DOI:10.1021/jf9005998

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

# Effect of Postharvest Temperature and Ethylene on Carotenoid Accumulation in the Flavedo and Juice Sacs of Satsuma Mandarin (*Citrus unshiu* Marc.) Fruit

Hikaru Matsumoto,*,† Yoshinori Ikoma,† Masaya Kato,‡ Naoko Nakajima,† and Yoshinori Hasegawa†

†Okitsu Citrus Research Station, National Institute of Fruit Tree Science, National Agriculture and Food Research Organization, Okitsunakacho, Shimizu-ku, Shizuoka 424-0292, Japan, and ‡Faculty of Agriculture, Shizuoka University, Ohya, Suruga-ku, Shizuoka 422-8529, Japan

The effect of postharvest temperature (5, 20, and 30 °C) and ethylene at different temperatures (20 and 5 °C) on carotenoid content and composition and on the expression of the carotenoid biosynthesis-related genes was investigated in the flavedo and juice sacs of Satsuma mandarin (*Citrus unshiu* Marc.) fruit. Under an ethylene-free atmosphere, storage at 20 °C rapidly increased the carotenoid content in flavedo and maintained the content in juice sacs. In contrast, storage at 5 and 30 °C gradually decreased the content in juice sacs but slowly increased that in flavedo. Under an ethylene atmosphere, storage at 20 °C enhanced the carotenoid accumulation in flavedo more dramatically than found under an ethylene-free atmosphere with distinct changes in the carotenoid composition but did not noticeably change the content and composition in juice sacs. In contrast, storage at 5 °C under an ethylene atmosphere repressed carotenoid accumulation with changes in the carotenoid composition in flavedo but did not clearly change the carotenoid content in juice sacs. Under an ethylene-free atmosphere, differences in the gene expression profile among the temperatures were observed but were not well-correlated with those in the carotenoid content in flavedo and juice sacs. Under an ethylene atmosphere, in flavedo, the gene expression of phytoene synthase (PSY) and phytoene desaturase (PDS) was slightly higher at 20 °C but lower at 5 °C than under an ethylene-free atmosphere. At 20 °C, the gene expression of several carotenoid biosynthetic enzymes promoted by ethylene seemed to be responsible for the enhanced accumulation of carotenoid in flavedo. In contrast, at 5 °C, the repressed gene expression of PSY and PDS by ethylene seemed to be primarily responsible for the repressed accumulation of carotenoid in flavedo. In juice sacs, the small response of the gene expression to ethylene seemed to be responsible for small changes in carotenoid accumulation under an ethylene atmosphere.

**KEYWORDS: Carotenoids; citrus; temperature; ethylene; gene expression; Satsuma mandarin**

## INTRODUCTION

Carotenoids are important for fruit quality in citrus fruit because the color orange in the peel and juice sacs is mainly due to the presence of these pigments (*1*). In addition, specific carotenoids are important as human nutrients because they serve as precursors for vitamin A and reduce the risk for chronic diseases, such as cardiovascular diseases and carcinogenesis (*2*, *3*).

Carotenoid accumulation in citrus fruit during fruit maturation was studied in the flavedo and juice sacs of different cultivars (*4*−*9*). Recently, the relationship between carotenoid accumulation and the gene expression of carotenoid biosynthesis-related enzymes in citrus fruit was investigated during natural ripening. At the green stage of the flavedo, $\beta$,$\varepsilon$-carotenoids (mainly lutein) are the major carotenoids in the flavedo of mandarins and oranges (*5*−*8*). With the transition of peel color from green to orange, the change from $\beta$,$\varepsilon$-carotenoid accumulation to $\beta$,$\beta$-carotenoid accumulation occurs with a decrease in the gene expression of lycopene $\varepsilon$-cyclase (LCYe) and $\beta$-ring hydroxylase (HYb) in the flavedo of mandarins and oranges (*6*−*8*). In the flavedo of Satsuma mandarin and Valencia orange, a concomitant increase in the gene expression of lycopene $\beta$-cyclase (LCYb) also occurs (*6*). As fruit maturation progresses, $\beta$,$\beta$-xanthophyll (e.g., $\beta$-cryptoxanthin and violaxanthin) accumulates massively with a simultaneous increase in the gene expression of phytoene synthase (PSY), phytoene desaturase (PDS), $\zeta$-carotene desaturase (ZDS), lycopene $\beta$-cyclase (LCYb), HYb, and zeaxanthin epoxidase (ZEP) in the flavedo and juice sacs of Satsuma mandarin and oranges, although, in the case of the flavedo of Navelate orange, the gene expression of LCYb and ZEP is constitutive (*6*−*9*). In addition to carotenoid biosynthetic genes, it has been reported that several genes in another upstream metabolic pathway, the methyl erythritol phosphate (MEP) pathway, play an important role in carotenoid accumulation in the flavedo and juice sacs of oranges during

*Corresponding author (telephone +81-543-69-7100; fax +81-543-69-2115; e-mail hikaruoo@affrc.go.jp).

 Published on Web 05/14/2009 

natural ripening (*8*, *10*). On the other hand, several studies have reported that, in the flavedo of orange, ethylene-induced carotenoid accumulation was correlated with a simultaneous increase in the gene expression of carotenoid biosynthetic enzymes (*11*), and ethylene-induced accumulation of abscisic acid (ABA) was also correlated with an increase in the gene expression of 9-*cis*-epoxycarotenoid dioxygenase, NCED (*12*), which catalyzes the cleavage of 9-*cis*-violaxanthin or 9′-*cis*-neoxanthin to form xanthoxin, a precursor of ABA (*13*). These results suggest that carotenoid accumulation during natural and ethylene-induced maturation is highly regulated by the coordination of the expression among carotenoid biosynthetic genes.

In a previous study, the effect of a postharvest ethylene treatment on carotenoid accumulation was investigated in the flavedo of citrus fruit (*11*, *14*−*18*). These studies focused on the flavedo of citrus fruit because a postharvest ethylene treatment is a common horticultural practice for the degreening of citrus fruit, especially from early-harvested cultivars in which the peel is green (*18*). A previous study investigated the optimal temperature for ethylene treatment for the degreening of citrus peel within the range of 15−30 °C in several cultivars (*16*). The study suggested that the optimal temperature for carotenoid accumulation in flavedo is in the range of 15−25 °C and that an ethylene treatment at these temperatures noticeably accelerates carotenoid accumulation in the flavedo of citrus fruit (*16*). Recently, the effect of postharvest ethylene treatment on the gene expression of carotenoid biosynthetic enzymes and on carotenoid accumulation was investigated in the flavedo of mature green citrus fruit at around 20 °C, the optimal temperature for degreening of citrus peel (*11*, *12*, *19*).

Postharvest citrus fruit is exposed to various temperatures (low to high) in a storage room, on a store shelf, and during transportation (*20*). In addition, postharvest citrus fruit may be exposed to ethylene at different temperatures when the citrus fruit and ethylene-producing fruits and vegetables coexist in a storage room, on a store shelf, or during transportation (*20*, *21*). However, few studies have focused on the effect of low temperature (5 °C) on carotenoid accumulation and the difference in the effect of ethylene on carotenoid accumulation among different temperatures. To understand the carotenoid accumulation of citrus fruit under the various postharvest conditions, extensive research on the effect of postharvest temperature and ethylene at different temperatures on carotenoid accumulation in citrus fruit is required. Furthermore, in juice sacs, research on carotenoid biosynthesis is important because some carotenoids have nutritional and health-promoting effects for humans (*2*, *3*). However, in edible juice sacs, the effect of postharvest temperature and ethylene on carotenoid biosynthesis has been scarcely studied.

