detection of phytofluene and ζ-carotene in red grapefruit juice, but data on the contents were not given. Rouseff et al. (*10*) analyzed the contents of five carotenes in the pulp of Ruby Red and Star Ruby grapefruits, but carotenoids in peel tissues were not studied. Two orange (*Citrus sinensis* Osbeck) cultivars were reported to accumulate lycopene. Monselise and Halvey (*11*) identified lycopene from Sarah fruit, a mutant of Shamouti orange, but other carotenoids were not mentioned, and no further reports can be found. The carotenoid composition in juice of the other red orange mutant, Cara Cara, which shares the same origin (Washington navel orange) with Bonanza orange applied in this study, was analyzed in comparison with ordinary orange (*12*), but the peel tissue was not studied. Pummelo (*Citrus grandis* Osbeck and *Citrus maxima* Merr.) normally has a golden yellow peel and white or yellowish pulp, but some cultivars were reported to have a pink pulp (*13*). An Indian red pummelo was reported to contain lycopene in 1934 (*14*). A preliminary study suggested that the red pigment of Chuzhou Early Red, a red flesh pummelo mutant originating in the Zhejiang Province of China with unidentified origin but probably very close to Yuhuan pummelo, is lycopene. Lycopene-accumulating mutants have only been reported in the three above-mentioned species in citrus. In addition, an orange mutant accumulating phytoene, phytofluene, and ζ-carotene, but not lycopene or *β*-carotene, was reported recently (*15*).

The aim of the present study was to establish the metabolic basis for the accumulation of lycopene in fruits of citrus red mutants, by comparing the carotenoid compositions in peel and

Table 1. List of Citrus Analyzed in This Study

common name	Latin name	cultivar	harvest place	year
ordinary navel orange	*C. sinensis* (L.) Osbeck	Bonanza	Zhejiang, China	2004
red navel orange	*C. sinensis* (L.) Osbeck	Cara Cara	Zhejiang, China	2004
ordinary pummelo	*C. grandis* (L.) Osbeck (*C. maxima* Merr.)	Yuhuan	Zhejiang, China	2004
red pummelo	*C. grandis* (L.) Osbeck (*C. maxima* Merr.)	Chuzhou Early Red	Zhejiang, China	2004
ordinary white grapefruit	*Citrus paradisi* Macf.	Marsh	South Africa	2005
red grapefruit	*C. paradisi* Macf.	Ruby Red	South Africa	2005
red grapefruit	*C. paradisi* Macf.	Star Ruby	South Africa	2005

Table 2. Separation and Characterization of Carotenoids in Citrus Tissues by HPLC–Photodiode Array Detection

