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（カンキツ培養砂じょうにおけるアスコルビン酸、フラボノイドおよびカロテノイド代謝に及ぼす温度の影響）

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ABBREVIATIONS

ABA abscisic acid

ANS anthocyanidin synthase

AO ascorbate oxidase

APX ascorbate peroxidase

AsA ascorbic acid

CHI chalcone isomerase

CHS chalcone synthase

DFR dihydroflavonal reductase

DHA dehydroascorbate

DHAR dehydroascorbate reductase

F3H flavanone-3-hydroxylase (F3H)

FLS flavonol synthase

FNS flavone synthase

GaLDH L-galactose dehydrogenase

GLDH L-galactono-1,4-lactone dehydrogenase

GME GDP-D-mannose 3′,5′-epimerase

GR glutathione reductase

GSH reduced glutathione

GSSG oxidized glutathione

HYb β ring-hydroxylase

HYe ε ring-hydroxylase

LCYb Lycopene-β-cyclase

LCYe lycopene-ε-cyclase

MDA monodehydroascorbate

MDAR monodehydroascorbate reductase

NCED2 Nine-*cis*-epoxycarotenoid dioxygenase

OMT *O*-methyltransferase

PDS phytoene desaturase

PSY phytoene synthase

VDE violaxanthin de-epoxidase

VTC1 GDP-D-mannose pyrophosphorylase (GMP)

VTC2 GDP-L-galactose phosphorylase (GGP)

VTC4 L-galactose-1-phosphate phosphatase (GPP)

ZDS ζ-carotene desaturase

ZEP zeaxanthin epoxidase

CONTENTS

INTRODUCTION

1 The accumulation of bioactive compounds in citrus fruits

The bioactive components is received much more interest in the past few years. Many bioactive compounds are highly accumulated in fruits and vegetables, which are consider as a natural antioxidants such as, ascorbic acid, flavonoids, and carotenoids. They have long been valued for wholesome nutritious and associated with human's health promotion (1). The nutritional studies have reported the positive effects of bioactive compounds intake to reduce the risk of many chronic diseases such as some type of cancers, Alzheimer's disease, and cardiovascular disease (34). These results have attracted attention on the study of bioactive compounds accumulated in fruits and vegetables in a past few years, particularly in citrus fruits.

Citrus is one of the most popular fruits because of its delicious taste, attractive color, and high nutritional values. Many different citrus species are the second most consumed fruits worldwide, which are commercially important in the word market both for fresh produce and food processing industry (87). The prevention of health problems through nutrition in citrus fruits is intensively promoted due to the contribution of many antioxidant compounds including ascorbic acid, flavonoid, and carotenoid. The accumulation of those compounds in citrus can be influenced by various factors such as genetic differences, environmental conditions, and postharvest processes (12,108). Among them, the environmental factors, especially temperature, were proposed as one of an important factor regulating the accumulation of ascorbic acid, flavonoid, and carotenoid in citrus fruits (18). However, the effects of temperature on the

accumulation of those compounds are not fully understood. The biochemical and molecular studies is still needed to understand the regulation of those bioactive compounds in citrus fruits.

1.1 Ascorbic acid

Ascorbic acid (AsA) has been revealed to participate in several physiological processes in plants, such as co-factor of many key enzymes, and regulating plants defense mechanism (35). In addition, AsA is also beneficial to human health. The consumption of AsA improves human immunity and reduces the risk of oxidative stress related illness, including some cancers and cardiovascular disease (25). Plants are able to synthesize AsA. In contrast to most plants, humans cannot synthesize AsA. The major source of AsA for human diet is fruits and vegetables. Some attempts have been made to increase nutritional value in fruits and vegetables by enhancing the AsA content (10, 12). Citrus fruits and related products are rich in AsA as compare to other fruits. They provide an important source of AsA for human nutrition. Generally, oranges contain the highest AsA level among the citrus species, follow by lemons, grapefruits, and mandarins (118).

The biosynthesis of AsA has long been studied in several fruits and vegetables including in citrus fruits (2, 118, 124). AsA biosynthesis consists of four distinct pathways. As shown in Fig. 1, L-galactose pathway is a main AsA biosynthetic route in higher plants (111). This pathway comprises six enzymatic steps to synthesize AsA including, GDP-D-mannose pyrophosphorylase (GMP, VTC1), GDP-D-mannose 3′,5′-epimerase (GME), GDP-L-galactose phosphorylase (GGP, VTC2), L-galactose-1-phosphate phosphatase (GPP, VTC4), L-galactose dehydrogenase (GalDH) and L-galactono-1,4-lactone dehydrogenase (GLDH), respectively. Then, AsA are further oxidized by ascorbate oxidase (AO) and ascorbate peroxidase (APX) into

monodehydroascorbate (MDA). MDA can either be regenerated back into AsA by monodehydroascorbate reductase (MDAR) or non-enzymatic disproportionate to dehydroascorbate (DHA). After that, DHA can be recycled back into AsA by dehydroascorbate reductase (DHAR), using reduced glutathione (GSH) as a reducing substance. In ascorbate-glutathione cycle, the oxidized glutathione (GSSG) is recycled back into reduced glutathione (GSH) by glutathione reductase (GR) (82,61).

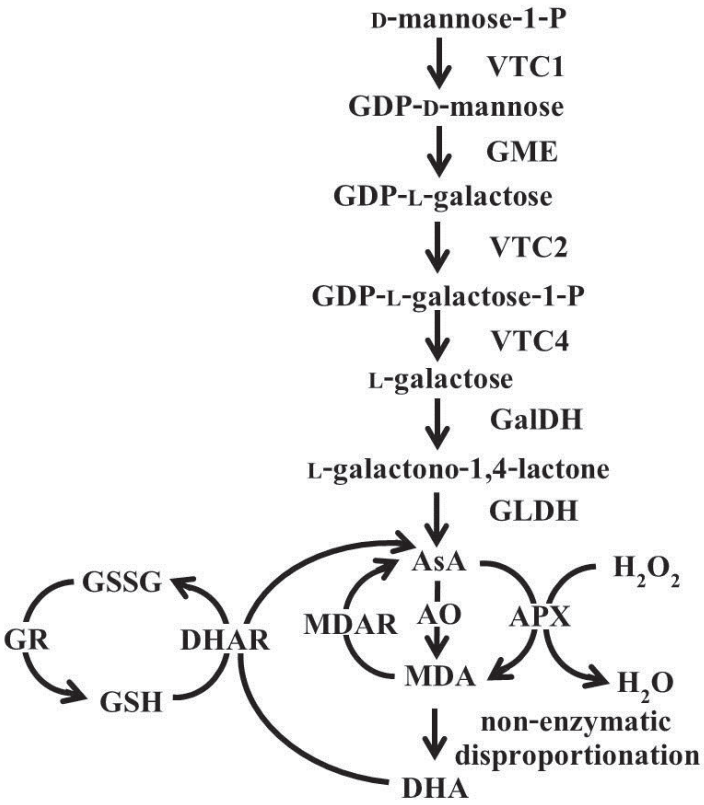

Fig. 1. The biosynthetic, oxidation, and regeneration pathway of AsA metabolism in plants. VTC1, GDP-D-mannose pyrophosphorylase; GME, GDP-D-mannose 3′,5′-epimerase; VTC2, GDP-L-galactose phosphorylase; VTC4, L-galactose-1-phosphate phosphatase; GalDH, L-galactose dehydrogenase; GLDH, L-galactono-1,4-lactone dehydrogenase; AO, ascorbate oxidase; APX, ascorbate peroxidase; MDA, monodehydroascorbate; MDAR, monodehydroascorbate reductase; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase.

