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Carotenoid Accumulation in Japanese Apricot (*Prunus mume* Siebold & Zucc.): Molecular Analysis of Carotenogenic Gene Expression and Ethylene Regulation

MASAYUKI KITA,*,† MASAYA KATO,§,# YUSUKE BAN,†,⊥ CHIKAKO HONDA,† HIDEAKI YAEGAKI,† YOSHINORI IKOMA,§ AND TAKAYA MORIGUCHI†,⊥

National Institute of Fruit Tree Science, Tsukuba, Ibaraki 305-8605, Japan; Okitsu Citrus Research Station, National Institute of Fruit Tree Science, Okitsunakamachi, Shimizu, Shizuoka 424-0292, Japan; Faculty of Agriculture, Shizuoka University, Ohya, Suruga, Shizuoka 422-8529, Japan; and Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Ibaraki 305-8572, Japan

To elucidate the regulatory mechanisms of carotenogenesis in Japanese apricot (*Prunus mume* Siebold & Zucc.), the relationships between carotenoid accumulation and the expression of the carotenogenic genes, phytoene synthase (*PmPSY-1*), phytoene desaturase (*PmPDS*), ζ-carotene desaturase (*PmZDS*), lycopene β-cyclase (*PmLCYb*), lycopene ε-cyclase (*PmLCYe*), β-carotene hydroxylase (*PmHYb*), and zeaxanthin epoxidase (*PmZEP*), were analyzed in two cultivars with different ripening traits, 'Orihime' and 'Nanko.' In 'Orihime' fruits, large amounts of carotenoids accumulated on the tree, concomitant with the induction of *PmPSY-1* and the downstream carotenogenic genes *PmLCYb*, *PmHYb*, and *PmZEP*. In 'Nanko' fruits, carotenoids accumulated mainly after harvest, correlating with an appreciable induction of *PmPSY-1* expression, but the downstream genes were not notably induced, which may explain the lower total carotenoid content in 'Nanko' than in 'Orihime.' In both cultivars, a decrease in *PmLCYe* expression and increased or constant *PmLCYb* expression could cause the metabolic shift from β,ε-carotenoid synthesis to β,β-carotenoid synthesis that occurs as ripening approaches. Next, the effects of ethylene on the expression of *PmPSY-1* and carotenoid accumulation were investigated in 'Nanko' fruits treated with propylene or 1-methylcyclopropene (1-MCP). Propylene treatment induced both ethylene production and carotenoid accumulation. *PmPSY-1* was constitutively expressed, but propylene treatment accelerated its induction. 1-MCP treatment caused a slight inhibition of carotenoid accumulation along with the repression, although not complete, of *PmPSY-1*. Collectively, although *PmPSY-1* expression was not exclusively regulated by ethylene, both the notable induction of *PmPSY-1* accelerated by ethylene and the subsequent induction of the downstream carotenogenic genes, especially *PmLCYb*, could be necessary for the massive carotenoid accumulation that occurs during ripening. Furthermore, the switch from *PmLCYe* expression to *PmLCYb* expression could cause β,β-carotenoid accumulation in both Japanese apricot cultivars.

KEYWORDS: Carotenoid; ethylene; gene expression; Japanese apricot (*Prunus mume*); phytoene synthase (*PmPSY-1*); fruit ripening

INTRODUCTION

Carotenoids are natural isoprenoid pigments that are synthesized by photosynthetic organisms and some non-photosynthetic bacteria and fungi (*1*). Plant carotenoids are mainly 40-carbon isoprenoids with polyene chains that contain up to 15 conjugated double bonds (*2*). These compounds not only are essential components for the photosynthetic antenna and reaction center complexes but also have essential roles in photoprotective activities in the chloroplast (*3*). In animals and humans, some carotenoids containing β-rings are precursors of vitamin A, which helps to prevent some types of human cancer and degenerative diseases (*4*). In addition, some plant carotenoids are precursors of the important plant hormone abscisic acid (*5*).

Carotenoids are formed via the mevalonate-independent pathway. This well-defined pathway of carotenoid biosynthesis in plants is a series of desaturation, cyclization, hydroxylation, and epoxidation steps (**Figure 1**). Geranylgeranylpyrophosphate

* Corresponding author (e-mail mkita@affrc.go.jp).
† National Institute of Fruit Tree Science, Tsukuba.
§ Okitsu Citrus Research Station, National Institute of Fruit Tree Science.
# Shizuoka University.
⊥ University of Tsukuba.

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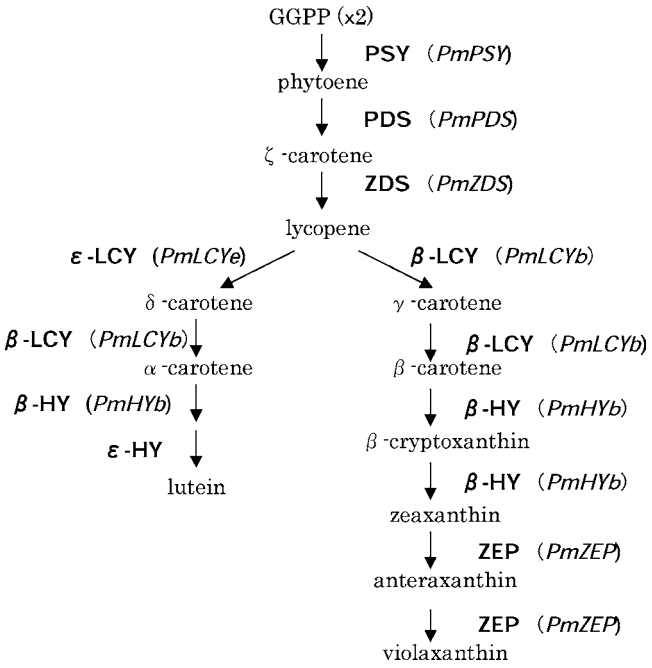