				occurrence[e]	
peak no.[a]	carotenoids	R_t[c] (min)	λ_{max} [d] (nm)	peel	juice vesicle
1	unidentified[b]	9.6	418, 446, 474	B, C, Y	B, C, Y
2	violaxanthin	11.6	415, 439, 468	M, R, S	B, C
3	luteoxanthin	13.3	399, 422, 448	M, R, S, Y	B, C
4	auroxanthin	13.9	380, 400, 425	B, C, M	B, C
5	unidentified	14.5	401, 425, 453	M	M
6	β-citraurin	14.6	458	B, C, S	
7	9-*cis*-violaxanthin	15.1	411, 435, 464	M, R, S, Y	B, C
8	unidentified	15.8	396, 417, 444	B, C	B, C
9	unidentified	15.9	399, 422, 442	M	
10	auroxanthin	16.3	380, 400, 425	B, C	
11	unidentified	16.4	(404), 427, 453	M	B, C, M, R, S
12	lutein	17.0	(420), 445, 473	Ch, M, R, S, Y	Y
13	unidentified	17.1	(404), 427, 451	B, C	B, C, M, R, S
14	zeaxanthin	18.8	(427), 450, 478	all samples	all samples
15	unidentified	19.8	416, 440, 469	Ch, M, R, S, Y	B, C
16	unidentified	20.3	402, 424, 450	B, C, M	
17	phytofluene	21.6	331, 348, 365	all samples but S	Ch, S
18	phytofluene	23.5	331, 348, 365	all samples but S	C, Y
19	phytoene	24.2	276, 286, 297	all samples	all samples
20	phytofluene	24.3	331, 348, 365	Ch, R, Y	
21	β-cryptoxanthin	25.1	(426), 451, 479	all samples but S	B, C, M, R, Y
22	phytofluene	25.3	331, 348, 365	All samples	C, M, R, S
23	unidentified	25.8	410, 434, 463		C
24	phytofluene	26.9	331, 348, 365	all samples	all samples
25	phytofluene	27.4	331, 348, 365	M, R, S, Y	C, S
26	unidentified	27.6	413, 435, 464		C
27	unidentified	27.8	(428), 452, 479		R, S
28	α-carotene	29.3	(424), 446, 474	Ch, Y	Ch, Y
29	unidentified	30.1	411, 434, 462		C, Ch, S
30	ζ-carotene	30.4	380, 400, 425	all samples but Ch	B, C, M, R, S
31	β-carotene	30.7	(427), 452, 479	Ch, R, S, Y	all samples but M
32	ζ-carotene	31.1	380, 400, 425	all samples but Ch	M, R, S
33	lycopene *cis*-isomer	35.7	440, 464, 495	S	C, Ch, R, S
34	lycopene *cis*-isomer	36.4	440, 464, 495	S	C, Ch, R, S
35	unidentified	37.7	421	B, C	B, C
36	lycopene	42.5	446, 473, 503	Ch, R, S	C, Ch, R, S

[a] The numbers are the same as those in **Figure 2**. [b] Unidentified compounds with carotenoid spectra. [c] R_t = retention time. [d] Obtained with photodiode array detection in mobile solvents. Values in parentheses indicate shoulders instead of peaks. [e] B, Bonanza; C, Cara Cara; Ch, Chuzhou Early Red; M, Marsh; R, Ruby Red; S, Star Ruby; and Y, Yuhuan.

juice vesicles of ordinary and red mutants of orange, grapefruit, and pummelo.

MATERIALS AND METHODS

Reagents and Standards. Chemicals and solvents were obtained from VWR International (Lutterworth, Leicestershire, United Kingdom). Ammonium acetate and all solvents used in high-performance liquid chromatography (HPLC) were of HPLC-grade. Other chemicals were of analytical grade. Carotenoid standards other than β-citraurin, auroxanthin, and luteoxanthin were obtained or prepared as previously described (*16*). β-Citraurin was isolated from peels of clementine fruit, a citrus reported to be abundant in this carotenoid (*17*), and identified by the same spectral characteristics and spectral changes to β-citraurinenol after $NaBH_4$ reduction as reported in the literature (*17*−*19*). Auroxanthin and luteoxanthin were prepared by HCl-catalyzed isomerization of a violaxanthin standard according to Britton (*18*) and Asai et al. (*20*).

Samples. Three ordinary citrus and four red mutants (**Table 1**) were used in this study. Fully ripe orange fruits and pummelo fruits were harvested from the Zhejiang Province of China, and ripe fruits of grapefruit were obtained from U.K. supermarkets. The ordinary and mutant citrus were harvested in the same year and from the same origin.

Preparation of Samples. *Extraction*. Peel and juice sacs were separated and cut into small pieces before freeze drying. The dried materials were finely ground, weighed (500 mg for Marsh grapefruit samples and Yuhuan pummelo juice vesicle samples and 10 mg for the others), extracted with chloroform/methanol/50 mM Tris buffer, pH 7.5, containing 1 M NaCl (2:1:1, v/v/v), and finally dried under nitrogen using the method described by Fraser et al. (*16*).

