

Carotenoid Accumulation and Carotenogenic Genes Expression During Two Types of Persimmon Fruit (*Diospyros kaki* L.) Development

Daqiu Zhao · Chunhua Zhou · Jun Tao

Published online: 27 November 2010
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Abstract Persimmon (*Diospyros kaki* L.), belonging to the Ebenaceae family, has been used not only as a fresh fruit, but also for many medicinal uses. Carotenoids are the main pigment in persimmon fruit, which contribute significantly to fruit color and nutritional quality due to their composition and content. In this study, fruit quality indices, carotenoid contents and expression of carotenogenic genes were analyzed in two types of persimmon fruit. The results demonstrated that there was a positive correlation between fruit color and the contents of main composition and total carotenoids. Carotenoid accumulation in persimmon fruit resulted from the interaction of carotenogenic genes, but the molecular mechanisms responsible for accumulation of carotenoids in two types of persimmon fruit had a few differences. As a complete unit, the relatively low expression level of phytoene synthase gene (*DkPSY*) in “Niuxin-shi” resulted in low carotenoid contents or even under the detection limit at the early fruit developmental stages; but low carotenoid contents in “Nishimurawase” were due to the relatively low expression level of carotenogenic genes other than *DkPSY*. At the late fruit developmental stages, increased expression levels of *DkPSY*, phytoene desaturase gene and beta-carotene hydroxylase gene (*DkBCH*) induced elevated carotenoid contents; because all carotenogenic genes strongly expressed in “Nishimurawase”, a large amount of carotenoids were accumulated. In addition, β -cryptoxanthin was the main composition whose content increased with the fruit maturity changes, which was

mainly because of *DkBCH* which might lead more conversion of β -carotene to β -cryptoxanthin.

Keywords Persimmon · Carotenoid accumulation · Expression analysis · Fruit development

Abbreviations

BCH	Beta-carotene hydroxylase
Ct	Threshold cycle
LCYB	Lycopene beta-cyclase
LCYE	Lycopene ϵ -cyclase
PCR	Polymerase chain reaction
PDS	Phytoene desaturase
PSY	Phytoene synthase
Q-PCR	Quantitative real-time PCR
RT-PCR	Reverse-transcription polymerase chain reaction
ZEP	Zeaxanthin epoxidase
ZDS	ζ -Carotene desaturase
HPLC	High-performance liquid chromatography

Introduction

Carotenoids constitute an abundant and widespread group of pigments occurring naturally in plants, fungi, bacteria, and animals, with more than 600 different carotenoids identified to date (Rock 1997; Cunningham and Gantt 1998). They not only affect the external appearance and internal quality of fruits (Tao and Zhang 2003) but also have numerous and varied beneficial bioactivities on human health and disease such as singlet oxygen quenching, free radicals scavenging (Miki 1991), vitamin A precursor (Matsuno 1991), immunoregulatory (Hughes 2001; Nagao 2009), anti-cancer (Rock 1997; Ito et al. 2009), anti-aging

D. Zhao · C. Zhou · J. Tao (✉)
College of Horticulture and Plant Protection,
Yangzhou University,
Yangzhou 225009, People's Republic of China
e-mail: taojun@yzu.edu.cn

effects (Fraser and Bramley 2004), and so on. With the improvement of people's living standards, the demand of carotenoids is becoming larger and larger. But in human body, carotenoids cannot be biosynthesized denovo, whose existence is due to oral ingestion via food and accumulation in certain tissues (Schieber and Carle 2005). Therefore, more and more people focus on searching for natural plant resources being rich in carotenoids and studying regulatory mechanisms of carotenoid biosynthesis and metabolism.

Persimmon (*Diospyros kaki* L.) which could be divided into two types, namely, astringent persimmon and non-astringent persimmon, is widely cultivated in East Asia such as China, Korea, and Japan (Yonemori et al. 2000; Li et al. 2009). China has always represented a substantial percentage in the persimmon world production. In 2009, its cultivation area and production is 703,869 Ha and 2,683,899 t, which occupy 89.75% and 70.48% of global cultivation area and production, respectively (FAO 2010). Persimmon has been used not only as a fresh fruit but also for many medicinal uses, with almost all parts of this plant. For example, the leaf is a natural antioxidant source (Sakanaka et al. 2005); the seed extracts have a strong radical-scavenging activity (Ahn et al. 2002); the fruit possesses hypolipidemic and antioxidant properties (Shela et al. 1998; Gorinstein et al. 2001) and is useful in promoting the immune system and antitumor activity (Achiwa et al. 1997; Kawase et al. 2003); the calyx extracts are used as anticonvulsants and to alleviate the side effects of barbituric acid compounds (Fukuda and Shibata 1994).