The objective of the present study was to investigate the effect of postharvest temperature and ethylene at different temperatures on carotenoid accumulation in the flavedo and juice sacs of Satsuma mandarin (*Citrus unshiu* Marc.) fruit. For this purpose, the content and composition of carotenoids and the gene expression of carotenoid biosynthesis-related enzymes were investigated because a relationship between the carotenoid accumulation and the gene expression of those enzymes during fruit maturation has been reported. In the present study, the effect of postharvest temperature was investigated at low (5 °C), ambient (20 °C), and high (30 °C) temperatures, and the effect of postharvest ethylene was investigated at ambient (20 °C) and low (5 °C) temperatures. Information on the effect of temperature and ethylene at different temperatures on carotenoid accumulation in flavedo and juice sacs will contribute to a better understanding of the interaction between fruit storage conditions and the maintenance of fruit quality in Satsuma mandarin fruit.

## MATERIALS AND METHODS

**Plant Materials and Storage Conditions.** Fruits of a late-maturing cultivar, Aoshima Satsuma mandarin (*C. unshiu* Marc.), were harvested on December 4, 210 days postanthesis, from a tree at the National Institute of Fruit Tree Science, Okitsu Citrus Research Station, Okitsu (Shizuoka, Japan). Fruits that were uniform in size and color were selected. The maturation stage of the sampled fruit was almost fully colored, but the peel of the fruit had partially green areas.

In the present study, two different experiments were conducted, that is, on the temperature effect and on the effect of ethylene at different temperatures. For the experiment on the temperature effect, the fruits were divided into three groups and incubated in containers under ethylene-free air for up to 3 weeks in the dark at 5, 20, and 30 °C. The samples were incubated in the dark under 85−90% relative humidity with continuous ventilation. At 1, 2, and 3 weeks after incubation, at least three fruits per treatment were collected. For the experiment on the effect of ethylene at different temperatures, the fruits were divided into two groups, and each was incubated at 20 and 5 °C in the dark for 24 h to equilibrate the fruit temperature to each storage temperature. The fruits equilibrated to each temperature were divided into two groups and subsequently shut tightly in airtight 19 L glass desiccators and incubated under the following conditions: at 20 °C under an atmosphere of 1000 $\mu$L/L ethylene; at 20 °C under an ethylene-free atmosphere; at 5 °C under an atmosphere of 1000 $\mu$L/L ethylene; and at 5 °C under an ethylene-free atmosphere. The entire treatment was performed in the dark for up to 16 days under 85−90% relative humidity. The fruits were ventilated every day. Just after ventilation, 19 mL of ethylene was spiked into the desiccators every day for the ethylene treatment. After 1 (just before ethylene treatment), 8, and 16 days of incubation, at least three fruits of each treatment were collected. The flavedo and juice sacs were separated from the sampled fruit (*5*), immediately frozen in liquid nitrogen, and stored at −80 °C until use. In both experiments, at least 30 fruits were used for each treatment, and at least 3 fruits per treatment and incubation time were sampled to conduct all experiments.

**Quantification of Ethylene in Juice Sacs.** Thirty fruits were divided into three groups and incubated in airtight containers under the following conditions: an atmosphere of 1000 $\mu$L/L ethylene, an atmosphere of 10 $\mu$L/L ethylene, or an ethylene-free atmosphere at 20 °C for 24 h. Immediately after the fruit was peeled and the segment membrane was removed, sample juice sacs were immersed in a saturated solution of $(NH_4)_2SO_4$, and the intracellular ethylene gas in the juice sacs was extracted according to a method described previously (*22*). The quantification of ethylene was performed by GC using an alumina column and flame ionization detection (FID). The quantification limit of ethylene was 0.01 $\mu$L/L. At least five fruits per treatment were analyzed. GC analysis was performed in two replicates for each sample.

**Carotenoid Analysis.** The carotenoid in the tissues was extracted according to a method described previously (*5*). The carotenoid concentration of each sample was analyzed using HPLC (Agilent 1100, Agilent Technologies, Palo Alto, CA) with a 250 mm × 4.6 mm i.d., 5 $\mu$m, $C_{30}$ carotenoid column (YMC Europe GMBH, Germany) with a ternary gradient elution of water, MeOH, and MTBE pumped at a flow rate of 1 mL/min and photodiode array detection (set to scan 220−250 nm). Three different gradient elution schedules were used to quantify the carotenoids (*6*). The carotenoids, phytoene, ζ-carotene, β-carotene, β-cryptoxanthin, zeaxanthin, 9-*cis*-violaxanthin, *all-trans*-violaxanthin, α-carotene, and lutein, were identified by comparing the retention time and spectra with authentic standards or literature data (*5*, *6*). The concentrations of the carotenoids were determined by reference to standard curves prepared for each carotenoid. The value of total carotenoids was the sum of the identified carotenoids. Extraction and analysis were performed at least in two replicates for each sample.

**Total RNA Extraction and Real-Time Quantitative RT-PCR.** Total RNA was extracted from the flavedo and juice sacs according to a previously reported method (*23*). The total RNA was cleaned up with the RNeasy Mini Kit (Qiagen, Hilden, Germany) with on-column DNase digestion. The reactions of reverse transcription (RT) were performed with 2 $\mu$g of purified RNA and a random hexamer at 37 °C for 60 min using TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA). TaqMan MGB probes and sets of primers designed on Satsuma

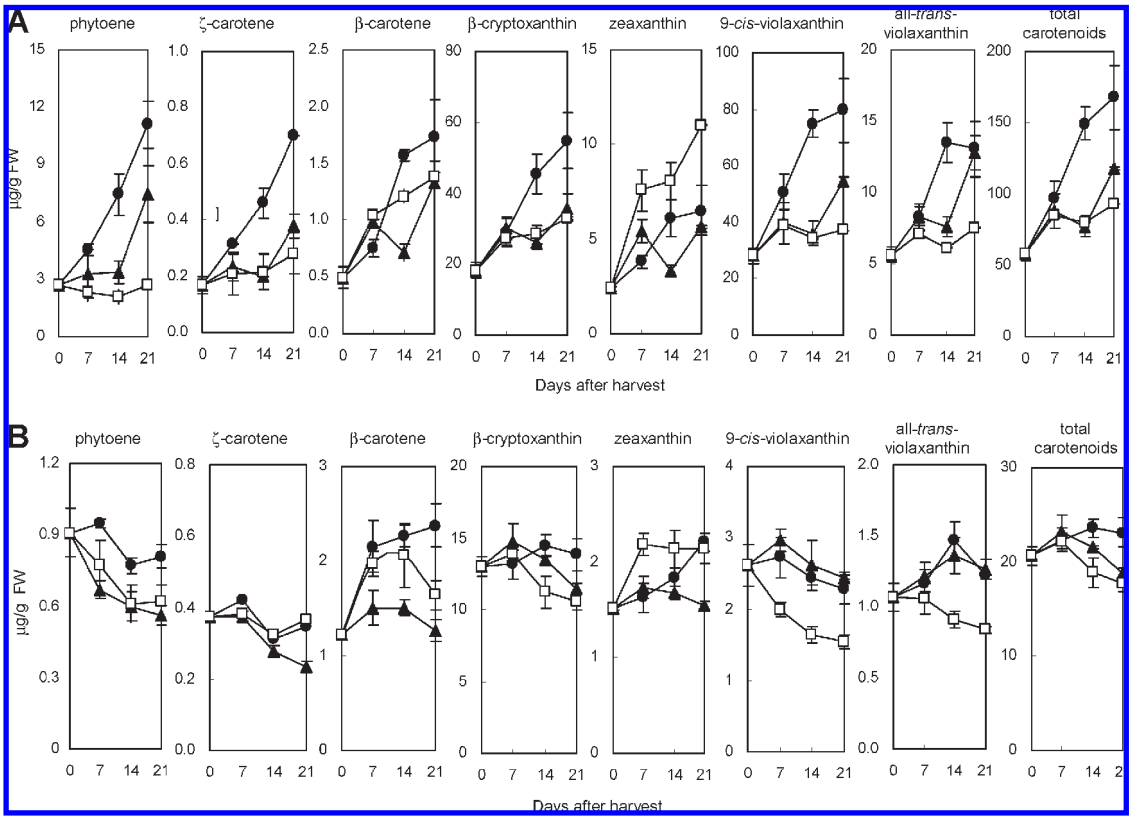


**Figure 1.** Effect of temperature on the carotenoid content in flavedo and juice sacs: (**A**) flavedo of fruit stored at 20 °C (●), 5 °C (▲), or 30 °C (□); (**B**) juice sacs of fruit stored at 20 °C (●), 5 °C (▲), or 30 °C (□). Content is expressed on a fresh weight basis ($\mu$g/g). Values are the mean ± SE of three fruits.