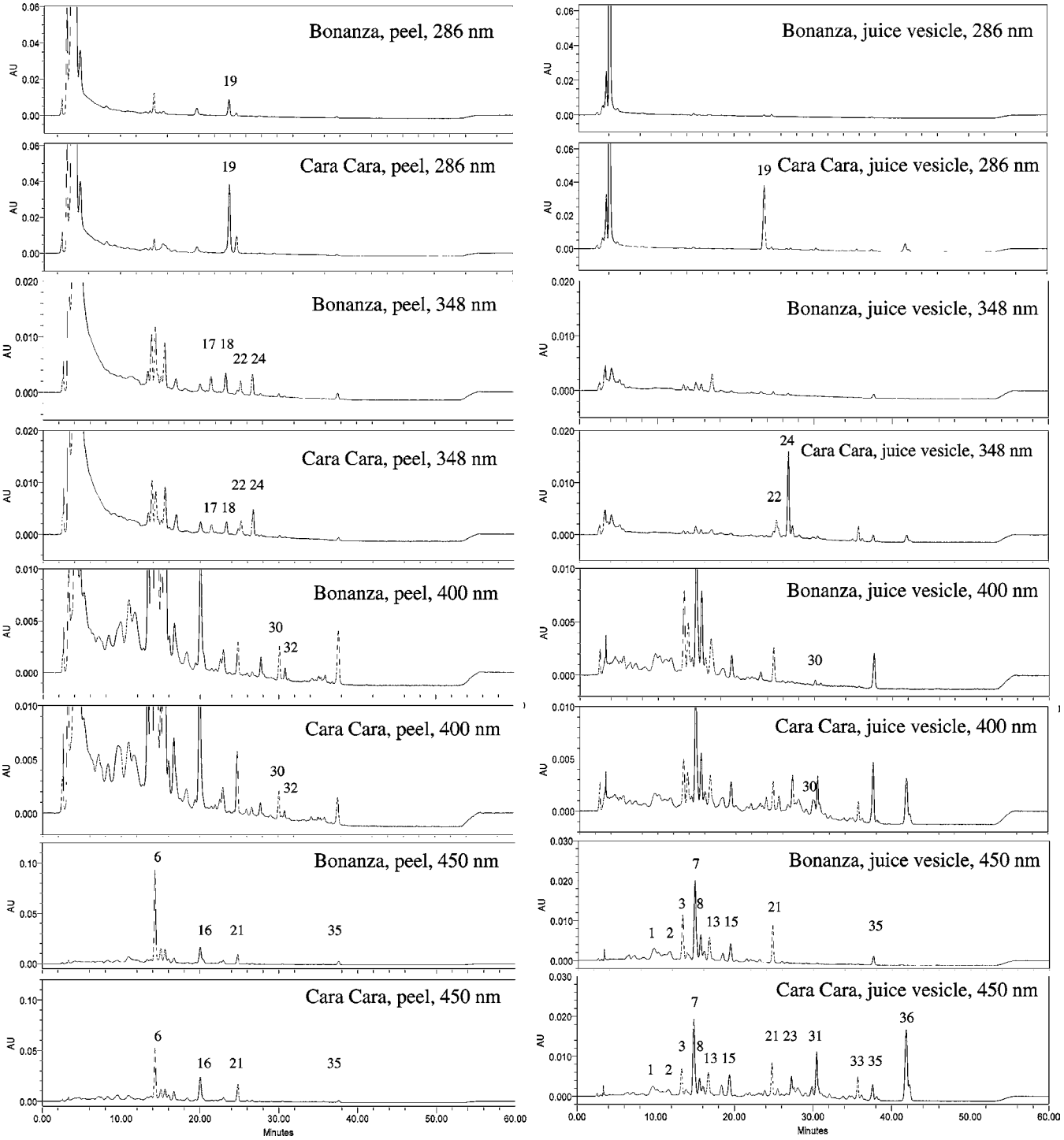

Figure 2. HPLC of carotenoids in peel and juice vesicle tissues of Bonanza orange and Cara red orange, monitored at different wavelengths. The peaks are numbered according to the elution sequence as detailed in **Table 2**.

Saponification. For saponification, the residue was first dissolved in 20 μL of diethyl ether, to which 200 μL of 6% (w/v) KOH in methanol was added. After it was mixed, the mixture was incubated at 60 °C for 30 min in darkness. Tris buffer was then added, and the extraction was performed as described above. For each sample, three replicates were performed.