The color of persimmon fruit ranges from yellow, orange, to deep red (Yonemori et al. 2000), which mainly results from carotenoids accumulation (Thomas and Chen 1998). Therefore, high carotenoids content, not only play an important role in biological property, but also are favored from the marketing perspective (Nakagawa et al. 2008; Robert et al. 2010). In recent years, with the clarification of carotenoids biosynthesis pathway in some model plants (Bartley and Scolnik 1995; Tao et al. 2002; Galpaz et al. 2006), the researches on carotenoids accumulation regulation of other plants are gradually deepening, and many carotenogenic have been cloned and manipulated (Ma et al. 2010; Rodríguez-Ávila et al. 2010; Sun et al. 2010; Zhao et al. 2010) (Fig. 1). Previously, we had cloned *DkPSY*, *DkBCH*, and *DkLCYB*, and studied their fruit tissue-specific expression patterns (Zhao et al. 2010). In order to clarify regulatory mechanism of persimmon fruit carotenoid accumulation, we analyzed carotenoid accumulation and carotenogenic genes expression during two types of persimmon fruit development in this study, which are important to improve the eating and appearance quality of persimmon fruit, and to regulate the further biosynthetic pathway of its carotenoids.

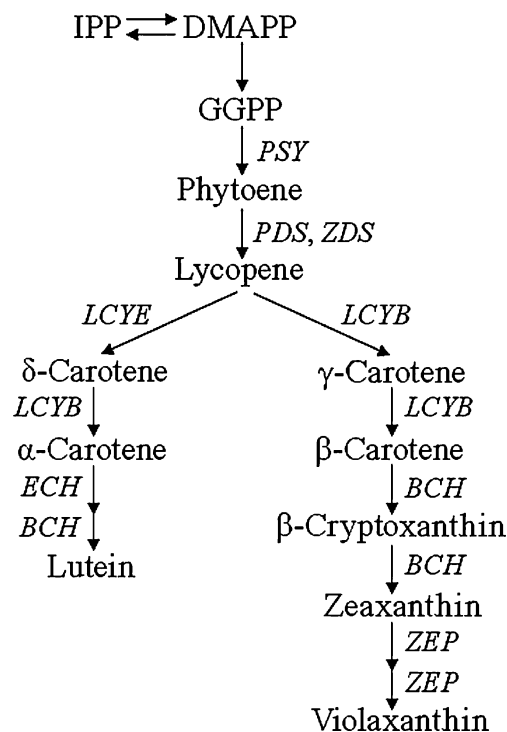


Fig. 1 Carotenoid biosynthetic pathway in plants (Niikawa et al. 2007; Rodríguez-Ávila et al. 2010). *IPP* isopentenyl pyrophosphate, *DMAPP* dimethylallyl pyrophosphate, *GGPP* geranylgeranyl pyrophosphate, *PSY* phytoene synthase, *PDS* phytoene desaturase, *ZDS* ζ-carotene desaturase, *LCYB* lycopene β-cyclase, *LCYE* lycopene ε-cyclase, *BCH* β-carotene hydroxylase, *ECH* ε-carotene hydroxylase, *ZEP* zeaxanthin epoxidase

Materials and Methods

Plant Materials

Two types of persimmon varieties, “Niuxinshi” (astringent) and “Nishimurawase” (non-astringent), were obtained from the Yizheng Science and Technology Exhibition Garden of Hilly and Mountainous District Production Forest and Fruit Tree, Jiangsu Province, China (32°25' N, 119°08' E). Fruits were collected at five developmental stages with an interval of about 15 days from August 21 to October 22, 2008 (Fig. 2). After transportation to laboratory, ten unblemished fruits from each variety were selected for some main fruit quality indices measurement, including size, shape and color. Then all fruits sampled were peeled, the flesh were cut into small pieces and immediately frozen in liquid nitrogen, and stored at −80°C until analysis.