mandarin cDNAs, *CitPSY* (accession no. AB114648), *CitPDS* (accession no. AB114649), *CitZDS* (accession no. AB114650), *CitLCYb* (accession no. AB114652), *CitHYb* (accession no. AB114653), *CitZEP* (accession no. AB114654), *CitNCED2* (accession no. AB219169), which is similar to AtNCED5 in *Arabidopsis* on the deduced amino acid level, and *CitNCED3* (accession no. AB219174 ), which is similar to AtNCED3 in *Arabidopsis* on the deduced amino acid level (*24*, *25*), were used for the analysis of the gene expression of PSY, PDS, ZDS, LCYb, HYb, ZEP, NCED2, and NCED3, respectively. For an endogenous control, the TaqMan Ribosomal RNA Control Reagents VIC Probe (Applied Biosystems) was used. TaqMan real-time PCR was carried out with the TaqMan Universal PCR Master Mix (Applied Biosystems) according to the manufacturer's instructions. The thermal cycling conditions were 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. The levels of gene expression were analyzed with ABI PRISM 7000 Sequence Detection System Software (Applied Biosystems) and normalized with the results of 18S rRNA. Real-time quantitative RT-PCR was performed in three replicates for each sample.

**ABA Quantification.** ABA was extracted according to a previously reported method (*25*, *26*). Extracted ABA was methylated with trimethylsilyldiazomethane (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and analyzed by GC-MS (GCMS-QP2010, Shimadzu, Kyoto, Japan) using a 30 m × 0.25 mm i.d., 0.25 $\mu$m, InertCap1 capillary column (GL Science, Tokyo, Japan) and MS detection. The column was held at an initial temperature of 80 °C for 1 min, ramped at 30 °C/min to 245 °C, and then ramped at 5 °C/min to 280 °C. Helium was used as the carrier gas. The MS was operated at 70 eV in the electron impact ionization (EI) mode and at an ion source temperature of 200 °C. The MS chromatograms were evaluated with total ion monitoring. The content of endogenous *cis*-ABA was calculated from the ratio of the peak area for 190(ABA)/194(d6ABA) and expressed on a fresh weight basis (ng/g). Extraction and analysis were performed at least in two replicates for each sample.

## RESULTS AND DISCUSSION

**Effect of Postharvest Temperature on Carotenoid Accumulation in Flavedo and Juice Sacs.** The effect of the postharvest temperature on carotenoid content was investigated at 5, 20, and 30 °C for 3 weeks. In the present study, total carotenoid content was calculated as the sum of the content of carotenoids, phytoene, ζ-carotene, β-carotene, β-cryptoxanthin 9-*cis*-violaxanthin, and *all-trans*-violaxanthin. In the flavedo, the total carotenoid content increased at all temperatures, and the increase was rapid and noticeable at 20 °C (**Figure 1A**). At 20 °C, the total carotenoid content dramatically increased with an increase in the content of most carotenoids such as β-cryptoxanthin and 9-*cis*-violaxanthin (**Figure 1A**). At 21 days after harvest, the total carotenoid content was higher at 20 °C than at other temperatures. In juice sacs, the total carotenoid content increased slightly at all of the temperatures at 7 days after harvest (**Figure 1B**). Afterward, the content remained at a similar level at 20 °C but gradually decreased at 5 and 30 °C. At 30 °C, the total carotenoid content decreased rapidly with a decrease in the content of β-cryptoxanthin, 9-*cis*-violaxanthin, and *all-trans*-violaxanthin. Thus, storage at 20 °C distinctly promoted carotenoid accumulation in flavedo and maintained the content in juice sacs at least above that just after harvest. In contrast, storage at 5 and 30 °C promoted carotenoid accumulation in flavedo, although more slowly at 5 and 30 °C than at 20 °C, but gradually decreased carotenoid content in juice sacs during 3 weeks of storage. These results suggest that carotenoid biosynthesis in citrus fruit is temperature-sensitive. Moreover, the effect of the temperature on carotenoid accumulation is tissue-dependent.

At 30 °C, specific changes in the accumulation of individual carotenoids were observed. In the content of 9-*cis*-violaxanthin and *all-trans*-violaxanthin, a rapid decrease was observed in juice sacs (**Figure 1B**). In flavedo, the increase was slower at 30 °C than at other temperatures (**Figure 1A**). Regarding the content of zeaxanthin, a rapid accumulation was observed in flavedo and juice sacs specifically at 30 °C (**Figure 1**). The content of phytoene in flavedo increased at 5 and 20 °C but did not clearly increase at

30 °C (**Figure 1A**). On the other hand, obvious differences in the accumulation of β-carotene between these temperatures were not observed in flavedo (**Figure 1A**). These results suggest that the effect of storage at 30 °C on carotenoid accumulation in Satsuma mandarin fruit may be different among individual carotenoids. Similar results have been reported in tomato and watermelon (*27*−*29*). In tomato, increasing the fruit temperature to 32 °C inhibited the accumulation of phytoene and lycopene but did not affect the content of β-carotene (*27*).

A previous study reported that the optimal temperature for carotenoid accumulation in citrus peel is in the range of 15−25 °C (*16*). The results in the present study also showed that postharvest storage at 20 °C distinctly enhanced the carotenoid accumulation in the flavedo of Satsuma mandarin fruit. Furthermore, the present study provided additional information that postharvest storage at 20 °C would be able to maintain the carotenoid content in edible juice sacs of Satsuma mandarin fruit.

**Effect of Ethylene at 20 and 5 °C on Carotenoid Content and Composition in Flavedo and Juice Sacs.** The effects of ethylene on carotenoid content and composition in the flavedo and juice sacs of Satsuma mandarin fruit were investigated at 20 and 5 °C because postharvest fruit may be exposed to ethylene during storage at both temperatures.

First, the concentration of ethylene in juice sacs was determined in the presence of ethylene of 1000 and 10 μL/L. Under an atmosphere of 10 μL/L ethylene, the ethylene concentration in the juice sacs of the fruit was 0.41 ± 0.04 μL/L. In contrast, that of the fruit under an atmosphere of 1000 μL/L ethylene was 43.9 ± 3.6 μL/L. In previous studies, to investigate the effect of ethylene on carotenoid accumulation in flavedo, treatments with 10 μL/L were usually administered (*11*, *12*). The purpose of the present study was to investigate the effect of ethylene on carotenoid accumulation not only in flavedo but also in juice sacs. Thus, an atmosphere of 1000 μL/L ethylene was selected because the concentration of ethylene in both fruit tissues was at least 10 μL/L under that condition.

Under air (an ethylene-free atmosphere), the content of total carotenoids in flavedo increased during storage at 20 and 5 °C, and the increase was rapid at 20 °C (**Figure 2**). At 16 days after harvest, the contents of total carotenoids at both temperatures were higher than that on harvest day. Under ethylene (an atmosphere of 1000 μL/L ethylene) at 20 °C, the contents of carotenes (phytoene, ζ-carotene, and β-carotene) and β-cryptoxanthin in flavedo increased dramatically (**Figure 2A**). At 16 days after harvest, the content of these carotenoids under ethylene was much higher than that under air. On the other hand, the contents of zeaxanthin, 9-*cis*-violaxanthin, and *all-trans*-violaxanthin in flavedo under ethylene were similar to those under air (**Figure 2A**). Thus, the enhanced accumulation of carotenes and β-cryptoxanthin by ethylene led to a distinct increase in the content of total carotenoids in flavedo under ethylene at 20 °C. In contrast, at 5 °C, the contents of carotenes and xanthophylls, except for zeaxanthin, in flavedo under ethylene were lower than those under air at 16 days after harvest (**Figure 2B**). The repressed accumulation of all carotenoids, except for zeaxanthin, by ethylene led to a lower content of total carotenoids in flavedo under ethylene at 5 °C. These results suggested that, under an ethylene-free atmosphere, storage at 20 and 5 °C enhanced carotenoid accumulation in flavedo. Under an ethylene atmosphere, storage at 20 °C dramatically enhanced the carotenoid accumulation, but storage at 5 °C repressed the carotenoid accumulation in flavedo. It is known that ethylene treatment at around 20 °C increases the carotenoid content in the flavedo of citrus fruit (*11*, *14*−*17*). The results in the present study suggested that the effect of ethylene on the carotenoid content in flavedo varied with the temperature condition.