1.2 Flavonoid

Flavonoids represent polyphenolic secondary metabolites that are mainly accumulated in a wide range of crop plants. More than 6,000 kinds of flavonoids were identified in plants (1). The basic structure of flavonoids consists of two aromatic rings and conjugates with heterocycle. The differences between individual flavonoids relate to the number of hydroxyl groups and attached sugars, and the position of hydroxyl groups and sugars attachment. The modification of their basic skeleton leads to the diversity of flavonoids in nature. Generallly, flavonoids can be classified into six groups, including, flavanones, flavones, flavonols, iso-flavones, flavanols, and anthocyanidins (12).

Citrus fruits are considered as one of the major source of flavonoids intake for human diet. A large number of flavonoids were observed among citrus species. The most abundance flavonoid accumulated in citrus fruits is flavanones, which is usually presented in diglycoside form and responsible for the taste of citrus (112). Among commercial citrus, the flavonoid content is highly accumulated in mandarins and sweet oranges, with flavanones being a main component, and followed by flavones at very low level. In contrast to mandarins and oranges, flavones are particularly abundant in lemons and limes. Although flavones are found at low level, the roles of their antioxidant activities to reduce the risk of chronic diseases are actively studied (34). Flavonoid exhibits crucial roles in many biological functions in plants including, plant pigmentation, and defense mechanism. In addition, the pharmaceutical activity of flavonoids as an antioxidant has been scientifically proven to ameliorate various diseases, such as minimized the risk of cardiovascular disease, cancers, obesity, type 2 diabetes, and inflammation (114).

In the past few years, the biochemical and molecular studies were performed to elucidate flavonoid biosynthesis pathway. Many genes encoded enzymes in flavonoid biosynthesis have been isolated (45, 58, 77, 109, 117). As shown in Fig. 2, the substrates derived from general phenylpropanoid pathway initiate the biosynthesis of flavonoids. The condensation between *p*-coumaroyl-CoA and melanyl-CoA catalyzed by chalcone synthase (CHS) produces chalcone, a substrate used in subsequently synthesis of all flavonoids. Then, it can be converted to flavanone by chalcone isomerase (CHI). At this step, flavanone can be modified to various flavonoid derivatives. Flavanone is converted to flavone and polymethoxylflavone by flavone synthase (FNS) and *O*-methyltransferase (OMT). Furthermore, flavanone can also be converted to dihydroflavonol by flavanone-3-hydroxylase (F3H). Then, it is sequentially converted to flavonol catalyst by flavonol synthase (FLS), and/or converted to anthocyanidin by dihydroflavonal reductase (DFR) and anthocyanidin synthase (ANS).

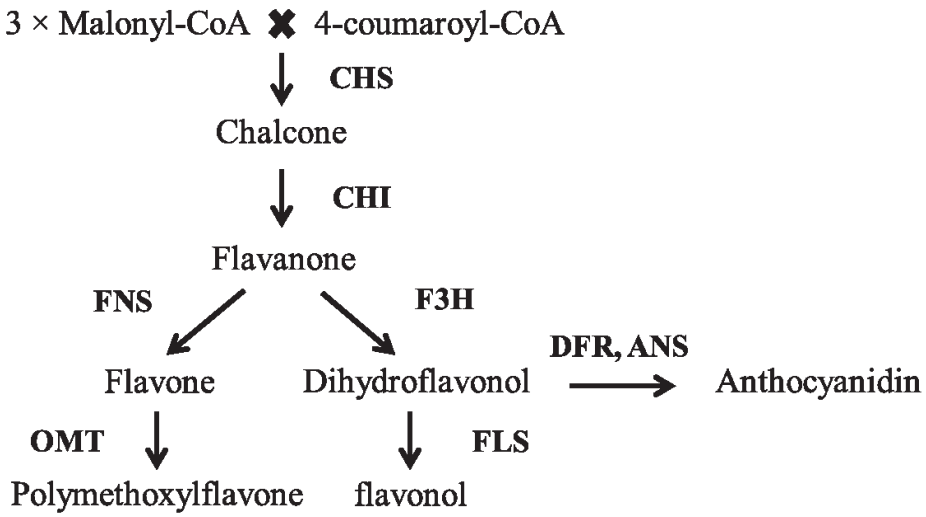

Fig. 2 The biosynthesis pathway of flavonoid in plants. CHS, chalcone synthase; CHI, chalcone isomerase; FNS, flavone synthase; OMT, *O*-methyltransferase; F3H, flavanone-3-hydroxylase; FLS, flavonol synthase; DHR, dihydroflavonal reductase; ANS, anthocyanidin synthase

1.3 Carotenoid

Carotenoids are considered as a major component in citrus fruit responsible for bright orange pigment, which determine quality and appearance of fruits. Carotenoids are natural isoprenoid substances that play an important role in many physiological processes in plants (7, 9, 84). In plant tissues, carotenoids are major agronomic quality for a number of fruits and vegetables, such as the provision of a diverse range of pigments, aromas, and scent compounds (36, 122). In addition, the important roles of carotenoids in human health cannot be neglected. The antioxidant activities of carotenoids have attracted attention for a long time. Carotenoids have been suggested to reduce the damaging effects of oxidative stress in various chronic diseases, such as eye-related disorders, cardiovascular diseases, and cancers (69, 47, 89).