HPLC Analysis of Carotenoids. *Equipment and Chromatographic Conditions*. HPLC analysis was carried out on a Waters system (Watford, Hertfordshire, United Kingdom) consisting of a no. 616 pump, no. 996 photodiode array detector, and no. 717 autosampler equipped with a reverse phase 5 μm (250 mm × 4.6 mm) C_{30} column and a 20 mm × 4.6 mm C_{30} guard (YMC Inc., Wilmington, NC). Chromatography was carried out at 25 °C, as previously described (*16*), with a modification to the elution program. Mobile phases A (methanol), B (80% methanol containing 0.2% ammonium acetate), and C (*tert*-butyl methyl ether) were applied as follows: 95% A, 5% B for 6 min, a linear gradient to 80% A, 5% B, and 15% C by 7 min, held until 12 min, gradient changed to 30% A, 5% B, and 65% C by 32 min, held until 48 min, then changed to 95% A and 5% B by 50 min, and then held to the end of analysis (60 min).

Carotenoid Identification and Quantification. Carotenoids were identified on the basis of the same retention times and same spectral characteristics with standards. Peak areas were recorded at 286, 348, 400, 473, and 450 nm for phytoene, phytofluene, ζ-carotene and auroxanthin, lycopene, and the others, respectively. Carotenoids with available standards other than auroxanthin, β-citraurin, and luteoxanthin