Changes in the composition (%) of major carotenoids, 9-*cis*-violaxanthin and β-cryptoxanthin, in flavedo at 16 days after

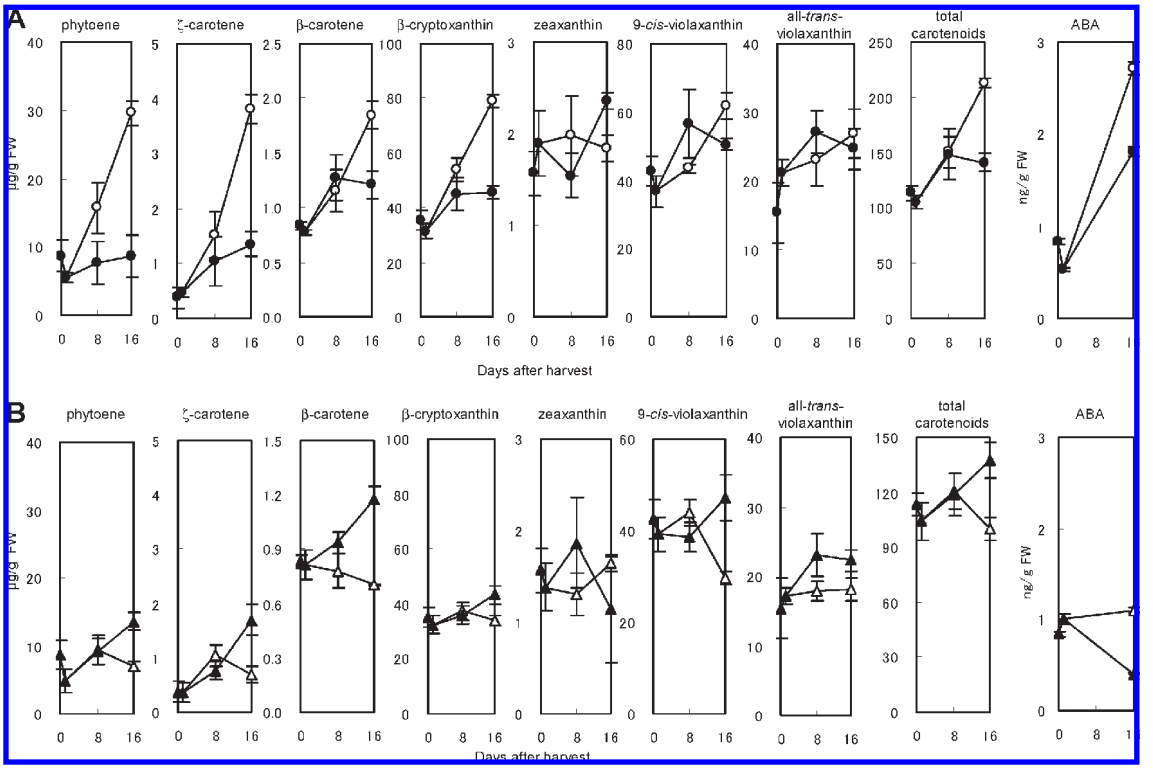


**Figure 2.** Effect of ethylene at 20 and 5 °C on the contents of carotenoids and ABA in flavedo: (**A**) fruit exposed to air (●) or 1000 μL/L ethylene (○) at 20 °C; (**B**) fruit exposed to air (▲) or 1000 μL/L ethylene (△) at 5 °C. Harvested fruits were incubated at 20 or 5 °C for 24 h to equilibrate the fruit temperature and then exposed to ethylene. Content is expressed on a fresh weight basis (μg/g in carotenoids and ng/g in ABA). Values are the mean ± SE of three fruits.

harvest are shown in **Figure 3**. Each carotenoid composition was calculated as a ratio (%) of its content to the total carotenoid content. Under air, no obvious changes in the composition of 9-*cis*-violaxanthin and $\beta$-cryptoxanthin were observed at either temperature (**Figure 3**). In contrast, under ethylene, the composition of 9-*cis*-violaxanthin decreased, but that of $\beta$-cryptoxanthin increased at both temperatures (**Figure 3**). The increase in the content of upstream carotenoids was mainly responsible for the decrease in the composition of 9-*cis*-violaxanthin by ethylene at 20 °C (**Figures 2A** and**3**). On the other hand, under ethylene at 5 °C, the composition of 9-*cis*-violaxanthin decreased without increases in the content of other carotenoids (**Figures 2B** and **3**),

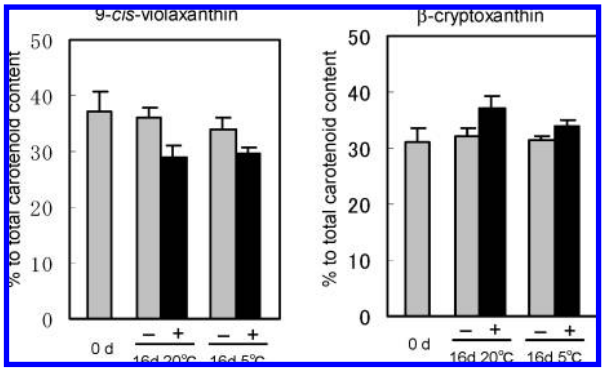


**Figure 3.** Effect of ethylene at 20 and 5 °C on the changes in the carotenoid composition in flavedo. Each carotenoid composition was calculated as a ratio (%) of its content to the total carotenoid content. Composition (%) of 9-*cis*-violaxanthin and $\beta$-cryptoxanthin is shown in freshly harvested fruit (0 d) and in the fruit under air (−) and ethylene (+) at 20 or 5 °C at 16 days after harvest. Values are the mean ± SE of three fruits.

suggesting that the metabolism of violaxanthin to ABA might be enhanced by ethylene.

In juice sacs, at 20 °C, the contents of phytoene, ζ-carotene, and $\beta$-carotene under ethylene were slightly higher, but the contents of zeaxanthin and 9-*cis*-violaxanthin were slightly lower than those under air (**Figure 4A**). At 5 °C, the content of all carotenoids in juice sacs under ethylene was slightly lower than that under air (**Figure 4B**). However, the difference in the content of all carotenoids between air and ethylene was small at 20 and 5 °C (**Figure 4B**). The distinct changes in the composition of 9-*cis*-violaxanthin and $\beta$-cryptoxanthin by ethylene observed in flavedo were not observed in juice sacs at either temperature (**Figure 5**). Thus, irrespective of the presence or absence of ethylene, storage at 20 and 5 °C did not noticeably change the carotenoid content and composition of 9-*cis*-violaxanthin and $\beta$-cryptoxanthin in juice sacs during 16 days of storage.

**Relationship between the Expression of Carotenoid Biosynthesis-Related Genes and Carotenoid Accumulation in Flavedo and Juice Sacs.** The gene expression of PSY, PDS, ZDS, LCYb, HYb, ZEP, NCED2, and NCED3, which was correlated with the carotenoid accumulation during maturation in Satsuma mandarin fruit (*6*, *25*), was examined in the present study. The gene expression of LCYe (the enzyme for $\beta$,$\varepsilon$-xanthophyll synthesis) was not examined because it was negligible in Satsuma mandarin fruit in December (*6*).