A large number of carotenoids are found in citrus fruits. The concentration and composition differ among citrus species. Generally, mandarin fruits predominately accumulated β-cryptoxanthin, while sweet orange fruits mainly accumulated 9-*cis*-violaxanthin as a major carotenoid. In contrast, lemon fruits accumulated much lower concentration of carotenoids than other citrus species. Carotenoid metabolism has been extensively studied in citrus fruits, and an almost complete metabolic pathway has been elucidated. Previous studies reported that the accumulation of carotenoid was transcriptionally regulated by genes involved in carotenoid metabolic pathway (43, 44, 63, 66, 92, 123). As shown in Fig.1, the first step in the biosynthesis pathway is the conversion of two geranylgernyl pyrophosphate (GGPP) molecules derived from the MEP (2-*C*-methyl-*D*-erythritol 4-phosphate) pathway to form phytoene, catalyzed by phytoene synthase (PSY). A series of desaturation by phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS) converts phytoene into lycopene. The cyclization of lycopene by lycopene-β-

cyclase (LCYb) and lycopene-ε-cyclase (LCYe) produces a diverse range of carotenoids. Lycopene is cyclized with one ε-ring and one β-ring by LCYe and LCYb to produce α-carotene. α-Carotene is then converted into lutein catalyzed by β-ring hydroxylase (HYb) and ε-ring hydroxylase (HYe). In addition, lycopene may be cyclized with two β-rings by LCYb to produce β-carotene. β-Carotene is then hydroxylated by HYb to form β-cryptoxanthin and zeaxanthin. A reversible reaction, called xanthophyll cycle, subsequently occurred. Zeaxanthin is converted into all-*trans*-violaxanthin and 9-*cis*-violaxanthin catalyzed by zeaxanthin epoxidase (ZEP). At this step, the reversal of all-*trans*-violaxanthin into zeaxanthin may be activated by violaxanthin de-epoxidase (VDE). Futhermore, 9-*cis*-violaxanthin may be enzymatically catabolized by 9-cis-epoxycarotenoid dioxygenases (NCED) to produce C_{25} epoxy-apocarotenoid and xanthoxin, which is modified for the synthesis of the phytohormone, abscisic acid (ABA).

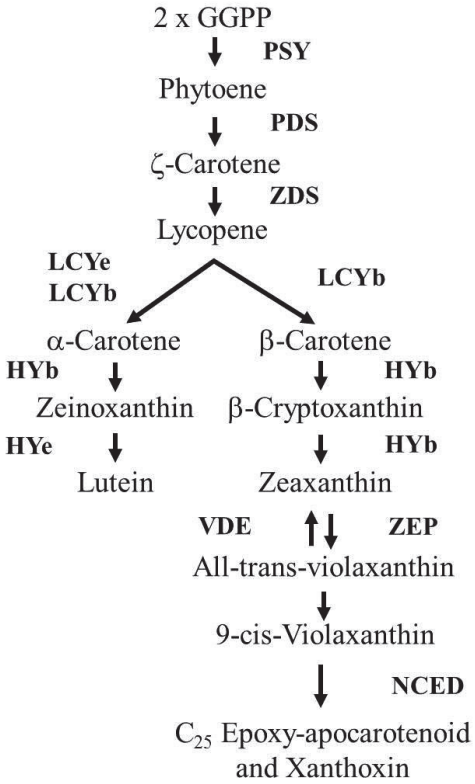

Fig. 3 The metabolism pathway of carotenoid in plants. GGPP, geranylgernyl pyrophosphate; PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, ζ-carotene desaturase; LCYb, lycopene by lycopene-β-cyclase; LCYe, lycopene-ε-cyclase; HYb, β-ring hydroxylase; HYe, ε-ring hydroxylase; ZEP, zeaxanthin epoxidase; VDE, violaxanthin de-epoxidase NCED, 9-cis-epoxycarotenoid dioxygenases

2. Factor affecting the accumulation of bioactive compounds in citrus fruits

The genetic and environmental factors were responsible for diversity in the contents and compositions of bioactive compounds in citrus fruits. In the past few decades, temperature was widely described as an important environmental factor affecting plant growth and development (31, 40, 45). In citrus, temperature does not only cause morphological, physiological, biochemical, and molecular changes, which adversely affects plant growth and productivity. It can lead to high variation in the biosynthesis and accumulation of bioactive compounds, especially ascorbic acid, flavonoid, and carotenoid in citrus (53, 60, 67). The accumulation of those compounds in citrus fruits is considered as an important agricultural trait, which determines quality and attracts consumer interest due to the health benefits. Therefore, the modification of ascorbic acid, flavonoid, and carotenoid in citrus fruits has become one of the biggest challenges for researchers.

The effects of temperature on many kinds of bioactive compounds have been received an attention for a long time. However, the study that investigated the effects of temperature on bioactive compounds accumulation during the maturation process in citrus fruits has been limited because the difficulties associated with controlling temperature in the open field. In our previous study, we successfully established an *in vitro* culture system using citrus juice sacs, which is an efficient technique for controlling undesirable variations during the experimental period. In the present study, the effects of temperatures on AsA, flavonoid, and carotenoid accumulation and the expression of those metabolic genes were investigated by using citrus juice sacs *in vitro* culture system at three different temperatures (10, 20, and 30°C). A better understanding of AsA, flavonoid, carotenoid regulation in response to different temperatures during citrus fruit

maturation will led to novel approaches in the molecular breeding of AsA, flavonoid, and carotenoid biosynthetic pathway in citrus fruits in the future.

CHAPTER 1

Effect of temperature on ascorbic acid metabolism in citrus juice sac *in vitro*

1. Introduction

Citrus fruits are rich in AsA content as compared to other commercial fruits and vegetables (19, 27). In citrus fruit, a major AsA biosynthetic route is L-galactose pathway (2, 118). A wide variation of AsA content in the fruits was depended on various factors, such as species, tissues, and stages of fruit development (53, 55, 56). The previous research in citrus studied AsA accumulation in Valencia orange, Lisbon lemon, and Satsuma mandarin during the ripening progress (124). It was found that AsA accumulation during ripening process was different in the three citrus species. AsA content remained constant and significantly lower in Satsuma mandarin than that of the other two citrus species. The transcriptional balances in AsA biosynthesis, oxidation, and regeneration contributed to the different patterns of AsA accumulation during the ripening process. In addition, it was previously found that AsA accumulation was increased in the flavedo, whereas it was decreased in the pulp during fruit ripening. The results indicated that the accumulation of AsA in citrus fruits was independently regulated in each fruit tissues (2).

AsA content was also affected by several environmental factors. AsA accumulation was strongly affected by climatic conditions, including light and temperature (70). In citrus, light stimulated the accumulation of AsA, and the level of AsA was also depended on the quality of light as well. The red LED light was not efficient to increased AsA amount, whereas the blue LED light notably boosted up AsA amount in juice sacs of citrus fruits. It was revealed that the

up-regulation of genes in AsA biosynthetic and regeneration pathway by the blue LED light enlarged the AsA amount in juice sacs of citrus fruits (124). Temperature significantly influenced on a number of physiological processes in plants. Low temperature has been reported to promote the plant growth rate in species of temperate fruits and vegetables. In addition, it also induced the accumulation of secondary metabolites in plants, including AsA (86). A number of plants accumulated more AsA when they were grown under low field temperature than high field temperature, such as kiwifruit (90), broccoli (97), spinach (86) and tomato (31). The previous researches reported that temperature strongly affected the biosynthetic, oxidation, and regeneration pathway in AsA metabolism. It was found that low temperature triggered the expression of AsA biosynthetic genes, whereas high temperature did not in tomato fruit during off-vine ripening (70). High temperature triggered the oxidation process, which was responsible for the loss of AsA content in tomato fruits when the temperature increased (91). Furthermore, in the last decade, some researches revealed the important of regeneration pathway in maintaining AsA level in plants (15, 30). The previous study found that the limitation of AsA regeneration by high temperature contributed to the reduction of AsA amount in tomato fruits (70).