Fruit Quality Indices

Fruit weight was the average value of ten fruits by balances (Gandg Testing Instrument Factory, China). The vertical and horizontal diameters were measured by

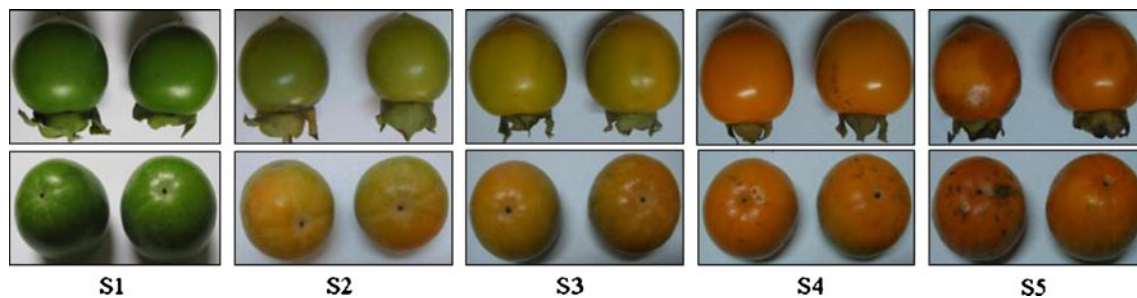


Fig. 2 Pictures of persimmon fruit development stages. *S1–S5* five sampled stages during fruit development. The upper fruits are “Niuxinshi” and the down fruits are “Nishimurawase”

micrometer scale (Taizhou Xinshangliang Measuring Tools Co., Ltd., China).

Fruit color was evaluated using a TC-P2A chroma meter (Beijing Optical Instrument Factory, China) using three color parameters including L^* , a^* and b^* values at opposite sides of each fruit. The hue angle ($H^\circ = \arctangent(b^*/a^*)$) and chroma ($C^* = (a^{*2} + b^{*2})^{1/2}$) were calculated according to the methods reported previously (McGuire 1992; Voss 1992).

Extraction and Quantification of Carotenoids

Carotenoids were extracted according to the method of Zhou et al. (2007); 200 mg flesh was ground into fine powder with liquid nitrogen, and extracted with 1.4 mL of chloroform/methanol/50 mM Tris buffer pH 7.5, containing 1 M NaCl (2:1:1 v/v/v) in a 2-mL tube. After centrifugation, the chloroform phase was recovered and the aqueous phase re-extracted with 700 μ L of chloroform. The pooled chloroform extracts were dried under nitrogen and dissolved in 350 μ L of 6% (w/v) KOH in methanol. The mixture was incubated at 60°C for 30 min in darkness, and 350 μ L of water and 700 μ L of chloroform were then added. After centrifugation, the chloroform phase was recovered and partitioned with water until the aqueous phase became neutral. The chloroform extracts were dried under nitrogen and stored at –20°C until high-performance liquid chromatography (HPLC) analysis. Extraction was carried out under dim-light conditions to prevent carotenoids photodegradation, isomerization, and structural changes. For each sample, three replicates were performed.

Before HPLC analysis, the dried extract was dissolved in 120- μ L ethyl acetate. HPLC analysis of carotenoids was carried out on Waters Alliance 2695 system (Waters Corp., Milford, MA) consisting of a 2695 separation module and a 2996 PDA detector, equipped with a 250 \times 4.6 mm i.d., 5 μ m, YMC reverse-phase C₃₀ column and a 20 \times 4.6 mm i.d., YMC C₃₀ guard (Waters, Milford, MA). Chromatography was carried out at 25°C with elution program as previously described (Xu et al. 2006). Carotenoids were identified on the basis of the same retention times and the

same spectroscopic characteristics as standards. Total carotenoid content was β -carotene equivalent which was converted by all chromatographic peak areas at a wavelength of 450 nm. Carotenoid standards β -cryptoxanthin and β -carotene were purchased from Sigma Chemical Co. (St. Louis, MO, USA), and lycopene was prepared as previously reported (Xu et al. 2006). Methods for carotenoid quantification have been reported previously (Xu et al. 2006).