The effect of temperature (5, 20, and 30 °C) on the expression of carotenoid biosynthesis-related genes was investigated during 3 weeks of storage (**Figure 6**). A temperature-dependent response in the gene expression of several carotenoid biosynthesis-related enzymes was observed in flavedo and juice sacs (**Figure 6**). In flavedo, the gene expression of PSY was higher at 20 °C than at other temperatures (**Figure 6A**). The gene expression of NCED3 was distinctly enhanced at 5 °C but repressed at 20 and 30 °C (**Figure 6A**). In juice sacs, the gene expression of

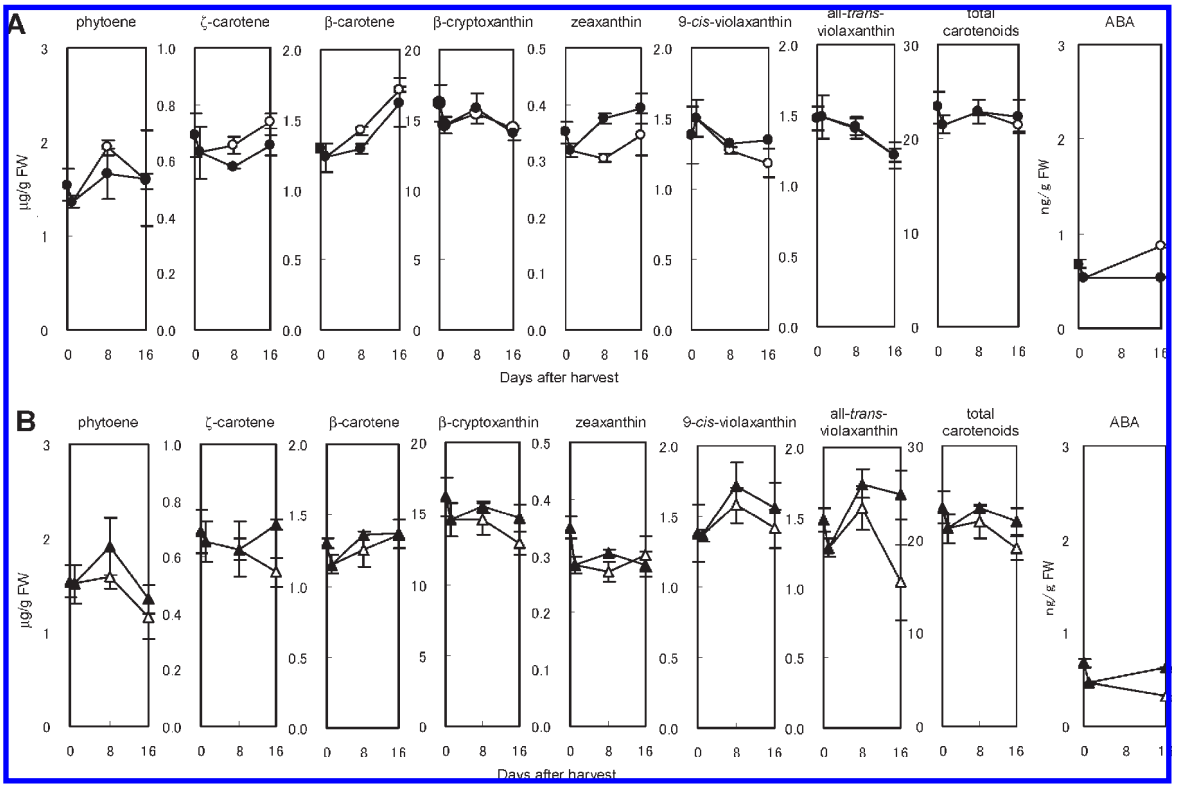


**Figure 4.** Effect of ethylene at 20 and 5 °C on the contents of carotenoids and ABA in juice sacs: (**A**) fruit exposed to air (●) or 1000 $\mu$L/L ethylene (○) at 20 °C; (**B**) fruit exposed to air (▲) or 1000 $\mu$L/L ethylene (△) at 5 °C. Harvested fruits were incubated at 20 or 5 °C for 24 h to equilibrate the fruit temperature and then exposed to ethylene. Content is expressed on a fresh weight basis ($\mu$g/g in carotenoids and ng/g in ABA). Values are the mean ± SE of three fruits.

PSY, PDS, HYb, ZEP, NCED2, and NCED3 was distinctly higher at 5 °C (**Figure 6B**). In contrast, the gene expression of most enzymes was lower at 30 °C than at other temperatures (**Figure 6B**). Thus, in juice sacs, low temperature showed promotive effects on the levels of the gene expression of PSY, PDS, ZEP, NCED2, and NCED3 (**Figure 6B**). However, differences among temperatures in the gene expression profile were not well-correlated with those in the carotenoid content in flavedo and juice sacs (**Figures 1** and **6**). Probably, some post-transcriptional factors, such as enzymatic properties, which are variable among different temperatures, and/or genes, which are not investigated in the present study, such as genes of 1-deoxy-D-xylulose-5-phosphate synthase, hydroxymethylbutenyl 4-diphosphate reductase, and hydroxymethylbutenyl 4-diphosphate synthase in the methyl erythritol phosphate (MEP) pathway, which is an upstream metabolic pathway, may also be involved in the regulation of the postharvest carotenoid accumulation in citrus fruit (*8*−*10*).

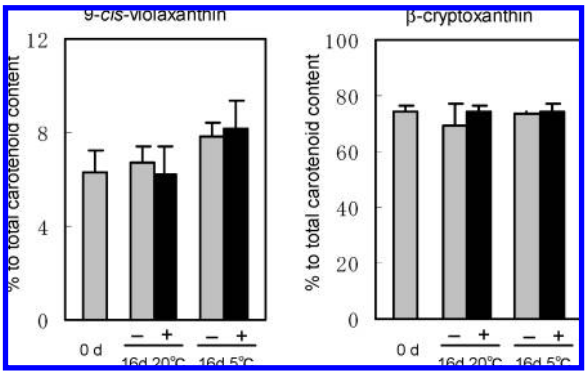


**Figure 5.** Effect of ethylene at 20 and 5 °C on the changes in the carotenoid composition in juice sacs. Each carotenoid composition was calculated as a ratio (%) of its content to the total carotenoid content. Composition (%) of 9-*cis*-violaxanthin and $\beta$-cryptoxanthin is shown in freshly harvested fruit (0 d) and in the fruit under air (−) and ethylene (+) at 20 or 5 °C at 16 days after harvest. Values are the mean ± SE of three fruits.

The effect of ethylene at different temperatures (20 and 5 °C) on the expression of carotenoid biosynthesis-related genes was investigated. In flavedo, the gene expression of HYb, ZEP, NCED2, and NCED3 was promoted by ethylene at 20 and 5 °C (**Figure 7**). In contrast, ethylene obviously promoted a reduction in the gene expression of PSY and PDS at 5 °C (**Figure 7B**), but at 20 °C ethylene slowed the reduction in the gene expression of PSY and enhanced the gene expression of PDS (**Figure 7A**). These results suggested that, in flavedo, the promotive effect of ethylene on the gene expression of HYb, ZEP, NCED2, and NCED3 was a common response at 20 and 5 °C. In contrast, the response of the gene expression of PSY and PDS to ethylene was different at 20 and 5 °C.