To date, molecular basis underlying the accumulation of secondary metabolites in response to different environmental factors is becoming an attractive scientific research in citrus fruits. However, the regulation of AsA accumulation in response to different temperature conditions in citrus fruits remains unclear. In this study, the influences of temperature on AsA metabolism were elucidated in citrus. AsA quantification and the gene expression in AsA metabolism pathway were carried out in the three citrus varieties (Valencia orange, Lisbon lemon, and Satsuma mandarin) in *in vitro* cultured system at different temperatures (10°C, 20°C, and 30°C).

2. Materials and methods

2.1 Plant materials

In the present study, three citrus species with different AsA, flavonoid, and carotenoid contents were used as plant materials; Satsuma mandarin (*Citrus unshiu* Marc), Valencia orange (*C. sinensis* Osbeck), and Lisbon lemon (*C. limon* Burm.f.). The fruits with diameters of approximately 4 – 5 cm at the immature green stage were randomly harvested from citrus trees. The Satsuma mandarin was harvested from Fujieda Farm, Shizuoka, Japan. The Valencia orange and Lisbon lemon were harvested from NARO Institute of Fruit Science, Department of Citrus Research, Okitsu, Shizuoka, Japan.

2.2 *In vitro* culture system and temperature treatments

The *in vitro* culture system was performed in accordance with a previously described method (123). Murashige and Skoog (MS) medium was used in the culture system. Medium was supplemented with sucrose (10% w/v) and agar (1% w/v), and pH was adjusted to 5.7. Medium was sterilized using an autoclave. Juice sacs were excised from citrus fruits and placed with the endocarp side up on 10 mL of medium in culture tubes (22 x 120 mm). Citrus juice sacs were cultured at 20°C during the first two weeks. They were then exposed to different temperatures of 10°C, 20°C, and 30°C for another two weeks in the culture system. Juice sacs were sampled twice, at the second week and the fourth weeks, in culture system. They were immediately frozen in liquid nitrogen and stored at -80°C until use in the three experiments.

2.3 Ascorbic acid extraction and quantification

AsA content in citrus juice sacs was measured by HPLC in three replications according to the published methods (64, 124). 0.5 g of juice sacs sample and 4 mL of extraction buffer (3% metaphospholic acid and 8% acetic acid) were homogenized, and centrifuged at 14,000 x *g* for 20 mins. The supernatant was filtered through Miracloth (Calbiochem, La Jolla, CA, USA), and 0.45 μM nylon filter (Advantec, Tokyo, Japan), respectively. Then aliquot (20 μL) was injected into HPLC with a J'sphere ODS-M80 column (YMC, Kyoto, Japan) and a LC-10AD pump (Shimadzu, Kyoto, Japan). The flow rate of mobile phase in the column (1.5% ammonium dihydrogen phosphate, pH 3.8) was 1.0 mL min^{-1}. The results of AsA content were detected by SPD-10A spectrophotometric detector (Shimadzu) at 245 nm and 2.6 mins of retention time. Peaks were converted to concentrations as μmol g^{-1} fresh weight using standard curve constructed by a serial dilutions of stock AsA.

2.4 RNA extraction

The total RNA in citrus juice sacs was extracted using phenol-chloroform, modified from the previously described method (38). Frozen juice sacs (1.8 g) were ground to powder in liquid nitrogen. The powder was added to 10 ml of phenol:chloroform:3-methy-l-butanol (25:24:1) and 10 ml of lysis buffer consisting of 0.5M EDTA and 1.5M Tris-borate buffer. The two phases were mixed and subsequently separated by centrifugation. The upper aqueous phase was pipetted to the new tube and this procedure was repeated three times. The upper aqueous phase containing total RNA was mixed with 0.25 volume of ethanol, 0.11 volume of 5M potassium acetate,

followed by 1:1 ratio of chloroform:3-methy-l-butanol (49:1), and separated by centrifugation. Total RNA was precipitated by 3M LiCl overnight at -30°C. Then, total RNA was subsequently pelleted by centifugation and resuspended in DEPC-treated water. The precipitation was repeated twice by 3M LiCl. Total RNA was purified by the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and stored as ethanol precipitation at -80°C.

2.5 cDNA synthesis and real time PCR analysis

In order to synthesize cDNA, 2 µg of purified RNA was used in the reverse transcription reactions with the cycle protocol of 37°C for 60 min with TaqMan Reverse Transcription Reagents (Thermo Fisher Scientific,Waltham, MA, USA) and random hexamer.

qRT-PCR was performed in the three replications. TaqMan MGB probes and the set of primers for *CitVTC1*, *CitVTC2*, *CitVTC4*, *CitGLDH*, *CitAPX1*, *CitAPX2*, *CitAPX3*, *CitchAPX*, *CitAO*, *CitMDAR1*, *CitMDAR2*, *CitDHAR*, *CitGR*, and *CitchGR* have been descried (121). A gene expression analysis was performed by StepOnePlus™ Real-Time PCR System (Applied Biosystems) according to the manufacturer's instructions. In the Real-Time PCR reaction mixture, 900nM of primers, 250 nM of TaqMan MGB Probe, and the cDNA template were used with the cycling protocol of 95°C for 10 mins, then 40 cycles of 95°C for 15 s and 60°C for 60 s. The results were calculated using StepOnePlus™ Real-Time PCR System Software (Applied Biosystems). In the present study, 18S ribosomal RNA was used as a reference gene in order to normalize the gene expression results.

2.6 Statistical analysis

Data were shown in the present study as the mean ± standard error for three replications. Statistical differences among temperature levels were evaluated with Tukey's HSD test at $P < 0.05$. Calculations were performed using JMP software (SAS Institute, Cary, NC).