Figure 1. Schematic representation of the carotenoid biosynthetic pathway in higher plants. Selected intermediates are shown. In this study, cDNAs corresponding to *PmPSY*, *PmPDS*, *PmZDS*, *PmLCYb*, *PmLCYe*, *PmHYb*, and *PmZEP* were isolated from the Japanese apricot cultivar 'Orihime.'

(GGPP) is synthesized through the ubiquitous isoprenoid pathway. The conversion of GGPP to phytoene by phytoene synthase (PSY) is the first committed step in the mevalonate-independent pathway, a head-to-head condensation of two GGPP molecules (*6*). Two functionally similar enzymes, phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS), convert phytoene to lycopene via phytofluene, ζ-carotene, and neurosporene. The cyclization of lycopene produces two types of carotenes that contain one or two rings of either the β- or ε-type. This cyclization of lycopene, catalyzed by the two cyclases, lycopene β-cyclase (β-LCY) and lycopene ε-cyclase (ε-LCY), is an important branching point (*7*). When only β-cyclase acts in this step, lycopene is converted to β-carotene with two β-end rings, whereas the actions of both ε-cyclase and β-cyclase lead to the conversion of lycopene to α-carotene with one ε and one β end ring. β-Carotene and β-cryptoxanthin are further metabolized to zeaxanthin via two hydroxylation steps catalyzed by β-ring hydroxylase (β-HY). Subsequently, zeaxanthin is converted to violaxanthin by zeaxanthin epoxidase (ZEP). α-Carotene is converted to lutein, a major xanthophyll, in a reaction catalyzed by two hydroxylases.

The carotenogenic genes have been isolated and their expression characterized during the carotenoid accumulation phase in many plant species, including tomato (*8*−*10*), bell pepper (*11*), *Arabidopsis* (*12*), and *Citrus* (*13*, *14*). In tomato, levels of the *PSY* and *PDS* mRNAs increase significantly at the breaker stage with the concomitant disappearance of both the β- and ε-cyclase mRNAs. Thus, lycopene accumulates in tomato fruit during ripening (*9*, *10*, *15*). In *Citrus* fruit, the composition of xanthophylls is regulated by the balance of expression between the upstream carotenogenic genes *PSY*, *PDS*, *ZDS*, and *LCYb* (encoding β-LCY) and the downstream genes *HYb* (encoding β-HY) and *ZEP* (*13*). These results show that carotenoid accumulation during fruit ripening is highly regulated at the transcriptional level.

Another interesting matter is the effect of ethylene on carotenoid accumulation. Ethylene promotes the ripening process in climacteric fruit such as tomato (*16*). Grierson (*17*) reported that ethylene induces the expression of *PSY* and other ripening-related genes. Recently, Marty et al. (*18*) analyzed the effect of ethylene on the expression of carotenogenic genes (*Psy-1*, *PDS*, and *ZDS*) and carotenoid accumulation in color-contrasted apricot fruit. The results showed that ethylene enhances *Psy-1* and *PDS* expression with a concomitant increase in the accumulation of phytoene and phytofluene. However, ethylene does not affect *ZDS* expression or β-carotene accumulation in apricot.

Japanese apricot (*Prunus mume* Siebold & Zucc.) is a typical climacteric fruit that produces a large amount of ethylene (*19*). Hence, Japanese apricot fruits accumulate massive amounts of carotenoid, thereby changing the skin color to yellow. However, the regulatory mechanisms of carotenogenesis in Japanese apricot are not well-known. Therefore, to reveal the key step(s) in carotenoid accumulation during ripening and after harvest in this species, the expression of the carotenogenic genes encoding PSY, PDS, ZDS, β-LCY, ε-LCY, β-HY, and ZEP (designated *PmPSY-1*, *PmPDS*, *PmZDS*, *PmLCYe*, *PmLCYb*, *PmHYb*, and *PmZEP*, respectively) was analyzed in the cultivars 'Orihime' and 'Nanko.' Fruits of the former accumulate large amounts of carotenoids on the tree, whereas those of the latter do so mainly after harvest. Furthermore, to clarify the relationships between *PmPSY-1* expression and ethylene in 'Nanko,' fruits were exposed to the ethylene analogue propylene or the ethylene receptor inhibitor 1-methylcyclopropene (1-MCP). Overall, the results indicated that the notable induction of *PmPSY-1* expression, which is accelerated by ethylene, and the subsequent induction of the downstream carotenogenic genes, especially *PmLCYb*, could be important factors in the massive accumulation of carotenoids. Furthermore, a decrease in *PmLCYe* expression and an increase in *PmLCYb* expression in the Japanese apricot cultivars could cause a metabolic shift from β,ε-carotenoid synthesis to β,β-carotenoid synthesis as ripening approaches.