RNA Extraction and Purification

Total RNA was extracted from flesh tissues according to a modified CTAB extraction protocol (Xu et al. 2004). Prior to reverse-transcription, RNA samples were purified with DNase using DNase I kit (TaKaRa, Japan), according to the manufacturer’s guidelines, and then quantified by a spectrophotometer (Eppendorf, Germany) at 260 nm.

First Strand cDNA Synthesis and Primer Design

Reverse-transcription polymerase chain reaction (RT-PCR) was performed on 1 μ g total RNA using PrimeScript™ First Strand cDNA Synthesis Kit (TaKaRa, Japan) with Oligo-dT primers and the conditions were 60 min at 42°C, 15 min at 70°C, and 1 min at 4°C.

Primers used for gene expression analysis were designed according to cDNA sequence of carotenogenic genes we have submitted to GenBank (Table 1), the amplified fragment length was about 150 bp.

Gene Expression Analysis

Quantitative real-time RT-PCR (Q-PCR) was performed using an ABI 7500 fluorescence quantitative PCR instrument (Applied Biosystems, USA). The *D. kaki Actin* (GenBank Accession No.AB219402) was used as an internal control, whose primers were listed in Table 1. Q-PCR was performed with the SYBR® *Premix Ex Taq*™ (Perfect Real Time; TaKaRa, Japan) and contained 2 \times SYBR® *Premix Ex Taq*™ 12.5 μ L, 50 \times ROX Reference

Table 1 Primers used for gene expression analysis

Primer name	Primer sequence (5'-3')	
Actin _F /Actin _R	CACCACTCAACCCAAAGG	CCAGAATCCAGGACAATAC
PSY _F /PSY _R	AACTATGGCTCTTTCTATGC	AACTTTCTGCTCCGATGA
PDS _F /PDS _R	TTCTATTAGCCGGTGAC	ACCATACTTGCTTCCACC
ZDS _F /ZDS _R	GAGGCATTGGTGGCATTAA	AGGCTGGCGTTTGTATCA
LCYB _F /LCYB _R	AGACTGCCTTGATACTACCT	TATGACCTTTGCCTGATG
BCH _F /BCH _R	GCGGTGTAGAAACGGATAA	GCGATAGTAGACAGCCAAAA

Dye II 0.5 μ L, 2 μ L cDNA solution as a template, 1 μ L mix solution of target gene primers, and 9 μ L ddH₂O in a final volume of 25 μ L. The amplification was carried out under the following conditions: 50°C for 2 min followed by an initial denaturation step at 95°C for 5 min, 40 cycles at 95°C for 15 s, 50°C for 15 s, and 72°C for 40 s. The Ct values of the triplicate reactions were gathered by the 7500 System Sequence Detection Software version 3.0.

Statistical Analysis

Fruit diameter, shape index, and weight were the average value of ten fruits. All the other data were means of three replicates with standard deviations. Microsoft Excel (Microcal Software Inc., Northampton, MA, USA) was used to calculate standard deviations. Figures were drawn by SigmaPlot 10.0 (SPSS Inc., USA).

Results

Fruit Quality Indices

“Niuxinshi” and “Nishimurawase” were used as representatives of astringent and non-astringent varieties, respectively. Their vertical and horizontal diameters and fruit weights of

five developmental stages were shown in Table 2. Fruit quality indices trended similarly and increased constantly in two types of cultivars with the development of fruit, but there were only some differences in quantity.

In the uniform color space, L^* represented the lightness, a^* represented ratio of red/magenta and green and b^* represented ratio of yellow and blue. A comparison of fruit color between “Niuxinshi” and “Nishimurawase” showed that they had similar tendency, but only had differences in quantity (Fig. 3). With the development of fruit, L^* value was constantly increased, but declined when fruit color deepened. a^* was negative value and constantly growth which showed that the fruit color changed from green to red. b^* value trended similarly with L^* value, which revealed that the fruit color was yellow and gradually deepening, and C^* also represented the lightness which trended similarly with L^* value.