3. Results

3.1 AsA accumulation in citrus juice sacs at different temperatures *in vitro*

The effects of temperature on AsA accumulation were carried out in the juice sacs of Valencia orange, Lisbon lemon, and Satsuma mandarin in *in vitro* culture system. Juice sacs were culture under the same condition at 20°C for two weeks and then cultured under different temperature treatments at 10°C, 20°C, and 30°C for another two weeks. AsA content was measured in the second week and the fourth week. To evaluate the effects of low and high temperature on AsA accumulation, the temperature treatment at 20°C in the fourth week was used as a control. The results showed that Valencia orange contained the highest AsA content among the three species (Fig. 4), followed by Lisbon lemon (Fig. 5), and Satsuma mandarin (Fig. 6). After four weeks cultures *in vitro*, AsA content at 10°C was significant higher than the control at 20°C, whereas AsA content at 30°C was not significant different as compared to the control at 20°C in the three citrus species (Fig. 4-6).

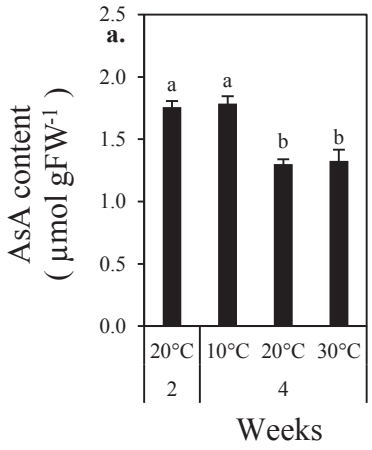

Fig. 4 AsA content in citrus juice sacs at different temperatures *in vitro* in Valencia orange. The data shown are the mean ± standard error in the three replications. Tukey's HSD test was performed by JMP software (SAS Institute, Cary, NC) at $P < 0.05$. The means indicated by the same letter was not significant different.

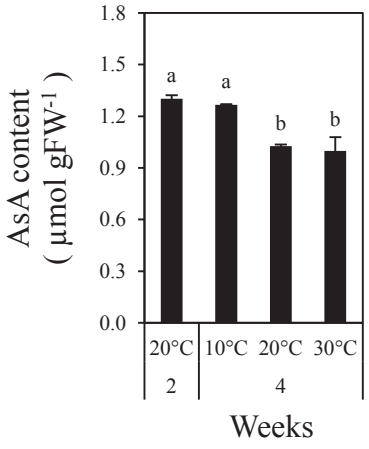

Fig. 5 AsA content in citrus juice sacs at different temperatures *in vitro* in Lisbon lemon. The data shown are the mean ± standard error in the three replications. Tukey's HSD test was performed by JMP software (SAS Institute, Cary, NC) at $P < 0.05$. The means indicated by the same letter was not significant different.

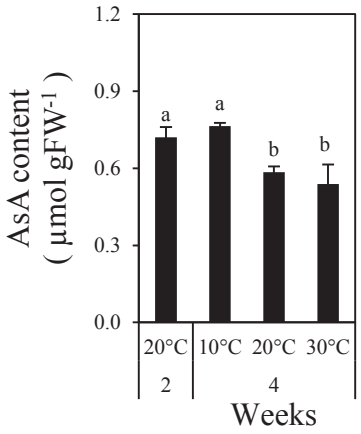

Fig. 6 AsA content in citrus juice sacs at different temperatures *in vitro* in Satsuma mandarin. The data shown are the mean ± standard error in the three replications. Tukey's HSD test was performed by JMP software (SAS Institute, Cary, NC) at $P < 0.05$. The means indicated by the same letter was not significant different.

3.1 The expression of genes involved in AsA metabolism pathway in citrus juice sacs at different temperatures *in vitro*

In the present study, the molecular mechanism regulating AsA accumulation in response to temperature was studied in Valencia orange, Lisbon lemon, and Satsuma mandarin in *in vitro* culture system. The changes of gene expression in biosynthetic pathway (*CitVTC1*, *CitVTC2*, *CitVTC4*, and *CitGLDH)*, oxidation pathway (*CitAPX1*, *CitAPX2*, *CitAPX3*, *CitchAPX*, and *CitAO*), and regeneration pathway (*CitMDAR1*, *CitMDAR2*, *CitDHAR*, *CitGR*, and *CitchGR*) were measured in the second week and the fourth week (Fig. 7-9). To evaluate the effects of low temperature and high temperature on the expression of AsA metabolic genes, the treatment at 20 °C in the fourth week was used as a control.

As for AsA biosynthetic genes, in Valencia orange and Lisbon lemon, the expression of *CitVTC1*, *CitVTC2*, and *CitVTC4* genes at 10°C was higher than the control at 20°C (Fig. 7 and 8). In Satsuma mandarin, the expression of *CitVTC4* gene at 10°C was higher, whereas the expression of other biosynthetic genes at 10°C was not significantly affected as compared with the control at 20°C (Fig. 9). Furthermore, in Valencia orange, the expression of *CitVTC2* gene at 30°C was lower, and the expression of other biosynthetic genes at 30°C was not significantly different as compared with the control at 20°C (Fig. 7). In contrast to Valencia orange, the higher expression of *CitVTC1* and *CitGLDH* genes in Lisbon lemon, and the higher expression of *CitVTC2* and *CitGLDH* genes in Satsuma mandarin were observed at 30°C (Fig. 8 and 9).

As for AsA oxidation genes, the expression of *CitAO* gene was significantly lower at 10°C in the three citrus species. In contrast, the expression of *CitAPX1* and *CitchAPX* genes at 10°C was higher than the control at 20°C in the three citrus species (Fig. 7-9). In addition, the

higher expression of *CitAPX1* and *CitAO* genes in Valencia orange, (Fig. 7), the higher expression of *CitAPX1*, *CitAPX3*, *CitchAPX*, and *CitAO* genes in Lisbon lemon (Fig. 8), and the higher expression of *CitAPX1*, *CitAPX2*, and *CitAPX3* genes in Satsuma mandarin (Fig. 9) were observed at 30°C as compared with the control at 20°C.

As for AsA regeneration genes, the expression of *CitMDAR1* and *CitMDAR2* genes at 10°C was higher than the control at 20°C in Valencia orange and Satsuma mandarin (Fig. 7 and 9). In contrast, the expression of *CitMDAR1* and *CitMDAR2* genes was significantly lower at 10°C in Lisbon lemon (Fig. 8). The expression of regeneration genes at 30°C was different in the three species. The expression of *CitMDAR1*, *CitMDAR2*, *CitGR*, and *CitchGR* genes at 30°C was lower than the control at 20°C in Valencia orange (Fig. 7). In contrast, the expression of *CitMDAR1*, *CitMDAR2*, and *CitGR* genes at 30°C was higher than the control at 20°C in Lisbon lemon and Satsuma mandarin (Fig. 8 and 9).