MATERIALS AND METHODS

Plant Material. Two Japanese apricot (*P. mume* Siebold & Zucc.) cultivars with different ripening traits were used in this study. 'Orihime' fruits display bright yellow coloration on the tree before harvest, whereas 'Nanko' fruits remain green on the tree and rapidly turn yellow after harvest. Both were cultivated at the experimental field of the National Institute of Fruit Tree Science (Tsukuba, Ibaraki, Japan). Fruits were harvested at different developmental stages. 'Nanko' fruits were harvested at 123 days after flowering [DAF; also regarded as 0 days after harvest (0 DAH)] and held for 6 days at 23 °C. The pits were removed from the fruits. Because coloration occurs in both the skin and the flesh, fruits with flesh and skin were immediately frozen in liquid nitrogen. The samples were lyophilized and stored at −80 °C until use.

Ethylene and Skin Color Measurements. For ethylene detection, the fruits were placed in a plastic airtight 250 mL jar for 1 h at room temperature. Gas from the head space of the jar was sampled with a plastic 1 mL syringe and injected into a gas chromatograph (GC-14B; Shimadzu, Kyoto, Japan) equipped with a flame ionization detector and a column filled with active alumina maintained at 60 °C. Five fruits were used in each measurement, with three replications. The fruit skin color evaluation was based on a CIE *L**a**b** colorimetric system, using a spectrophotometer CM-2002 (Minolta, Tokyo, Japan). Ten fruits were used for the measurement.

Isolation of cDNAs of Carotenogenesis and Ethylene Biosynthesis Genes. To obtain cDNAs for carotenogenic genes, we prepared total RNA from 'Orihime' at 107 DAF, according to the method of Ikoma et al. (*20*), and used it as a template in reverse transcriptase PCR (RT-PCR). First-strand cDNA was synthesized from the total RNA using a

Table 1. Primers Used in the Amplification, Length of Amplified cDNA Fragments from *Prunus mume*, and Comparison of Nucleotide Sequences

	DDBJ accession no.	sense primers (upper) and antisense primers (lower)	length (bp)	% identity with the best score of other non-*Prunus* nucleotides (accession no.)	% identity with the best score of *Prunus* nucleotides (accession no.)
PmPSY-1	AB253628	GAAGGAATGMGWATGGACTT GCTTCRATCTCRTCYAGTAT	1305[a]	81.2 *Citrus unshiu* (AB037975)	99.5 (AY822067)
PmPDS	AB253629	CAARCCHGGRGARTTYAGYCG AGTTTTCTRTCRAACCA	660	82.4 *Citrus* × *paradisi* (AY494790)	98.5 (AY822065)
PmZDS	AB253630	TAYGCYYTNGGWTTYATWGACTG GCTCCTTCCATRCTRTCDATGTARTC	851	92.8 *Malus* × *domestica* (AF429984)	98.3 (AY822066)
PmLCYb	AB253631	AAYAAYTAYGGWGTTTGGWDGATGA YARRAANCCDTGCCA	993	83.7 *Citrus unshiu* (AY166796)	*b*
PmLCYe	AB253632	ACTGTTGCWTCKGGRGCAGC CCACATCCAHKTTGGMAMDCGRAA	714	82.4 *Citrus* × *paradisi* (AF486650)	*b*
PmHYb	AB253633	TGGMGAGRAAGMRATCSGAGAGG TCCTTRGGWCCNARRAASARBCCATA	568	86.3 *Citrus unshiu* (AF296158)	*b*
PmZEP	AB253634	GCTGCTTTGGAAGCYATTGAT CKAAAMCGMGCRGGAAARTT	1478	82.0 *Vitis vinifera* (AY337615)	98.2 (AF159948)

[a] PSY is full-length cDNA which was obtained by 5′-RACE and 3′-RACE. [b] No instances for gene isolation in *Prunus* species.

Ready-To-Go first-strand synthesis kit (Amersham Biosciences, Piscataway, NJ). To isolate partial cDNAs of the carotenogenic genes, degenerate primers were synthesized on the basis of the regions conserved among plants in the previously reported sequences of each gene (**Table 1**). At the same time, the cDNAs for ripening specific ACC synthase (*PmACS*) and ACC oxidase (*PmACO*) were also isolated by RT-PCR, on the basis of the known sequences (*21*). The amplified fragments were fused into the pCR2.1 vector with a TA cloning system (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions, and sequenced using model 373A and 310 sequencers (Perkin-Elmer Applied Biosystems, Foster City, CA). The sequences were compared with the nucleic acid sequences deposited in the DNA Data Bank of Japan (DDBJ) using the program FASTA. Homology searches were carried out with GENETYX-WIN software (Software Development, Tokyo, Japan).

RNA Extraction and RNA Gel Blot Hybridization. Total RNA was extracted according to the method of Ikoma et al. (*20*) from Japanese apricot fruits at different stages and subjected to different treatments. Total RNA (15 μg) was denatured, fractionated on 1.2% (w/v) denaturing agarose gels, and transferred to nylon membrane (Hybond-N; Amersham Biosciences). Hybridization was carried out at 42 °C under denaturing conditions with formamide. The membranes were washed twice with 2× SSC containing 0.1% SDS at room temperature for 5 min and twice in 0.5× SSC containing 0.1% SDS at 65 °C for 15 min and then exposed to X-ray film. For hybridization, probes were synthesized from amplified cDNA fragments of each carotenogenic gene from 'Orihime' using DIG-11-dUTP and a Random Primed DNA Labeling Kit (Roche Diagnostics, Mannheim, Germany).