In addition, the color was well described by a^*/b^* ratio which was negative for green fruits, zero for yellow fruits, and positive for orange fruits (Rodrigo et al. 2004). Alternatively, the color also could be depicted by hue angle (H°) as follows: 0° for reddish-purple, 90° for yellow, 180° for bluish-green and 270° for blue (Crisosto et al. 1994; Intelmann et al. 2005). These two values all showed that the fruit color changed from green to yellow, and “Nishimurawase” was more evident.

Table 2 Fruit size, weight, and shape index of persimmon fruit during developmental stages

Stage	Date	Vertical diameter (cm)	horizontal diameter (cm)	Fruit shape index	Fruit weight (g)
Niuxinshi					
S1	08–21	5.19	4.77	1.08	64.52
S2	09–05	5.25	4.84	1.08	66.59
S3	09–20	6.13	5.19	1.18	84.78
S4	10–05	6.28	5.50	1.14	100.20
S5	10–22	6.30	5.52	1.14	105.34
Nishimurawase					
S1	08–21	4.43	4.85	0.91	60.84
S2	09–05	4.47	5.06	0.88	67.18
S3	09–20	4.96	5.51	0.90	87.72
S4	10–05	5.20	6.16	0.84	119.37
S5	10–22	5.23	6.18	0.84	124.02