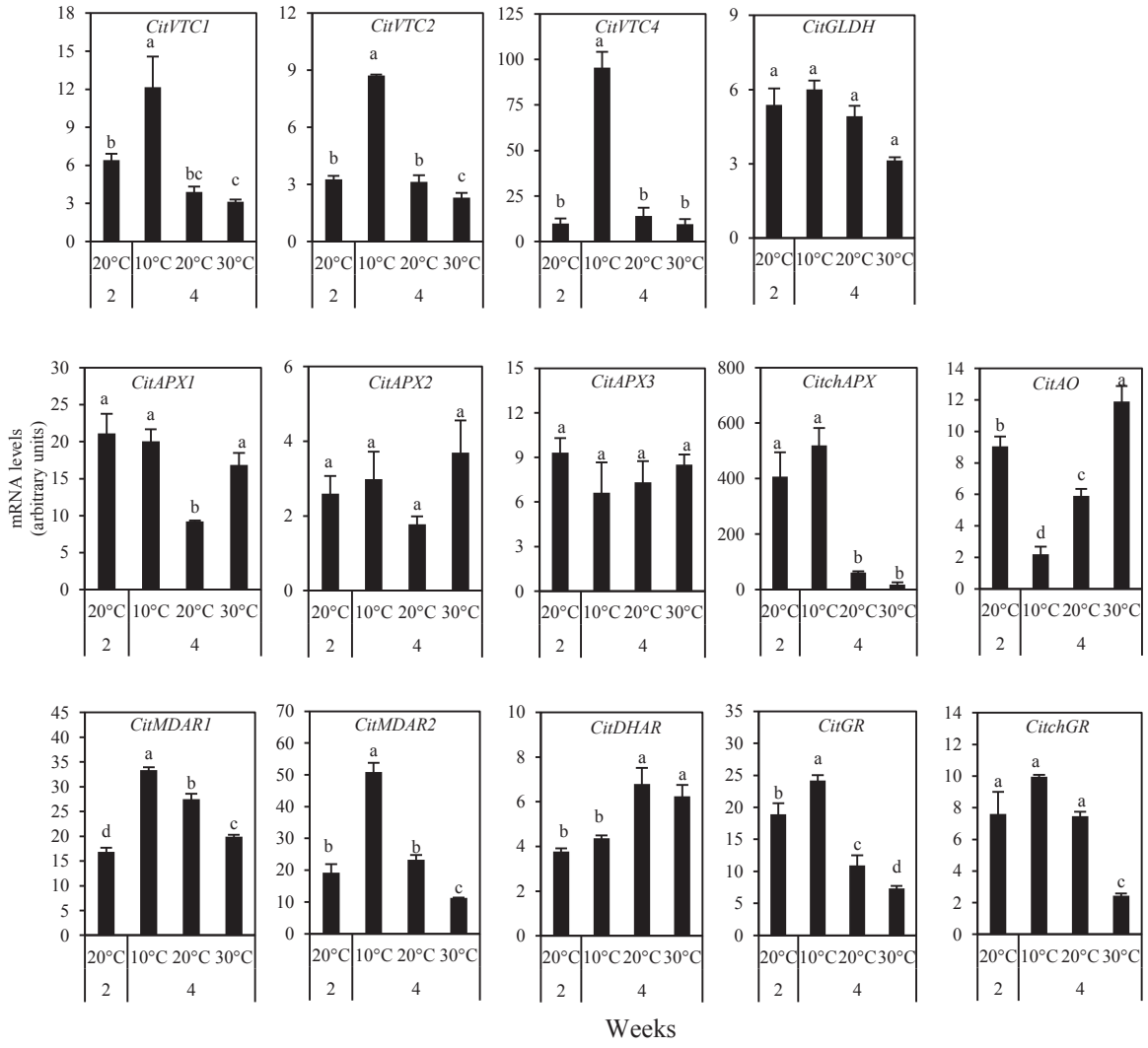

Fig. 7 The expression of genes involve in AsA metabolism pathway in Valencia orange at different temperatures *in vitro*. The data shown are the mean ± standard error in the three replications. Tukey's HSD test was performed by JMP software (SAS Institute, Cary, NC) at $P < 0.05$. The means indicated by the same letter was not significant different.