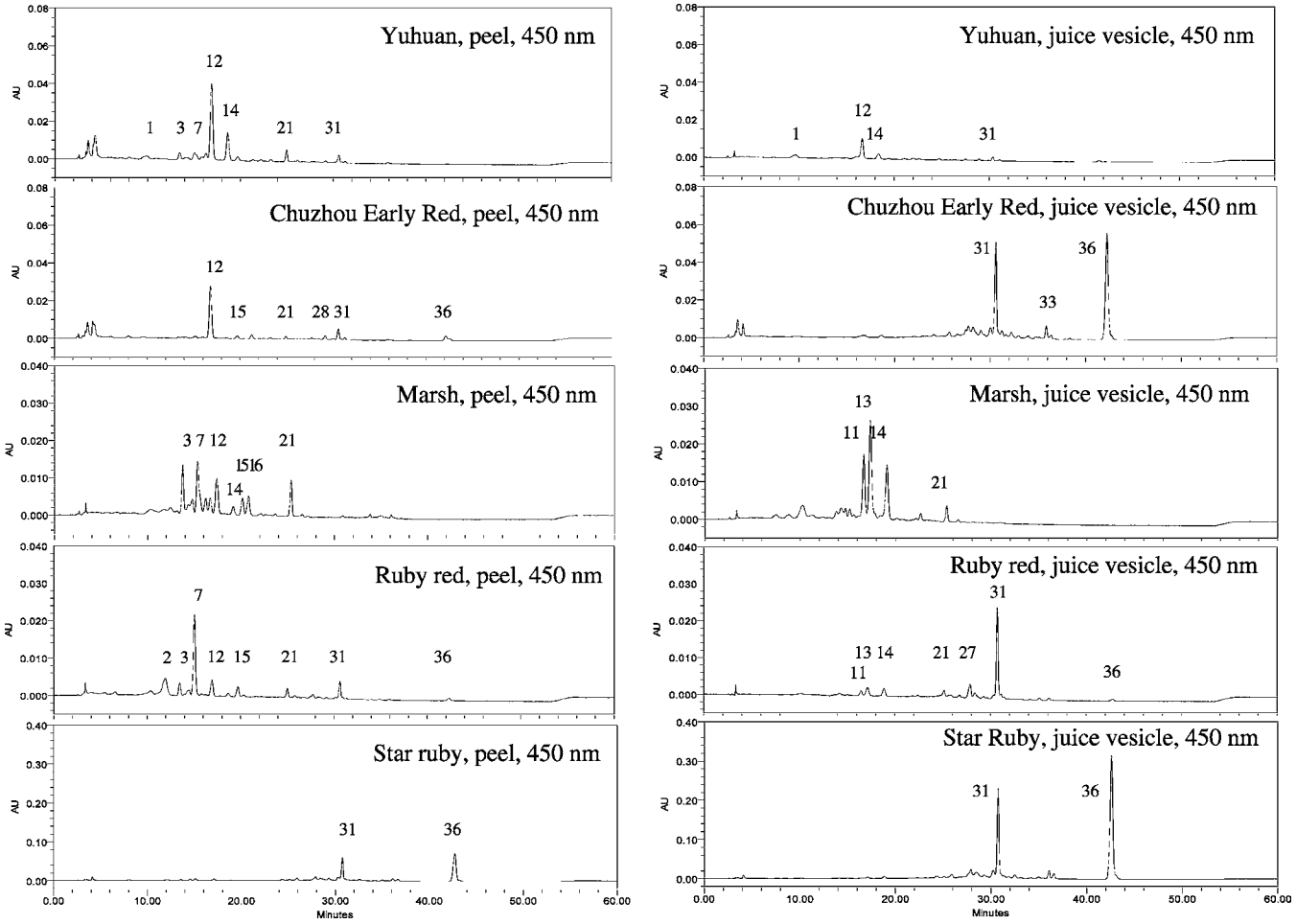

Figure 3. HPLC of carotenoids in peel and juice vesicle tissues of pummelos and grapefruits monitored at 450 nm. The peaks were numbered according to the elution sequence as detailed in **Table 2**.

were quantified according to a calibration curve of corresponding standards; auroxanthin and luteoxanthin were quantified as violaxanthin equivalents; β-citraurin and unidentified carotenoids were quantified as β-carotene equivalents.

Detection Limits of the Analysis. A peak with an area of 5000 was always clearly distinguished under the chromatographic conditions described above, which is equivalent to 1.26, 0.75, 0.52, and 0.43 ng of phytoene, phytofluene, ζ-carotene, and other carotenoids, respectively. On the basis of a starting material of 10 mg, a final volume of 50 μL for HPLC and an injection volume of 20 μL, the detection limits were 0.32, 0.19, 0.13, and 0.11 μg/g DW for phytoene, phytofluene, ζ-carotene, and the others, respectively. The limits were 50 times lower for Marsh grapefruit samples and Yuhuan pummelo juice vesicle samples since more starting material was applied.

RESULTS AND DISCUSSION

HPLC Carotenoid Profiles. In 1971, Stewart and Wheaton (*21*) applied HPLC to the separation of citrus carotenoids to reduce the analysis time and obtain a better separation. Further improvements to columns and replacement of MgO or $ZnCO_3$ by reversed phase C18 (*22*) or C30 (*1*) were made later. The C30 reversed phase HPLC column is able to separate carotenoid isomers and, when coupled with a photodiode array detector, achieves accurate identification and quantification of citrus carotenoids. This system was applied in this study.

A total number of 36 major (over 1% of total carotenoids in at least one sample) carotenoids, including some cis−trans isomers, were detected, and 23 of them were identified (**Table 2**). In addition, around 10 minor (less than 1% of total carotenoids in all samples) carotenoids were detected. Some abundant carotenoids are also shown in **Figures 2** and **3**. The system separated structural isomers such as lutein and zeaxanthin as well as cis−trans isomers such as violaxanthin and 9-*cis*-violaxanthin. Separation of phytofluene cis−trans isomers was successful, and a total number of six isomers were detected, with the isomer eluted at 26.9 min being the most abundant one and the only one found in all samples analyzed (**Table 2**). No cis−trans isomer of phytoene was observed. Two cis−trans isomers of ζ-carotene were detected, with the first eluted isomer as the main one.