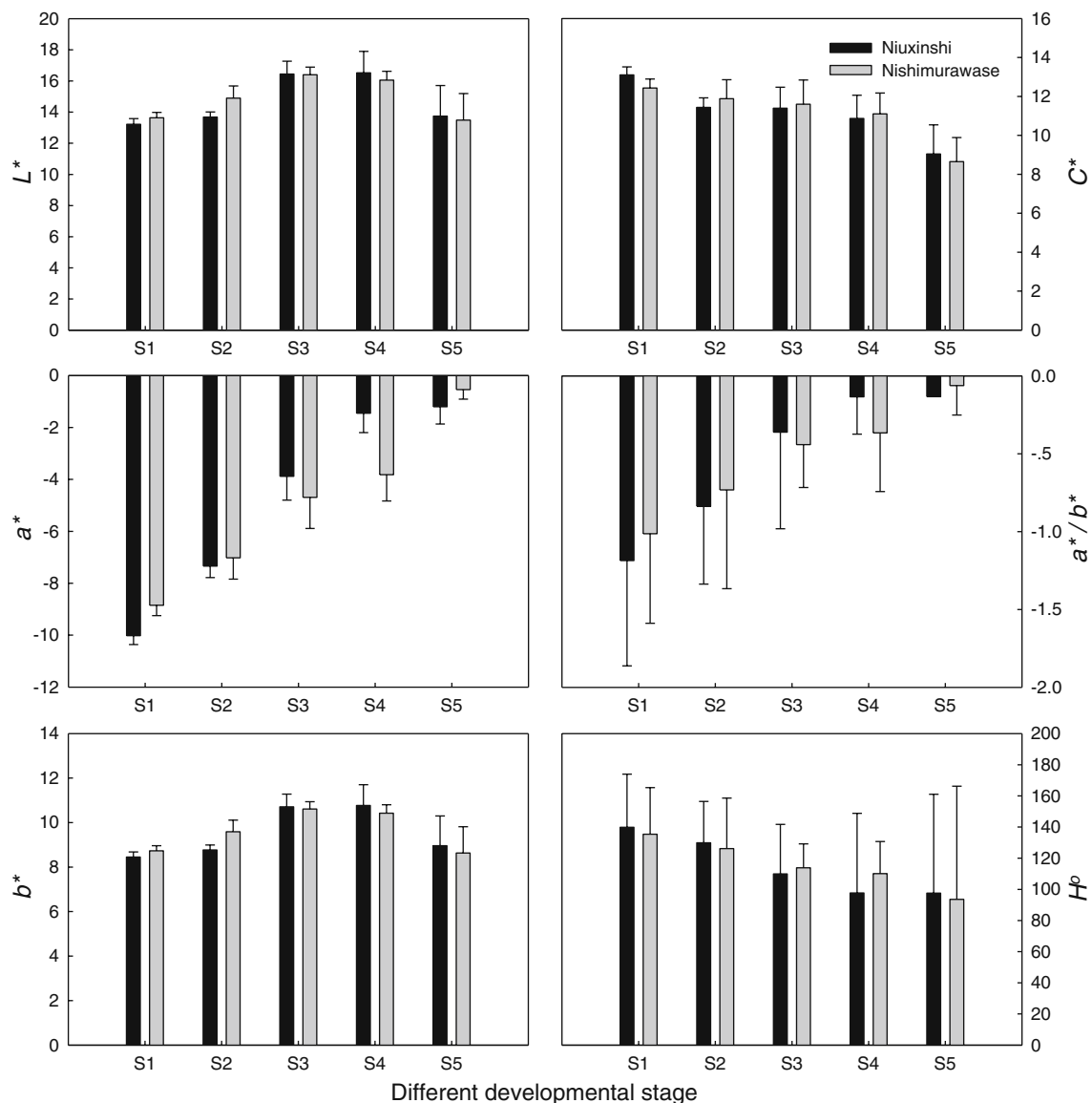


Fig. 3 Changes in peel color of persimmon fruit during development

Carotenoid Accumulation

We determined the contents of total carotenoid, β -cryptoxanthin, which was the main carotenoid composition of persimmon fruit (Kudritskaya et al. 1984; Yuan et al. 2006), β -carotene and lycopene, which were upstream products of carotenoid biosynthesis in the flesh of two types of persimmon by HPLC, with different accumulation patterns as listed in Table 3. Total carotenoid content was gradually increasing during fruit development, especially in “Nishimurawase” which increased by about 5.54 times from S1 to S5 stage, and total carotenoid content in “Nishimurawase” was higher than that in “Niuxinshi” at each stage, which was above twice as much as that in “Niuxinshi” when fruit was nearly maturity. With regard to developmental stages, three carotenoid compositions were

all under detection limit at S1 stage, but total carotenoid content in “Nishimurawase” was $16.44 \pm 1.27 \mu\text{g}/100 \text{ g FW}$, and it was not detected in “Niuxinshi”. At S2 stage, three carotenoid compositions in “Niuxinshi” were the same as that at S1. As far as concerned the carotenoid compositions, contents of individual carotenoid were differed. Lycopene only detected in “Nishimurawase” at the final stage. β -Cryptoxanthin and β -carotene were under the detection limit at early fruit developmental stages, but it was also found that maturity changes were accompanied by increased β -cryptoxanthin and β -carotene contents.

Carotenogenic Genes Expression Analysis

To examine whether carotenoid accumulation in flesh during persimmon fruit development and differences in

Table 3 Carotenoid content in the flesh of different cultivars during fruit development ($\mu\text{g}/100\text{ g FW}$)

Cultivar	β -cryptoxanthin	β -carotene	Lycopene	Total
Niuxinshi				
S1	–	–	–	–
S2	–	–	–	5.39 \pm 1.06
S3	–	1.76 \pm 0.24	–	24.16 \pm 3.27
S4	10.24 \pm 0.98	1.77 \pm 0.11	–	39.17 \pm 3.54
S5	25.99 \pm 2.33	2.51 \pm 0.26	–	50.24 \pm 5.22
Nishimurawase				
S1	–	–	–	16.44 \pm 1.27
S2	2.12 \pm 0.33	–	–	26.37 \pm 3.05
S3	5.86 \pm 0.49	4.00 \pm 0.33	–	49.59 \pm 3.12
S4	22.60 \pm 2.03	5.22 \pm 0.49	–	101.79 \pm 9.04
S5	28.55 \pm 2.08	5.47 \pm 0.49	5.39 \pm 0.52	107.62 \pm 9.22