Extraction and Determination of Carotenoids. Total carotenoids were extracted according to the method of Kato et al. (*13*), and the extracts were stored at −20 °C until HPLC analysis. Extraction was carried out under dim-light conditions to prevent photodegradation, isomerization, and structural changes occurring in the carotenoids. Twenty microliters of each sample was injected into a reverse-phase HPLC system consisting of a Jasco model PU-880 pump (Jasco, Tokyo, Japan), a Jasco 880-50 degasser, a Jasco CO-860 column oven, and a YMC Carotenoid S-5 column (250 mm × 4.6 mm i.d.). The eluent was separated at a flow rate of 1 mL min^{-1} on the system and monitored using the Jasco MD-910 multiwavelength photodiode array detector. The gradient elution method consisted of an initial 30 min of 95% methanol, 1% methyl *tert*-butyl ether (MTBE), and 4% H_2O, followed by a linear gradient of 6% methanol, 90% MTBE, and 4% H_2O for 60 min. Peaks were identified by comparison with authentic sample at individual retention times and the specific absorption spectra of 452 nm for α-carotene, lutein, β-carotene, β-cryptoxanthin, zeaxanthin, and violaxanthin and 286 nm for phytoene. Extractions and measurements were performed three times.

Propylene and 1-MCP Treatments. 'Nanko' fruits, which produce no ethylene on the tree, were harvested at 113 DAF, 1 week before the full-ripening stage. After harvest, the fruits were exposed to propylene or 1-MCP. For propylene treatment, fruits were exposed to 5000 ppm of propylene for 24 h at 23 °C, and for 1-MCP treatment, fruits were exposed to 1 ppm of 1-MCP for 24 h at 23 °C. Propylene, an analogue of ethylene, was added to the container using a plastic syringe. To generate 1-MCP, the required amount of SmartFresh (AgroFresh Inc., Spring House, PA), calculated on the basis of the container volume, was placed in a glass vial, and water was added according to the manufacturer's instructions. After the treatment, the fruits were exposed to fresh air. Ethylene detection was performed as described above. Each treatment was replicated three times.

RESULTS

Physiological Characterization during Fruit Ripening. In 'Orihime' and 'Nanko,' natural fruit dropping occurs approximately 105 and 120 DAF, respectively. In 'Orihime,' fruit on the tree produced ethylene after 92 DAF, which is about 2 weeks before the full ripening stage (natural dropping stage), with ethylene production increasing rapidly at 95 DAF (**Figure 2A**). In contrast, ethylene production by 'Nanko' fruit on the tree (i.e., before harvest) was below the detectable level. After harvest at 123 DAF, 'Nanko' showed high ethylene production of 285 nL gFW^{-1} h^{-1} at 3 DAH (**Figure 2B**). At harvest stage, the skin color of 'Orihime' and 'Nanko' was determined in terms of the a^* and b^* index by spectrophotometer, with ($-a$) indicating green, ($+a$) red, and ($+b$) yellow. At harvest, the color index of 'Orihime' was 7.3 ($+a$) and 46.8 ($+b$), whereas that of 'Nanko' was −10.5 ($-a$) and 33.1 ($+b$).

Isolation and Identification of cDNA Fragments of Carotenogenic Genes. Degenerate primers were synthesized on the basis of conserved regions of the deduced amino acid sequences of the carotenogenic genes (**Table 1**). RT-PCR was performed using first-strand cDNA isolated from ripe 'Orihime' fruit. Comparison of the sequences of the cDNAs isolated revealed strong similarities (81.2−92.8% homology) between the individual genes and the carotenogenic genes of other plant species (**Table 1**). We designated these genes *PmPSY-1* (DDBJ accession no. AB253628), *PmPDS* (AB253629), *PmZDS* (AB253630), *PmLCYb* (AB253631), *PmLCYe* (AB253632), *PmHYb* (AB253633), and *PmZEP* (AB253634).

cDNAs of *PSY* (*AprPsy-1*), *PDS* (*AprPds*), *ZDS* (*AprZds*), and *ZEP* (*PA-ZE* and *PA-ZE2*) have been isolated previously

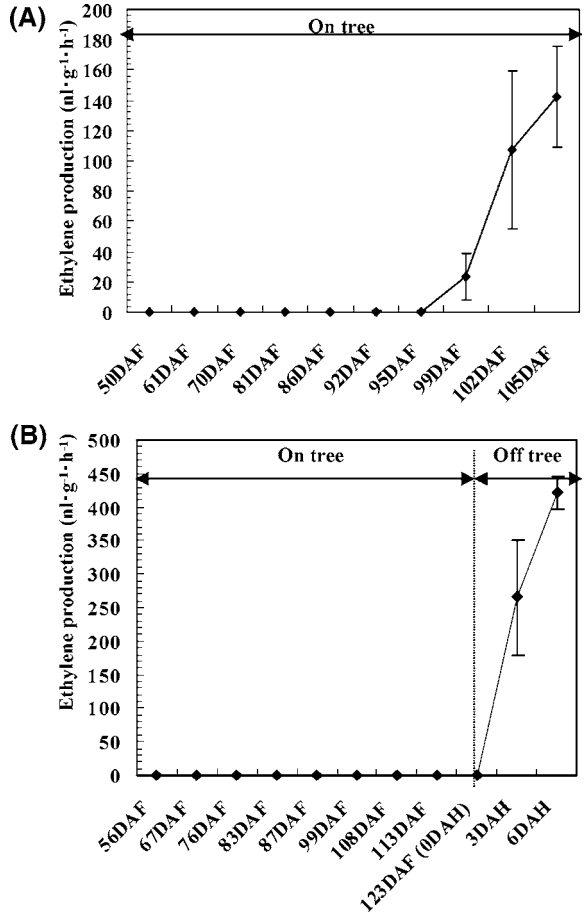

Figure 2. Ethylene production of 'Orihime' (**A**) and 'Nanko' (**B**) fruits during fruit ripening. The 105 and 123 DAF correspond to the full ripening stages (natural dropping stage) of 'Orihime' and 'Nanko,' respectively. After harvest, 'Nanko' fruit was held in fresh air at 23 °C. Values are means ± SE of three measurements.