In flavedo, at 20 °C, ethylene distinctly enhanced the accumulation of phytoene, $\zeta$-carotene, $\beta$-carotene, and $\beta$-cryptoxanthin with promotion in the gene expression of PSY, PDS, LCYb, and HYb (**Figures 2A** and **7A**). In contrast, ethylene did not clearly enhance the accumulation of 9-*cis*-violaxanthin and *all-trans*-violaxanthin in flavedo at 20 °C, although the gene expression of PSY, PDS, LCYb, HYb, and ZEP was promoted by ethylene (**Figures 2A** and **7A**). Probably, the biosynthesis of 9-*cis*-violaxanthin and *all-trans*-violaxanthin was stimulated by ethylene as massively as that of phytoene, $\zeta$-carotene, $\beta$-carotene, and $\beta$-cryptoxanthin was. However, a drastic increase in the gene expression of NCED2 and NCED3 by ethylene seemed to promote the metabolism from

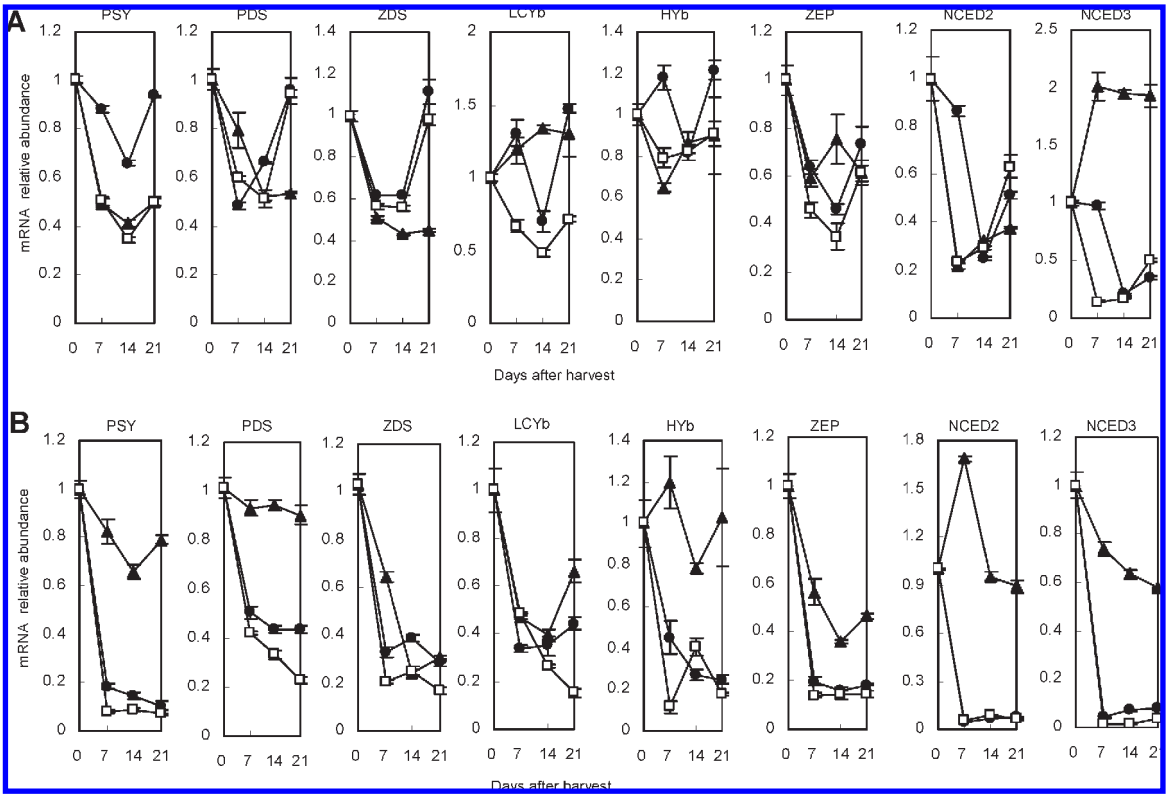


**Figure 6.** Effect of temperature on the expression of carotenoid biosynthesis-related genes in flavedo and juice sacs: (**A**) flavedo of the fruit stored at 20 °C (●), 5 °C (▲), or 30 °C (□); (**B**) juice sacs of the fruit stored at 20 °C (●), 5 °C (▲), or 30 °C (□). Levels of gene expression of PSY, PDS, ZDS, LCYb, HYb, ZEP, NCED2, and NCED3 were analyzed by real-time quantitative RT-PCR with TaqMan MGB probes and sets of primers designed on Satsuma mandarin cDNAs, *CitPSY*, *CitPDS*, *CitZDS*, *CitLCYb*, *CitHYb*, *CitZEP*, *CitNCED2*, and *CitNCED3*, respectively. An expression value of 1 was arbitrarily assigned to the sample immediately after harvest. Values are the mean ± SE of three measurements.

9-*cis*-violaxanthin to ABA (**Figures 2A** and **7A**). In fact, the content of ABA in flavedo under ethylene was higher than that under air (**Figure 2A**). Thus, ethylene appeared to enhance not only the biosynthesis of violaxanthin but also the metabolism from violaxanthin to ABA by NCED at 20 °C. Consequently, the enhanced accumulation of 9-*cis*-violaxanthin by ethylene was inconspicuous at 20 °C.

In contrast, in flavedo at 5 °C, ethylene repressed the accumulation of phytoene and ζ-carotene with repressed gene expression of PSY and PDS (**Figures 2B** and **7B**). The contents of β-carotene, β-cryptoxanthin, and violaxanthin in flavedo under ethylene were lower than those under air, although the gene expression of LCYb and HYb was promoted by ethylene. In addition, under ethylene at 5 °C, the content of 9-*cis*-violaxanthin in flavedo clearly decreased at 16 days after harvest (**Figure 2B**). Probably, the repressed gene expression of PSY and PDS by ethylene led to a low biosynthesis of β-carotene, β-cryptoxanthin, and violaxanthin (**Figures 2B** and **7B**). Under the condition, drastic increases in the gene expression of NCED2 and NCED3 by ethylene appeared to enhance the metabolism of 9-*cis*-violaxanthin to ABA by NCEDs and, consequently, decreased the content of 9-*cis*-violaxanthin (**Figures 2B** and **7B**). Thus, under ethylene at 5 °C, the repressed gene expression of PSY and PDS by ethylene seemed to be primarily responsible for the repressed accumulation of carotenoids. In addition, the promoted gene expression of NCEDs by ethylene seemed to encourage the reduction of 9-*cis*-violaxanthin content in flavedo under ethylene at 5 °C.

The effect of ethylene on the carotenoid accumulation and expression of carotenoid biosynthesis-related genes was also investigated in the flavedo of orange (*Citrus sinensis* L. Osbeck) at the stage of mature green and color break (*11*, *12*). Rodrigo et al. reported that, at 20 °C, ethylene clearly up-regulated the gene expression of PSY, ZDS, HYb, and NCEDs and sustained or transiently increased the gene expression of PDS, LCYb, and ZEP in the flavedo of orange. Enhancement in the gene expression of PSY, HYb, and NCEDs by ethylene treatment was also observed at 25 °C with microarray analysis in the flavedo of mature green Satsuma mandarin fruit (*19*). The present results regarding the response of carotenoid accumulation and the gene expression to ethylene at 20 °C in flavedo were similar to their results, but a clear up-regulation in the gene expression of PSY and ZDS was not observed in our present study. The maturation stage of the fruit in the present study progressed in comparison with that of the fruit used in previous studies (*11*, *12*). Therefore, differences in the maturation stage of the fruit may be responsible for the differences in the response of the gene expression to ethylene between the present study and previous studies.

In juice sacs, the responses of all gene expression to ethylene were similar at 20 and 5 °C (**Figure 8**). The differences in the response of the gene expression of PSY and PDS to ethylene between temperatures observed in flavedo were not observed in juice sacs (**Figures 7** and **8**). In contrast, as observed in flavedo, the gene expression of HYb, ZEP, NCED2, and NCED3 was promoted by ethylene at both temperatures, although the promotion was smaller in juice sacs than in flavedo (**Figures 7** and **8**). On the basis of the results in flavedo and juice sacs, it was suggested that the temperature-independent promotions in the gene expression of HYb, ZEP, NCED2, and NCED3 by ethylene