Diversity of Carotenoid Composition in Different Citrus Tissues and Species. Diversity of carotenoid composition in peel and juice vesicle tissues of citrus was found, as shown in **Table 2** and **Figures 2** and **3**. A higher content and more isomers of phytofluene and ζ-carotene were observed in orange peel tissue than juice vesicles (**Table 2**). β-Citraurin, which was one of the most abundant carotenoids in orange peel, was not detected in juice vesicle tissues, whereas the reverse was found for violaxanthin, 9-*cis*-violaxanthin, and luteoxanthin. The diversity of carotenoids in peel and juice vesicle tissues suggests that the biosynthesis of carotenoids in these two tissues is independent and that exchange of carotenoids between these tissues is unlikely. The difference in total carotenoids between peel and juice vesicle tissues of ordinary pummelo (**Table 3**) further supports this hypothesis. The presence of β-citraurin as a major carotenoid in orange peels has been reported previously (*17*, *23*, *24*), but the high percentage of total carotenoids found

Table 3. Content of Carotenoids in Ordinary and Mutant Citrus Peel and Juice Vesicle Tissues[a]

Bonanza (B) and Cara Cara (C) oranges

	contents (μg/gDW)				percentages in total carotenoids (%)			
	peel		juice vesicle		peel		juice vesicle	
carotenoids	B	C	B	C	B	C	B	C
phytoene	14.45	79.17	0.85	57.10	9.54	36.30	2.12	39.89
phytofluene[b]	10.95	9.83	0.22	17.45	7.23	4.51	0.55	12.19
ζ-carotene[b]	2.42	1.80	0.32	0.55	1.60	0.82	0.79	0.38
lycopene[b]	*c*			23.74				16.56
β-carotene			0.10	4.70			0.26	3.28
β-cryptoxanthin	4.40	7.80	5.05	4.45	2.91	3.58	12.66	3.10
zeaxanthin	0.20	1.47	1.12	1.41	0.13	0.67	2.80	0.99
violaxanthin			1.16	0.86			2.91	0.60
9-*cis*-violaxanthin			12.01	10.67			30.08	7.48
luteoxanthin			4.69	2.73			11.74	1.91
auroxanthin[b]	47.70	47.43	2.03	1.15	31.47	21.75	5.04	0.81
β-citraurin	35.25	20.02			23.25	9.18		
unidentified	36.20	50.52	12.41	18.28	23.87	23.19	31.05	12.81
total carotenoids	151.57	218.04	39.96	143.09	100	100	100	100
total carotenes	27.82	90.80	1.39	103.54	18.37	41.63	3.46	72.30
total xanthophylls	123.75	127.24	38.57	39.55	81.63	58.37	96.54	27.70

Yuhuan (Y) and Chuzhou Early Red (Ch) pummelos

	contents (μg/gDW)				percentages in total carotenoids (%)			
	peel		juice vesicle		peel		juice vesicle	
carotenoids	Y	Ch	Y	Ch	Y	Ch	Y	Ch
phytoene	30.01	5.19	0.0736	1.74	55.33	30.93	34.62	5.55
phytofluene[b]	7.26	4.27	0.0049	0.43	13.43	25.42	2.31	1.36
ζ-carotene[b]	1.03				1.90			
lycopene[b]		0.98		16.07	/	5.85		51.00
α-carotene	0.11	0.26	0.0044	0.47	0.20	1.53	2.09	1.40
β-carotene	0.54	0.77	0.0107	6.32	0.99	4.59	5.04	19.98
lutein	7.45	4.39	0.0739		13.71	26.23	34.75	
β-cryptoxanthin	1.07	0.19	0.0034		1.96	1.16	1.61	
zeaxanthin	2.78	0.06	0.0217	0.17	5.11	0.39	10.21	0.53
9-*cis*-violaxanthin	1.08				1.98			
luteoxanthin	0.61				1.11			
unidentified	2.31	0.66	0.0201	6.48	4.28	3.90	9.37	20.18
total carotenoids	54.25	16.77	0.2127	31.68	100	100	100	100
total carotenes	38.95	11.47	0.0936	25.03	71.85	68.32	44.06	79.29
total xanthophylls	15.30	5.30	0.1191	6.65	28.15	31.68	55.94	20.71