from apricot (*18*, *22*). The homologies of *PmPSY-1*, *PmPDS*, *PmZDS*, and *PmZEP* to the corresponding genes above are 99.5, 98.5, 98.3, and 98.2%, respectively (**Table 1**). Therefore, probes synthesized from the cDNAs isolated from 'Orihime' hybridized with genes from other *P. mume* cultivars, including 'Nanko,' and could be used to monitor the expression levels of carotenogenic genes in these cultivars. Similarly, amplified fragments of *ACS* (*PmACS*) and *ACO* (*PmACO*) showed high similarities to the ripening-related *ACS* (99.7% homology, AB031026) and *ACO* genes (96.7% homology, AF026793) of *Prunus* species.

Carotenoid Contents and Expression Profiles of Carotenogenic Genes during Fruit Ripening in 'Orihime' and 'Nanko.' In 'Orihime' and 'Nanko,' natural fruit dropping occurs at approximately 105 and 120 DAF, respectively. In 'Orihime,' ethylene production began on the tree after about 92 DAF, 2 weeks earlier than the full-ripening stage (natural dropping stage). In contrast, in 'Nanko,' ethylene production was below the detectable level on the tree, but was observed after harvest (data not shown).

'Orihime' fruit color changed from green to bright yellow after 95 DAF. The total carotenoid content decreased until 84 DAF and increased in the subsequent stage (**Figure 3**). The proportions of β,ϵ-carotenoids, α-carotene, and lutein among the total carotenoids were 42.9% at 56 DAF, 45.1% at 74 DAF, and 42.9% at 84 DAF. The levels of lutein, a major β,ϵ-carotenoid, decreased gradually throughout fruit ripening on the tree. In contrast, the levels of the β,β-carotenoids, β-carotene, β-cryptoxanthin, zeaxanthin, and violaxanthin increased along with the total carotenoid accumulation. The β,β-carotenoids comprised 77.5% of the total carotenoids at 95 DAF and 90.4% at 107 DAF. This increase in the proportion of β-carotene within the carotenoid species is a remarkable feature of 'Orihime.'

'Nanko' fruits remained green to pale green on the tree, changing to yellow mainly after harvest, concomitant with the increase in total carotenoids, especially zeaxanthin and violaxanthin (**Figure 3**). The β,ϵ-carotenoids in the fruits on the tree comprised 34.1% of the total carotenoids at 63 DAF and 45.8% at 103 DAF. The carotenoid components that increased after harvest were mainly β,β-xanthophylls, including β-cryptoxanthin, zeaxanthin, and violaxanthin; no increase in β,ϵ-carotenoid (lutein) was observed after harvest. The postharvest increase in β,β-xanthophylls in 'Nanko' was comparable to that occurring naturally in 'Orihime' at 95 and 107 DAF on the tree.

RNA gel blot analysis of carotenogenic genes in 'Orihime' revealed that all of the carotenogenic genes, except *PmPSY-1*, were expressed in young fruits. These expression levels were constant until 74 DAF and decreased once at 84 DAF (**Figure 4**). *PmLCYe* was highly expressed in young fruits at 56 DAF and decreased gradually as fruit matured. At 84 DAF, the total amount of carotenoids was at its lowest level (**Figure 3**), as was the expression level of all of the carotenogenic genes (**Figure 4**). After 84 DAF, fruit skin color began to turn yellow, and the expression of all of the carotenogenic genes, except *PmLCYe*, was induced. *PmPSY-1* induction was particularly strong at this stage. The expression patterns of *PmLCYe* and *PmLCYb* paralleled the metabolic shift from β,ϵ-carotenoids to β,β-carotenoids in 'Orihime.'

In 'Nanko,' the expression patterns of *PmPSY-1*, *PmPDS*, and *PmZDS* generally resembled those in 'Orihime' (**Figure 4**). A slight induction of *PmLCYb*, *PmHYb*, and *PmZEP* was observed upon harvest, but no notable induction of these genes was observed toward the final stage, unlike the situation with 'Orihime.' The expression levels of the two cyclase genes were high in the early stage and low at 103 DAF. Subsequently, the expression of *PmLCYe* decreased below the detectable level, whereas that of *PmLCYb* remained constant toward the later stages, albeit at a low level. Over all stages, there was little induction of the downstream carotenogenic genes *PmLCYb*, *PmHYb*, and *PmZEP* in 'Nanko,' unlike the situation in 'Orihime.'