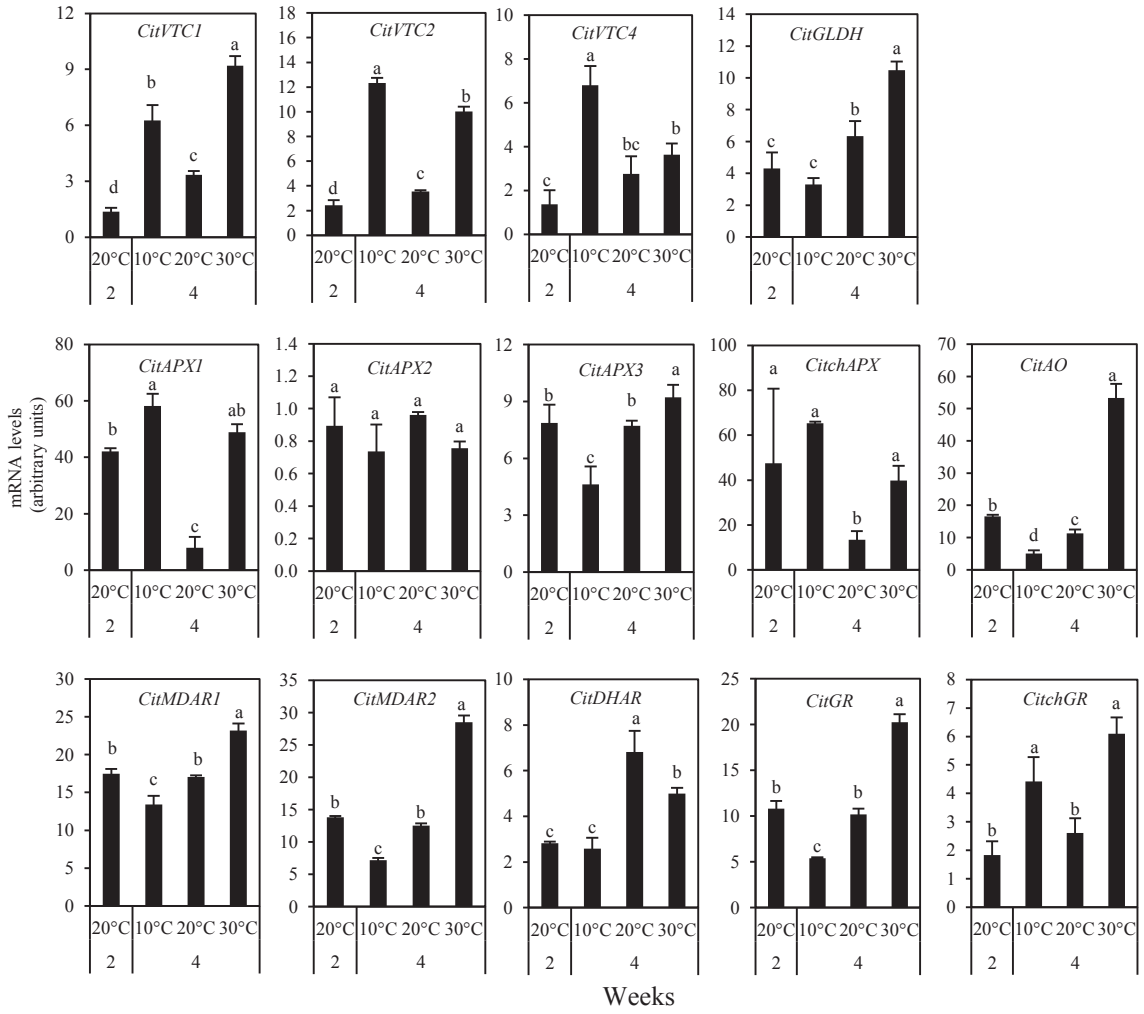

Fig. 8 The expression of genes involved in AsA metabolism pathway in Lisbon lemon at different temperatures *in vitro*. The data shown are the mean ± standard error in the three replications. Tukey's HSD test was performed by JMP software (SAS Institute, Cary, NC) at $P < 0.05$. The means indicated by the same letter was not significant different.

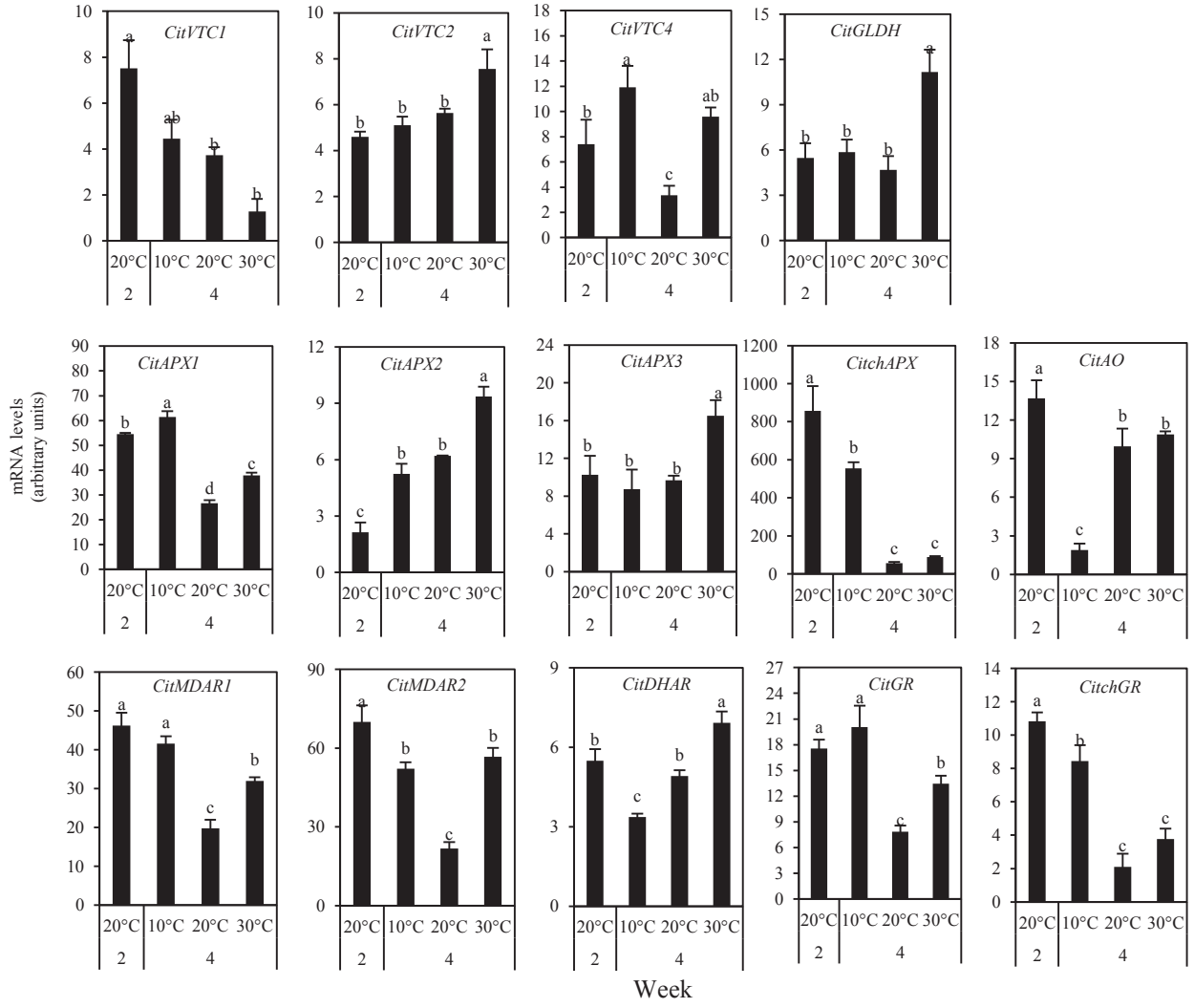

Fig. 9 The expression of genes involved in AsA metabolism pathway in Satsuma mandarin at different temperatures *in vitro*. The data shown are the mean ± standard error in the three replications. Tukey's HSD test was performed by JMP software (SAS Institute, Cary, NC) at $P < 0.05$. The means indicated by the same letter was not significant different.

4. Discussion

In citrus fruit, it has been reported that the transcriptional regulation of AsA metabolic genes is a major mechanism regulating AsA accumulation (2, 124). To date, the roles of temperature regulating AsA metabolism during fruit ripening is highly required. In the present study, the results showed that the decrease in AsA content at 20°C was observed during four weeks *in vitro* in the three citrus species. The previous study revealed that the reduction of AsA level in citrus juice sacs *in vitro* was similar to the reduction of AsA level in citrus juice sacs ripening under natural condition (120). By using this technique, the juice sacs grew normally with AsA accumulation similar to the fruits ripening on the tree and no callus form during the experimental period.