Marsh (M), Ruby Red (R) and Star Ruby (S) grapefruits

	contents (μg/gDW)						percentages in total carotenoids (%)					
	peel			juice vesicle			peel			juice vesicle		
carotenoids	M	R	S	M	R	S	M	R	S	M	R	S
phytoene	0.9949	10.34	101.77	0.0736	1.48	65.52	29.97	38.98	46.84	17.64	22.19	20.66
phytofluene[b]	0.1959	4.51	38.77	0.0170	0.33	33.28	5.92	16.96	17.87	4.07	5.01	10.44
ζ-carotene[b]	0.0543	0.86	10.72	0.0114	0.06	2.15	1.64	3.35	5.04	2.67	0.89	0.68
lycopene[b]		0.31	42.32		0.16	159.35		1.16	19.08		2.33	48.93
β-carotene	0.0082	0.84	13.97		2.93	43.11	0.24	3.14	6.35		43.39	13.44
lutein	0.2483	0.88	1.20				7.50	3.49	0.55			
β-cryptoxanthin	0.1610	0.45		0.0177	0.20		4.84	1.76		4.37	2.97	
zeaxanthin	0.0459	0.15	0.23	0.0735	0.31	1.13	1.38	0.59	0.11	18.05	4.59	0.36
violaxanthin	0.0051	1.66	0.10				0.15	6.61	0.05			
9-*cis*-violaxanthin	0.5207	4.50	1.52				15.69	17.82	0.70			
luteoxanthin	0.3116	0.63	0.57				9.39	2.43	0.27			
auroxanthin[b]	0.6085						18.39					
β-citraurin			0.92						0.42			
unidentified	0.1615	0.99	6.11	0.2148	1.23	18.07	4.89	3.71	2.72	53.20	18.63	5.49
total carotenoids	3.3159	26.12	218.20	0.4080	6.70	322.61	100	100	100	100	100	100
total carotenes	1.2533	16.86	207.55	0.1020	4.96	303.41	37.77	63.59	95.18	24.38	73.81	94.15
total xanthophylls	2.0626	9.26	10.65	0.3060	1.74	19.20	62.23	36.41	4.82	75.62	26.19	5.85

[a] Data are means of three replicates, with standard errors generally within 20 and 10% of means for contents expressed in definite contents (μg/gDW) and percentages in total carotenoids (%), respectively. [b] Sum of all isomers. [c] Under the detection limit.

in this study was quite extraordinary (**Table 3**), implying that the biosynthesis of β-citraurin might be affected by growth conditions.

The carotenoid composition also varied among species. Lutein, a major carotenoid in peels of grapefruits and pummelos, was not detected in fully ripe orange fruits. In addition, an

unidentified carotenoid (peak 13), which has a very similar retention time to lutein, appeared in orange tissues. The unidentified carotenoid has the same spectral characteristics as mutatoxanthin (*1*), which was also previously reported to be barely resolved from lutein by chromatography (*1*). The absence of lutein in orange tissues was contradictory to some previous reports (*1*, *12*). The reason was not clear but might be related to the developmental stage of the fruit analyzed. The existence of lutein as a major carotenoid in young fruits and its disappearance in full-colored Navelate and Pinalate orange fruits has been reported (*15*, *24*). Similarly, α-carotene was only detected in pummelo tissues.

Difference in the Contents of Carotenoids in Ordinary and Mutant Citrus. Table 3 summarizes the carotenoids in peel and juice vesicle tissues of seven citrus fruits. Xanthophylls accounted for most colored carotenoids in ordinary citrus tissues, while no lycopene and only low levels of α- and β-carotene were observed. As a result, ordinary citrus peel and pulp are generally yellow to orange in color. Orange peels have an orange red color because of high amounts of β-citraurin. As compared to ordinary citrus cultivars, lycopene and β-carotene accumulated in citrus red mutants, except for the peel tissue of the Cara Cara orange. However, traces (0.83 μg/g DW and 0.41% of total carotenoids) of lycopene were detected in the peel of Cara Cara orange from another year (data not shown). Although no lycopene and a low content of β-carotene were found, carotenoid metabolism in peel tissue was still affected by the mutation, as the content of phytoene increased more than 4-fold (**Table 3** and **Figure 2**). The accumulation of phytoene and phytofluene was even more obvious in Cara Cara juice vesicle tissues, where the content increased by more than 60-fold. Total carotenoids increased in mutant citrus, but total xanthophylls were not significantly affected (**Table 3**). All increases resulted from changes to carotenes.