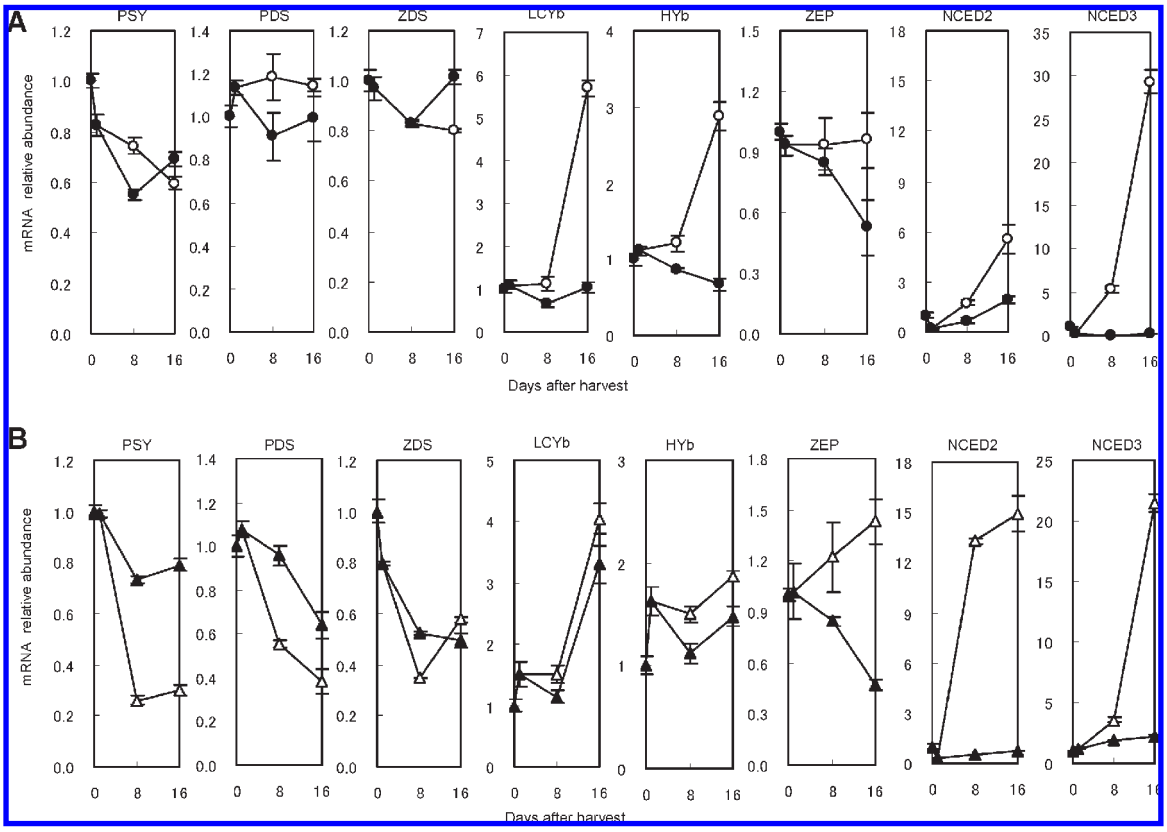


**Figure 7.** Effect of ethylene at 20 and 5 °C on the expression of carotenoid biosynthesis-related genes in flavedo: (**A**) fruit exposed to air (●) or 1000 μL/L ethylene (○) at 20 °C; (B) fruit exposed to air (▲) or 1000 μL/L ethylene (△) at 5 °C. Harvested fruits were incubated at 20 or 5 °C for 24 h to equilibrate the fruit temperature and then exposed to ethylene. Levels of gene expression of PSY, PDS, ZDS, LCYb, HYb, ZEP, NCED2, and NCED3 were analyzed by real-time quantitative RT-PCR with TaqMan MGB probes and sets of primers designed on Satsuma mandarin cDNAs, *CitPSY*, *CitPDS*, *CitZDS*, *CitLCYb*, *CitHYb*, *CitZEP*, *CitNCED2*, and *CitNCED3*, respectively. An expression value of 1 was arbitrarily assigned to the sample immediately after harvest. Values are the mean ± SE of three measurements.

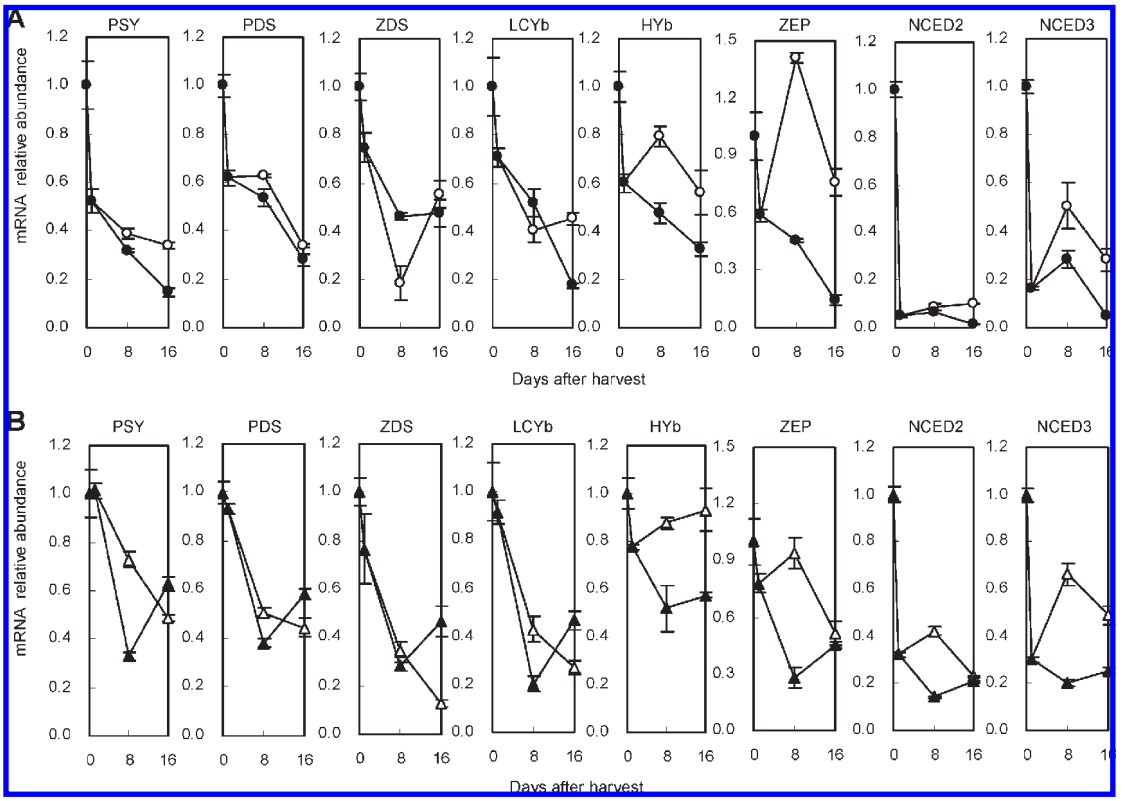


**Figure 8.** Effect of ethylene at 20 and 5 °C on the expression of carotenoid biosynthesis-related genes in juice sacs: (**A**) fruit exposed to air (●) or 1000 μL/L ethylene (○) at 20 °C; (**B**) fruit exposed to air (▲) or 1000 μL/L ethylene (△) at 5 °C. Harvested fruits were incubated at 20 or 5 °C for 24 h to equilibrate the fruit temperature and then exposed to ethylene. Levels of gene expression of PSY, PDS, ZDS, LCYb, HYb, ZEP, NCED2, and NCED3 were analyzed by real-time quantitative RT-PCR with TaqMan MGB probes and sets of primers designed on Satsuma mandarin cDNAs, *CitPSY*, *CitPDS*, *CitZDS*, *CitLCYb*, *CitHYb*, *CitZEP*, *CitNCED2*, and *CitNCED3*, respectively. An expression value of 1 was arbitrarily assigned to the sample immediately after harvest. Values are the mean ± SE of three measurements.

were similar in flavedo and juice sacs. In contrast, a difference in the response of the gene expression of PSY and PDS to ethylene between temperatures was specifically observed in flavedo. The response of the gene expression to ethylene was smaller in juice sacs than in flavedo (**Figures 7** and **8**), which seemed to be responsible for the small changes in the content and composition of carotenoids by ethylene treatment in juice sacs (**Figures 4** and **5**).