(–) under the detection limit

carotenoid composition between two types of persimmon could be related to the expression of carotenoid biosynthetic genes, transcript levels of five carotenogenic genes we had isolated from persimmon fruit, i.e. phytoene synthase gene (*DkPSY*, GenBank Accession No. FJ594485), phytoene desaturase gene (*DkPDS*, GenBank Accession No. GU112527), ζ -carotene desaturase gene (*DkZDS*, GenBank Accession No. GU075728), lycopene beta-cyclase gene (*DkLCYB*, GenBank Accession No. FJ940723), and beta-carotene hydroxylase gene (*DkBCH*, GenBank Accession No. FJ790215) were analysed by Q-PCR which was a highly sensitive, accurate, rapid and high-throughput technique had been developed (Feng et al. 2006; Qi et al. 2010) (Fig. 4), in the same samples as used in the HPLC analysis. During two types of persimmon fruit development, the expression levels of *DkPSY* and *DkBCH* were all relatively low at first four stages, but they were substantial increase at S5 stage, especially in “Niuxinshi” which were about 16 and 12 times what they were at the first stage, respectively. The expression level of *DkPDS* reached maximum value at S4 stage, which was slightly higher in “Niuxinshi” at former stages while the latter stage was contrary. The expression pattern of *DkZDS* was similar in “Niuxinshi” and “Nishimurawase”, which had been rising until reached maximum level, and then declined; their difference was that the highest expression level of “Niuxinshi” and “Nishimurawase” was at S2 and S4 stages, respectively. In addition, *DkLCYB* mRNA had been maintained at a low level.

Discussion

Persimmon (*D. kaki* L.) is a deciduous fruit tree which is predominantly cultivated in sub-tropical and warm tem-

perate regions. Over many years, some studies on persimmon fruit carotenoids have been investigated. Nakagawa et al. (2008) found the main carotenoid composition in yellow- to orange-colored fruit was β -cryptoxanthin, and that lycopene was the major pigment in red-colored fruit. In persimmon fruit, maturity changes were accompanied by an increase in β -cryptoxanthin content (Gross et al. 1984). But in the peel, total carotenoid content decreased until color break, then increased gradually and drastically at the last ripening stages (Ebert and Gross 1985), and the expression levels of *DkPSY*, *DkLCYB*, and *DkBCH* genes in peel tissue was higher than that in flesh and pedicel tissues (Zhao et al. 2010). In this study, all values of color evaluation revealed that the fruit color changed from green to yellow with the fruit development, and “Nishimurawase” was more evident. Meanwhile, β -cryptoxanthin and total carotenoid contents in “Nishimurawase” were higher than those in “Niuxinshi”, these revealed there was a positive correlation between fruit color and the contents of main composition and total carotenoids.

Carotenoids could be found in all plants chlorenchyma, but their content and composition are to some degree regulated by developmental stages of plant tissues (Xu and Zhang 2000). Ronen et al. (1999) found that during tomato fruit development, the mRNA levels of *PSY* and *PDS* increased, while the mRNA levels of *LCYB* and *LCYE* declined and completely disappeared, which led to accumulation of lycopene. Kato et al. (2004) found that a simultaneous increased in the expression of genes (*PSY*, *PDS*, *ZDS*, *LCYB*, *BCH*, and *ZEP*) led to massive β -cryptoxanthin, zeaxanthin, and violaxanthin accumulation in citrus fruit as maturation progressed.

In this study, total carotenoid content had been increased, and the content in “Nishimurawase” was always higher than that in “Niuxinshi” during fruit