Carotenoid Accumulation and Gene Expression in 'Nanko' Following Propylene or 1-MCP Treatment. To clarify the effects of ethylene on carotenoid accumulation and the expression of carotenogenic genes, 'Nanko' fruits were harvested at 113 DAF, 1 week before full ripening on the tree. After propylene or 1-MCP treatment of the harvested fruits, the carotenoid levels and *PmPSY-1* expression were analyzed until 3 DAH. Untreated fruits began to produce a small amount of ethylene at 1 DAH, increasing to 11 nL g of FW^{-1} h^{-1} at 2 DAH and 265 nL g of FW^{-1} h^{-1} at 3 DAH (**Figure 5A**). Much more ethylene was produced in propylene-treated fruits than in the control. In contrast, in 1-MCP-treated fruits, ethylene production was undetectable throughout the experiment.

Propylene treatment led to increased total carotenoid content, although the levels were not significant compared to the levels in the control and the 1-MCP-treated fruits (**Figure 5B**). This increase was accompanied by the accumulation of *PmPSY-1* mRNA. In contrast, the total carotenoid content remained at the initial level in 1-MCP-treated fruits, which also showed a low level of *PmPSY-1* expression. Thus, *PmPSY-1* expression was accelerated by propylene treatment.

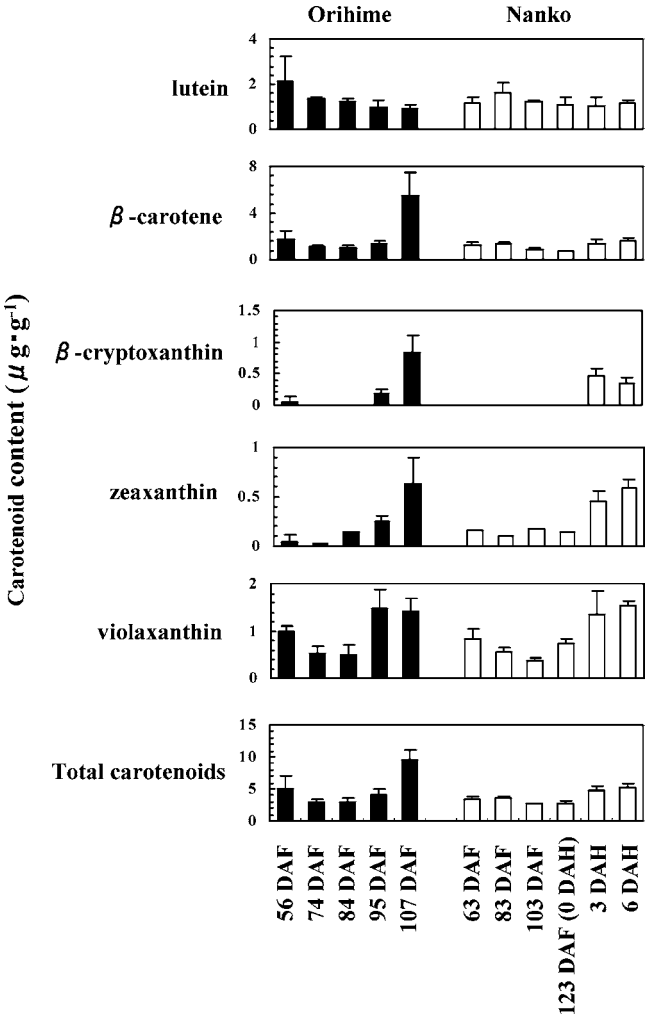

Figure 3. Carotenoid concentrations during fruit ripening in 'Orihime' and 'Nanko,' as determined by HPLC. 'Orihime' fruits were harvested from trees at each stage and analyzed immediately, whereas 'Nanko' fruits were harvested from trees at 63, 83, 103, and 123 DAF (0 DAH), and fruits held for 3 and 6 DAH were analyzed. Values are means ± SE of three measurements. Note the different content scale for each carotenoid.

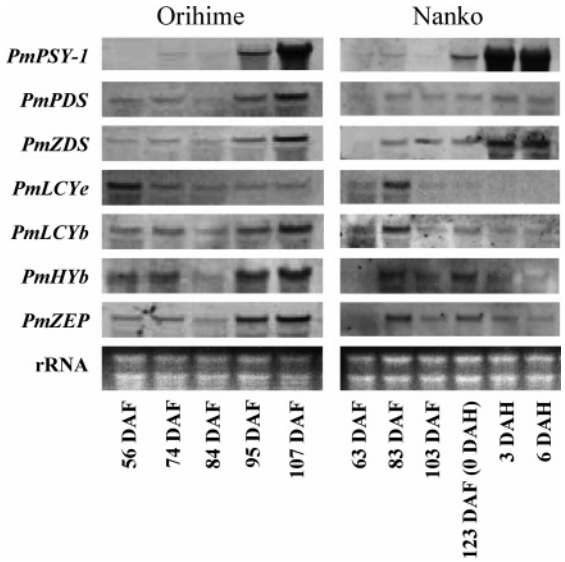

Figure 4. RNA gel blot analysis of *PmPSY-1*, *PmPDS*, *PmZDS*, *PmLCYe*, *PmLCYb*, *PmHYb*, and *PmZEP* in 'Orihime' and 'Nanko.' Fifteen micrograms of total RNA was loaded per lane. RNA in the gel was stained with ethidium bromide and transferred to nylon membrane. Hybridizations were performed under high-stringency conditions with DIG-labeled cDNAs corresponding to each carotenogenic gene.