In conclusion, under an ethylene-free atmosphere, storage at 20 °C enhanced carotenoid accumulation in flavedo and maintained the carotenoid content in edible juice sacs. On the other hand, storage at 5 and 30 °C slowly increased the carotenoid content in flavedo but gradually decreased the content in edible juice sacs. These results suggest that carotenoid biosynthesis in citrus fruit is temperature-sensitive. Moreover, the effect of temperature on carotenoid accumulation in citrus fruit is tissue-dependent. Under an ethylene atmosphere, in flavedo, carotenoid accumulation was enhanced more dramatically than under an ethylene-free atmosphere at 20 °C but repressed at 5 °C. These results suggest that the effect of ethylene on carotenoid accumulation in flavedo varied with temperature. In juice sacs, the carotenoid content did not noticeably change during storage at 20 and 5 °C under ethylene atmosphere.

## LITERATURE CITED

(1) Gross, J. *Pigments in Fruits*; Academic Press: London, U.K., 1987.
(2) Olson, J. A. Provitamin-A function of carotenoids: the conversion of β-carotene into vitamin-A. *J. Nutr.* **1989**, *119*, 105–108.
(3) Rao, A. V.; Rao, L. G. Carotenoids and human health. *Pharmacol. Res.* **2007**, *55*, 207–216.
(4) Lee, H. S.; Castle, W. S. Seasonal changes of carotenoid pigments and color in Hamlin, Earlygold, and Budd blood orange juices. *J. Agric. Food Chem.* **2001**, *49*, 877–882.
(5) Matsumoto, H.; Ikoma, Y.; Kato, M.; Kuniga, T.; Nakajima, N.; Yoshida, T. Quantification of carotenoids in citrus fruit by LC-MS and comparison of patterns of seasonal changes for carotenoids among citrus varieties. *J. Agric. Food Chem.* **2007**, *55*, 2356–2368.
(6) Kato, M.; Ikoma, Y.; Matsumoto, H.; Sugiura, M.; Hyodo, H.; Yano, Y. Accumulation of carotenoids and expression of carotenoid biosynthesis genes during maturation in citrus fruit. *Plant Physiol.* **2004**, *134*, 824–837.
(7) Rodrigo, M. J.; Marcos, J., F.; Zacarías, L. Biochemical and molecular analysis of carotenoid biosynthesis in flavedo of orange (*Citrus sinensis* L.) during fruit development and maturation. *J. Agric. Food Chem.* **2004**, *52*, 6724–6731.
(8) Alquezar, B.; Rodrigo, M. J.; Zacarías, L. Regulation of carotenoid biosynthesis during fruit maturation in the red-fleshed orange mutant Cara Cara. *Phytochemistry* **2008**, *69*, 1997–2007.
(9) Fanciullino, A.-L.; Cercós, M.; Dhuique-Mayer, C.; Froelicher, Y.; Talón, M.; Ollitrault, P.; Morillon, R. Changes in carotenoid content and biosynthetic gene expression in juice sacs of four orange varieties (*Citrus sinensis*) differing in flesh fruit color. *J. Agric. Food Chem.* **2008**, *56*, 3628–3638.
(10) Alós, E.; Cercós, M.; Rodrigo, M. J.; Zacarías, L.; Talón, M. Regulation of color break in citrus fruits. Changes in pigment profiling and gene expression induced by gibberellins and nitrate, two ripening retardants. *J. Agric. Food Chem.* **2006**, *54*, 4888–4895.
(11) Rodrigo, M. J.; Zacarías, L. Effect of postharvest ethylene treatment on carotenoid accumulation and the expression of carotenoid biosynthetic flavedo of orange (*Citrus sinensis* L. Osbeck) fruit. *Postharvest Biol. Technol.* **2007**, *43*, 14–22.
(12) Rodrigo, M. J.; Alquezar, B.; Zacarías, L. Cloning and characterization of two 9-*cis*-epoxycarotenoid dioxygenase genes, differentially

regulated during fruit maturation and under stress conditions, from orange (*Citrus sinensis* L. Osbeck). *J. Exp. Bot*. **2006**, *57*, 633–643.

(13) Schwartz, S. H.; Qin, X.; Zeevaart, J. A. D. Characterization of a novel carotenoid cleavage dioxyngenase from plants. *J. Biol. Chem*. **2001**, *276*, 25208–25211.

(14) Stewart, I.; Wheaton, T. A. Carotenoids in citrus: their accumulation induced by ethylene. *J. Agric. Food Chem*. **1972**, *20*, 448–449.

(15) Young, R.; Jahn, O. Ethylene-induced carotenoid accumulation in citrus fruit rinds. *J. Am. Soc. Hortic. Sci*. **1972**, *97*, 258–261.

(16) Wheaton, T. A.; Stewart, I. Optimum temperature and ethylene concentration for postharvest development of carotenoid pigments in *Citrus*. *J. Am. Soc. Hortic. Sci*. **1973**, *98*, 337–340.

(17) Sonnen, H. D. Carotenoid formation in ripening Satsuma fruit. *Proc. Int. Soc. Citricult*. **1977**, *3*, 1089–1092.

(18) Grierson, W.; Cohen, E.; Kitagawa, H. Degreening. In *Fresh Citrus Fruits*; Wardowski, W. F., Nagy, S., Grierson, W., Eds.; AVI Publishing: Westport, CT, 1986; pp 253−274.

(19) Fujii, H.; Shimada, T.; Sugiyama, A.; Nishikawa, F.; Endo, T.; Nakano, M.; Ikoma, Y.; Shimizu, T.; Omura, M. Profiling of ethylene-responsive genes in mature mandarin fruit using a citrus 22K oligoarray. *Plant Sci*. **2007**, *173*, 340–348.

(20) Kader, A. A. Postharvest biology and technology: an overview. In *Postharvest Technology of Horticultural Crops*, 3rd ed.; Kader, A. A., Ed.; University of California Agriculture and Natural Resources: Oakland, CA, 2002; pp 39−48.

(21) Saltveit, M. E. Effect of ethylene on quality of fresh fruit and vegetables. *Postharvest Biol. Technol*. **1999**, *15*, 279–292.

(22) Beyer, E. M.; Morgan, P. G. A method for determining the concentration of ethylene in the gas phase of vegetative plant tissues. *Plant Physiol*. **1970**, *46*, 352–354.

(23) Ikoma, Y.; Yano, M.; Ogawa, K.; Yoshioka, T.; Xu, Z. C.; Hisada, S.; Omura, M.; Moriguchi, T. Isolation and evaluation of RNA from polysaccharide-rich tissues in fruit for quality by cDNA library construction and RT-PCR. *J. Jpn. Soc. Hortic. Sci*. **1996**, *64*, 809–814.

(24) Kato, M.; Matsumoto, H.; Ikoma, Y.; Kuniga, T.; Nakajima, N.; Yoshida, T.; Yano, Y. Accumulation of carotenoids and expression of carotenoid biosynthetic genes and carotenoid cleavage dioxygenase genes during fruit maturation in the juice sacs of 'Tamami,' 'Kiyomi' tangor, and 'Wilking' mandarin. *J. Jpn. Soc. Hortic. Sci*. **2007**, *76*, 103–111.

(25) Kato, M.; Matsumoto, H.; Ikoma, Y.; Okuda, H.; Yano, Y. The role of carotenoid cleavage dioxygenases in the regulation of carotenoid profiles during maturation in citrus fruit. *J. Exp. Bot*. **2006**, *57*, 2153–2164.

(26) Okuda, H. A comparison of IAA and ABA levels in leaves and roots of two citrus cultivars with different degrees of alternate bearing. *J. Hortic. Sci. Biotechnol*. **2000**, *75*, 355–359.

(27) Gautier, H.; Diakou-verdin, V.; Bénard, C.; Reich, M.; Buret, M.; Bourgaud, F.; Poessel, J. L.; Caris-Veyrat, C.; Génard, M. How does tomato quality (sugar, acid, and nutritional quality) vary with ripening stage, temperature, and irradiance?. *J. Agric. Food Chem*. **2008**, *56*, 1241–1250.

(28) Vogele, A. C. Effect of environmental factors on the color of tomato and water melon. *Plant Physiol*. **1937**, *12*, 929–955.

(29) Perkins-Veazie, P.; Collins, J. K. Carotenoid changes of intact watermelons after storage. *J. Agric. Food Chem*. **2006**, *54*, 5868–5974.

**Received September 8, 2008. Revised manuscript received April 24, 2009.**