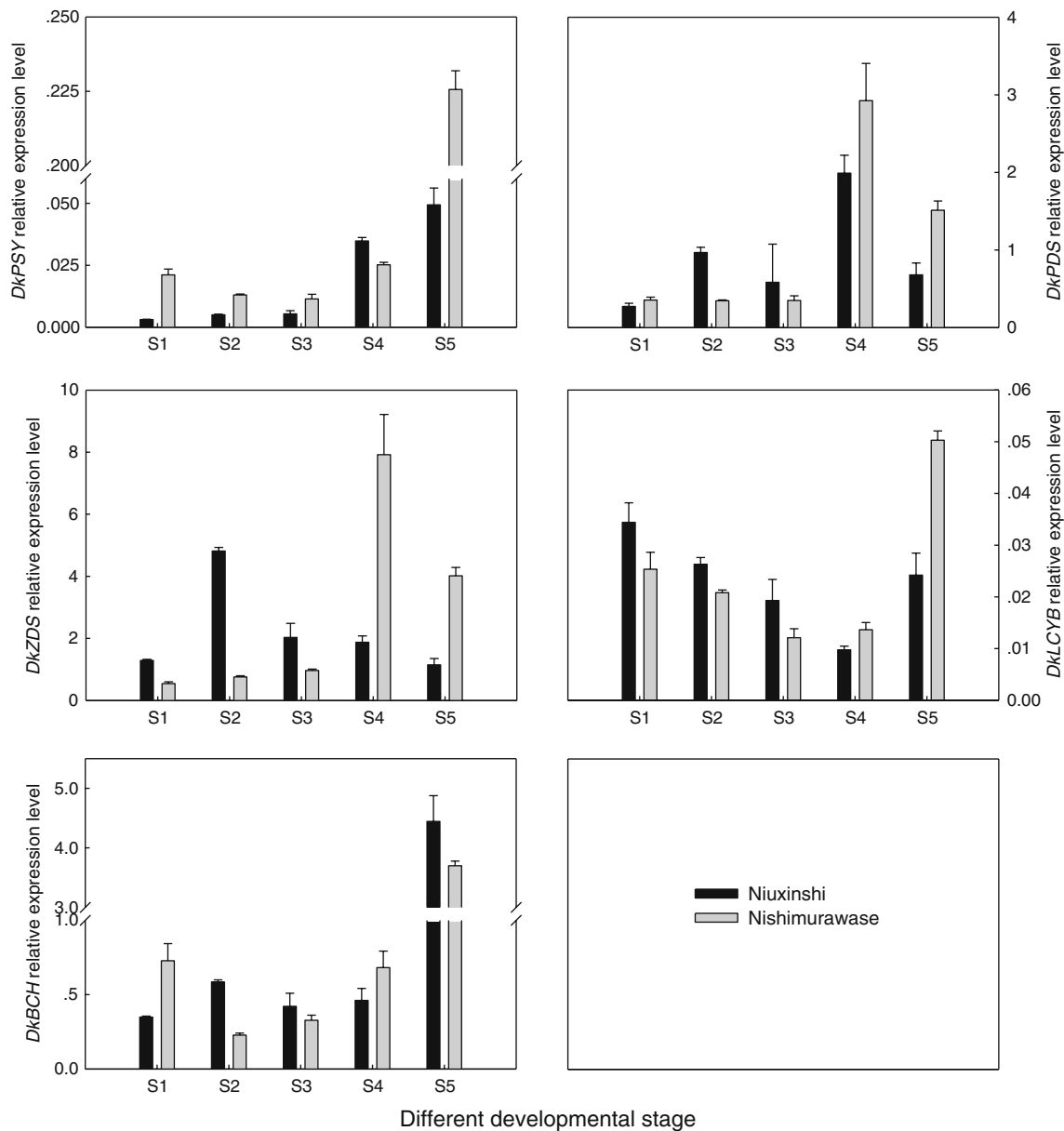


Fig. 4 Expression pattern of carotenogenic genes of persimmon fruit at different developmental stage

development, together with the results of carotenogenic genes expression analysis, which indicated that the carotenoid accumulation of persimmon fruit resulted from the interaction of these carotenogenic genes. For example, although the expression level of *DkPSY* in “Nishimurawase” was slightly lower than that in “Niuxinshi” at S4 stage, relatively higher total carotenoid content were found in “Nishimurawase” due to the higher expression levels of *DkPDS*, *DkZDS*, *DkLCYB* and *DkBCH*. But the accumulation mechanism had a few differences in two types of persimmon fruit. As a complete unit, the relatively low expression level of *DkPSY* in “Niuxinshi” resulted in low carotenoid contents or even

under the detection limit at the early fruit developmental stages; but low carotenoid contents in “Nishimurawase” were due to the relatively low expression level of carotenogenic genes other than *DkPSY*. At the late fruit developmental stages, increased expression levels of *DkPSY*, *DkPDS*, and *DkBCH* induced elevated carotenoid contents; because all carotenogenic genes strongly expressed in “Nishimurawase”, lots of carotenoids were accumulated. In addition, β -cryptoxanthin was the main component whose content increased with the fruit maturity changes, which was mainly because of *DkBCH* which might lead more conversion of β -carotene to β -cryptoxanthin (Niikawa et al. 2007).

Acknowledgements This work was financially supported by the Natural Science Foundation of China (30771491), China Postdoctoral Science Foundation (20100471401) and Postdoctoral Science Foundation of Jiangsu Province (0901055C).

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