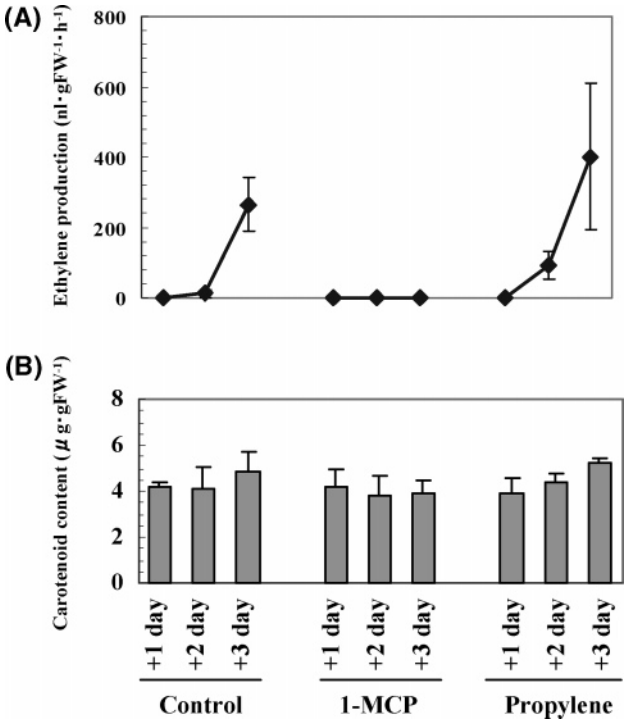

Figure 5. (**A**) Time course of the rate of ethylene production by 'Nanko' fruits after harvest. Fruits were harvested at 113 DAF (1 week before full ripening on the tree) and treated with 5000 ppm of propylene or 1 ppm of 1-MCP at 23 °C. After treatment, all fruits were held in fresh air at 23 °C. Values are means ± SE of three measurements. (**B**) Total carotenoid content in 'Nanko' fruits after treatment with propylene or 1-MCP. Values are means ± SE of three measurements. There were no significant differences in the results of the different treatments.

Simultaneously, the expression of *PmACS* and *PmACO* in 'Nanko' fruits was investigated (**Figure 6**). Propylene treatment induced *PmACS* and *PmACO* expression. Slight expression of *PmACO* was observed even after the 1-MCP treatment, but its expression was notably up-regulated concomitant with the ethylene production triggered by propylene. Unlike *PmACO*, *PmACS* expression was not detected in the fruits treated with 1-MCP, which could have resulted in a lack of ethylene production; thus, *PmACS* expression was fully correlated with ethylene production.

DISCUSSION

In general, Japanese apricot is a climacteric fruit that produces a large amount of ethylene after harvest. Inaba et al. (*19*) reported that the ethylene production was increased dramatically after harvest in 'Nanko.' However, up to now, there have been no reports concerning ethylene production by 'Orihime,' which shows a characteristic yellow coloration on the tree. We confirmed on-tree ethylene production in 'Orihime,' unlike other *P. mume* cultivars, including 'Nanko.' Therefore, to elucidate the regulatory mechanisms of carotenogenesis in Japanese apricot, we investigated the relationship between carotenoid accumulation and carotenogenic gene expression in 'Orihime' and 'Nanko,' which differ in fruit-ripening characteristics. In addition, we cloned and demonstrated the expression of seven putative carotenogenesis genes involved in the biosynthesis of

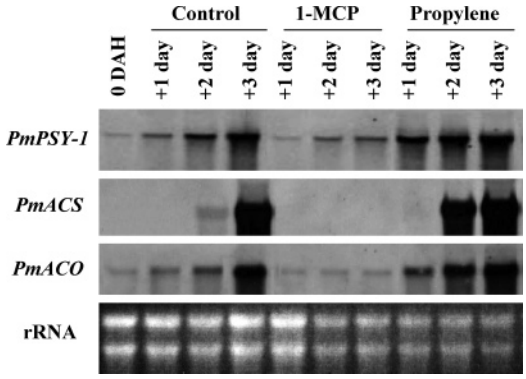

Figure 6. RNA gel blot analysis of *PmPSY-1*, *PmACS*, and *PmACO* in 'Nanko' fruits after treatment with propylene or 1-MCP. Fifteen micrograms of total RNA was loaded per lane. RNA in the gel was stained with ethidium bromide and transferred to nylon membrane. Hybridizations were performed under high-stringency conditions with DIG-labeled cDNAs corresponding to each gene.

carotenoid species, such as β-carotene, zeaxanthin, violaxanthin, and lutein, during ripening. To the best of our knowledge, this is the first expression analysis of these seven carotenogensis-related genes in *Prunus* species.

'Orihime' fruits began to accumulate large amounts of carotenoids, especially β,β-carotenoids, on the tree. In contrast, no significant carotenoid accumulation occurred in 'Nanko' fruits on the tree. However, after harvest, 'Nanko' fruits began to accumulate carotenoids. In both cultivars, the induction of *PmPSY-1* expression was a prerequisite for the massive carotenoid accumulation, both on and off the tree. Carotenoid accumulation during fruit ripening has been reported to coincide with the up-regulation of the *PSY* gene in the fruits of many species, including *Citrus* (*13*, *23*), pepper (*11*), and tomato (*8*). The remarkable increase in *PSY* expression in many species supports the important role of PSY as a regulating enzyme in carotenogenesis (*9*). However, total carotenoid content in 'Nanko' was lower than that in 'Orihime,' despite the notable induction of *PmPSY-1* in both cultivars. A comparison of downstream carotenogenic gene expression in 'Orihime' and 'Nanko' showed that the expression levels of *PmLCYb*, *PmHYb*, and *PmZEP* were lower in 'Nanko' than in 'Orihime.' This difference might explain the low level of carotenoid accumulation in 'Nanko'; that is, the induction of downstream carotenogenic genes might be required for the massive carotenoid accumulation, in addition to high *PmPSY-1* expression. Furthermore, a comparison of carotenoid species that accumulated in 'Orihime' and 'Nanko' revealed that 'Nanko' showed lower β-carotene and β-cryptoxanthin levels than 'Orihime.' Therefore, out of all of the downstream carotenogenic genes, the up-regulation of *PmLCYb* is probably an important factor for carotenoid accumulation in Japanese apricot fruits.