Unlike oranges, the total carotenoid and phytoene contents decreased in peel tissues of red pummelo. The amounts of β-cryptoxanthin and zeaxanthin, and especially the latter, decreased significantly (**Table 3** and **Figure 2**). Carotenoids were almost absent in ordinary pummelo juice vesicles, with a content 250 times less than peel. A mutation in red pummelo restored its capacity for carotenoid biosynthesis, as the content of any individual carotenoid in juice vesicles of red pummelo was higher than ordinary pummelo. Lycopene and β-carotene were predominant in red pummelo juice vesicles, accounting for 51.00 and 19.98% of total carotenoids, respectively.

Peels, and especially juice vesicles, of ordinary grapefruit were almost devoid of carotenoids. Therefore, the golden yellow color of Marsh peel must mainly results from other colored pigments such as flavonoids. Total carotenoids increased by about 10-fold in Ruby Red and even more in Star Ruby. Juice vesicles of Star Ruby had the highest total carotenoid contents among all samples analyzed, which was more than 800 times that of Marsh. Higher contents of lycopene (by near 1000 times) and β-carotene (by near 15 times) in juice vesicles of Star Ruby than Ruby Red well-explained the much deeper red color of the former. Moreover, differential accumulation of lycopene and β-carotene was observed between these two red grapefruits. A higher content of lycopene than β-carotene was observed in Star Ruby tissues, just as that in red pummelo and red orange, while the reverse was found in Ruby Red (**Table 3** and **Figures 2** and **3**). Xanthophylls were predominant carotenoids in Marsh fruit but minor component in red grapefruits, with carotenes accounting for about 95% of total carotenoids in Star Ruby fruits.

Although lycopene-accumulating citrus mutants have been reported previously (*6*−*12*), the underlying mutations have remained unsolved. The accumulation of phytoene and the increase in total carotenes of 2.26- and 73.49-fold in peels and juice vesicles of Cara Cara red orange suggest that the mutation might affect phytoene synthase or other enzyme(s) early in the plastid isoprenoid pathway (*25*). Mutations in lycopene β-cyclase and carotene β-hydroxylase are unlikely in red orange because of the simultaneous accumulation of β-carotene and no obvious changes in xanthophylls. For red pummelo and grapefruits, because lycopene and β-carotene accumulated, while the percentage of xanthophylls in total carotenoids was greatly reduced, defective carotene β-hydroxylation could be possible in this mutant. However, increases in total carotenoids were observed in all mutant tissues except for red pummelo peels, and in red pummelo juice vesicles and red grapefruit tissues, the increment was so dramatic that it is reasonable to speculate that mutation(s) in genes other than those encoding carotenoid biosynthetic enzymes might also be involved. Li (*26*) reported several 100-fold higher β-carotene levels in the curd of a mutant cauliflower, but expression of carotenoid biosynthetic genes was not obviously changed. Recently, their further study suggests that the *Or* gene encodes a plastid membrane protein (*27*), which might be involved in sequestration of carotenoids (*26*). Unlike the association with chlorophyll-binding proteins in chloroplasts, carotenoids in chromoplasts were sequestered to specific lipoproteins (*28*). Whether mutations related to carotenoid sequestration have occurred in red pummelo and grapefruits should be further investigated.

In conclusion, differences in carotenoid contents and profiles were observed between two tissue types, peels and juice vesicles; three species, orange, pummelo, and grapefruit; and ordinary citrus and lycopene-accumulating mutants. Aside from the increases in lycopene and β-carotene in mutant tissues, accumulation of phytoene was also observed. Changes in the total content of carotenoids and xanthophylls were different in mutants of three species, suggesting that different mechanisms might be involved.

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