Ripe fruits of both cultivars accumulated significant amounts of β,β-carotenoids. The branching from β,ϵ-carotenoid synthesis to β,β-carotenoid synthesis is controlled by two lycopene cyclases (*7*). RNA gel blot analysis indicated that the change from β,ϵ-carotenoid synthesis to β,β-carotenoid synthesis was roughly correlated with the switching of *PmLCYe* and *PmLCYb* expression. Although the two cyclase genes were also expressed at the early stage in both cultivars, higher expression of *PmLCYe* at this stage might induce the predominant accumulation of β,ϵ-carotenoids. In the late stage, low *PmLCYe* expression and high *PmLCYb* expression might lead to the massive β,β-carotenoid accumulation in 'Orihime.' This change from β,ϵ-carotenoid accumulation to β,β-carotenoid accumulation, accompanying the disappearance of *LCYe* transcripts and an increase in *LCYb* transcripts, was also observed in *Citrus* (*13*). In contrast, in 'Nanko,' neither *PmLCYe* nor *PmLCYb* was up-regulated in the late stage of maturation or after harvest. However, *PmLCYb* expression remained constant after harvest, albeit at a low level, concomitant with the disappearance of the *PmLCYe* signal. Therefore, the lack of *PmLCYe* expression plus consistent *PmLCYb* expression might be responsible for the increase in the proportion of β,β-carotenoids in the total carotenoid content of this cultivar. Thus, *PmLCYb* could play important roles not only in β,β-carotenoid accumulation but also in the massive carotenoid accumulation due to the downstream carotenogenic genes.

The expression of *PmPDS*, the second gene in the carotenoid biosynthetic pathway, was low in both cultivars, in contrast to the strong induction of *PmPSY-1*. Weak accumulation of *PDS* transcript has also been reported in other plants (*8*, *24*, *25*). This finding leads to the question of how this low *PmPDS* expression can be sufficient to support the massive carotenoid synthesis that actually occurs. Cunningham and Gantt (*6*) proposed that a multienzyme carotenogenic complex exists. If this is true, the signal for high *PmPSY-1* expression might be efficiently transduced to *PmPDS* through the complex. In consequence, the successive desaturations might be promoted, even under conditions of low *PmPDS* expression.

Ethylene played an important role in the strong induction of *PmPSY-1*, but *PmPSY-1* expression was not completely under the control of ethylene, as some expression was observed in the absence of ethylene production. However, this slight increase in *PmPSY-1* expression seemed to be insufficient to allow the accumulation of large amounts of carotenoids. On the other hand, in propylene-treated 'Nanko,' the carotenoid content was almost the same as in the control, despite the up-regulation of *PmPSY-1*. This result suggests that the *PmPSY-1* expression level and the ethylene production observed at 3 days after treatment in the control fruits were sufficient to trigger the carotenoid accumulation; therefore, no clear differences in carotenoid contents were observed between the control and propylene-treated fruits at 3 days after treatment. Alternatively, the possibility of insufficient amounts of substrates for carotenoid biosynthesis cannot be ruled out because the fruit was harvested before full ripening occurred on the tree. Indeed, Fraser et al. (*26*) reported that carotenoid formation in tomato depends on an accessible pool of GGPP.

In conclusion, we have provided an overview of the relationship between carotenoid accumulation and the expression of carotenogenic genes in two Japanese apricot cultivars that differ in their ripening traits. Our results suggest that (i) a significant induction of *PmPSY-1* and the simultaneous induction of downstream carotenogenic genes, especially *PmLCYb*, are required for the massive carotenoid accumulation that occurs during ripening, (ii) a decrease in *PmLCYe* expression and an increase in *PmLCYb* expression cause a metabolic shift from β,ϵ-carotenoid synthesis to β,β-carotenoid synthesis as ripening approaches, and (iii) ethylene production is a prerequisite for the primary induction of *PmPSY-1* in Japanese apricot. Because Japanese apricots are usually eaten in pickled form, improved carotenoid content could be important for human health as well as enhancing market value. The present work provides data that could be used to improve the carotenoid content of Japanese apricot.

ABBREVIATIONS USED

1-MCP, 1-methylcyclopropene; ACC, 1-aminocyclopropane-1-carboxylic acid; ACO, 1-aminocyclopropane-1-carboxylic acid

oxidase; ACS, 1-aminocyclopropane-1-carboxylic acid synthase; DAF, days after flowering; DAH, days after harvest; DDBJ, DNA Data Bank of Japan; GGPP, geranylgeranylpyrophosphate; HPLC, high-performance liquid chromatography; HYb, β-ring hydroxylase; LCYb, lycopene β-cyclase; LCYe, lycopene ϵ-cyclase; MTBE, methyl *tert*-butyl ether; PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, ζ-carotene desaturase; ZEP, zeaxanthin epoxidase.

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