The present results indicated that low temperature noticeably induced the expression of AsA biosynthetic genes in citrus juice sacs. In Valencia orange and Satsuma mandarin, the expression of *CitVTC4* gene at 10°C was significantly higher than the control at 20°C. In Lisbon lemon, the expression of *CitVTC1*, *CitVTC2*, and *CitVTC4* genes at 10°C was significantly higher than the control at 20°C. The higher expression of the *CitVTC* genes was coincident with higher AsA accumulation in citrus juice sacs at 10°C in the three citrus species. These results were correspondence with the previous studies. It was found that temperature significantly influenced on AsA accumulation in fruits and vegetables, such as kiwifruit (90), broccoli (107, 97), and tomato (31, 94). Presently, only a few studies were described on AsA biosynthetic pathway in response to different temperatures at transcriptional level. In tomato, it was reported that low temperature triggered the expressions of *VTC1*, *VTC2*, and *VTC4* genes in tomato fruits during off-vine ripening and it also increased the expression of VTC4 gene in tomato fruits

during post-harvest life (39, 70). In citrus, it was found that AsA biosynthetic genes played a major role in regulating AsA accumulation and the expression of those genes could be influenced by environmental factors. It was previously revealed that the increase in the expressions of *CitVTC1*, *CitVTC2*, and *CitVTC4* genes by the blue LED light enlarged the AsA amount in juice sacs of citrus fruits (124). In the present study, the higher expression of *CitVTC4* gene at 10°C was found in the three citrus species, which indicated that *CitVTC4* might be a key gene in regulating AsA accumulation at 10°C in citrus fruits. At 30°C, the higher expression of *CitVTC1* and *CitGLDH* genes in Lisbon Lemon and the higher expression of *CitVTC2* and *CitGLDH* genes in Satsuma mandarin were observed. In the meanwhile, the higher expression of *CitAPX3* and *CitAO* in Lisbon lemon and the higher expression of *CitAPX2* and *CitAPX3* in Satsuma mandarin were also observed at 30°C. The simultaneous increase in the biosynthetic genes and oxidation genes at 30°C might be attributed to maintain AsA content in Lisbon lemon and Satsuma mandarin. In addition, alternative biosynthetic pathways have been reported to be involved in AsA accumulation, such as L- gulose, the *myo*-inositol, and D-galacturonic acid pathways. D-Galacturonic acid pathway was revealed to play an important role in AsA accumulation in strawberry (20), grape (21), tomato (5), and citrus (2). In citrus fruits, it was found that the accumulation of AsA significantly dropped along with the decrease in expression of genes in D-galacturonic acid pathway in the flavedo of fruit in the dark condition treatment (49). Thus, the roles of alternative biosynthetic pathway in regulating AsA accumulation by different temperatures will be further investigated in the future research of citrus fruits.

Oxidation pathway has been suggested to regulating AsA accumulation under stresses environment (91). The expression of APX gene was triggered by various environmental stimulus, such as oxidative damage, high light intensity, cold, and high temperature (98, 99, 125). Previous

researches reported that APX gene was considered to be related to plant defense mechanism against oxidative damage, such as in sunflower (73), pea (88), and spinach (121). In this study, the expression of *CitAPX1* and *CitchAPX* genes was higher at 10°C in the three citrus species. In addition, the expression of *CitAPX1* gene was also higher at 30°C in the three citrus species. It might be assumed that the higher expression of *CitAPX1* and *CitchAPX* genes at 10 and 30°C triggered an antioxidant defense system for protecting plant against oxidative damage. Furthermore, the expression of *CitAO* gene was significantly lower at 10°C, whereas its expression was higher at 30°C in the three citrus species. These results suggested that the down-regulation of *CitAO* gene at 10°C contributed to increase AsA level in the juice sacs of the three citrus species.

In addition to biosynthesis and oxidation pathway, the important of AsA regeneration pathway was illustrated in maintaining the level of AsA in fruits and vegetables (126). In this study, the expression of *CitMDAR1* and *CitMDAR2* genes was higher at 10°C in Valencia orange and Satsuma mandarin. The higher expression of two *CitMDAR* genes was consistent with the higher AsA content at 10°C in those of Valencia orange and Satsuma mandarin. The present results were similar to the previous studies in other transgenic plants. It was revealed that the overexpression of AsA regeneration genes promoted the accumulation of AsA through improving AsA recycling ability (15, 26, 105, 120). In tomato, the overexpression of MDAR gene increased the AsA amount in tomato leaves (57). In citrus fruit, it was found that the coordinated expression of MDAR family genes contributed to increased recycling capacity in the pulp of citrus varieties contain larger AsA content (2). It could be suggested that *CitMDAR1* and *CitMDAR2* might be the key genes for regulating AsA accumulation at 10°C in Valencia orange and Satsuma mandarin. Contrastingly, the expression of *CitMDAR1* and *CitMDAR2* genes was

not affected by low temperature in Lisbon lemon. It might be concluded that the higher AsA content at 10°C in Lisbon lemon was only determined by the transcriptional regulation between biosynthetic and oxidation genes.

These results indicated that AsA accumulation was induced at 10°C, whereas it was not significantly affected at 30°C as compared with the control at 20°C in juice sacs of the three citrus species. The enhancement of AsA accumulation at 10°C was highly regulated at the transcriptional level. In Valencia orange and Satsuma mandarin, the higher expression of *CitVTC4* gene in biosynthetic pathway and the higher expression of *CitMDAR1* and *CitMDAR2* genes in regeneration pathway, together with the lower expression of *CitAO* gene in oxidation pathway contributed to enhance AsA content at 10°C. In Lisbon lemon, the higher expression of *CitVTC1*, *CitVTC2*, and *CitVTC4* genes in biosynthetic pathway, and the lower expression of *CitAO* gene in oxidation pathway contributed to enhance AsA content at 10°C. These results indicated that the changes at the transcriptional level of AsA biosynthetic, oxidation, and regeneration genes were different among the three citrus species in response to temperature. This information will be necessary for engineering the large amount of AsA in citrus fruits in the future research.

CHAPTER 2

Effect of temperature on flavonoid metabolism in citrus juice sac *in vitro*

1. Introduction

The diversities of flavonoid composition and quantity in citrus species were influenced by many environmental factors and affected to both the appearance and the taste of fruits (40). The previous findings reported that the macronutrients and water level influenced flavonoids accumulation. Then, the nutrient and water management have consequently become an attractive tool for improving flavonoids level in fruits and vegetables production (113).

In recent years, temperature has been elucidated as a major effector on flavonoid accumulation in many important economic crops. The poor coloration of apple fruits caused by high temperature was reported in several apple varieties and the coloration in response to temperature varied depending on varieties (116). The biosynthesis of flavonoid, particularly anthocyanin, has long been elucidated to promote by low temperature, but it was inhibited by high temperature (105). The study at the molecular level reported the transcript of genes related to anthocyanin synthesis was up-regulated at low temperature in apple (115). In grape, the effect of temperature from early maturation to harvest has been demonstrated in many studies (46, 74, 75, 117). Those results reported that low temperature accelerated anthocyanin accumulation in grape. The regulatory mechanism of anthocyanin biosynthesis under different temperature conditions was elucidated in grape. It was previously reported that the expression of genes and the activity of enzymes involved in the anthocyanin synthesis were higher in grape grown under

low night temperature than those grown under high night temperature (75). From the previous researches, it was found that the studies on the induction of flavonoid accumulation by low temperature have only been performed in some of economical fruits, particularly in apple and grape. A little information regarding to those issue is demonstrated in citrus fruits because its difficulty to control surrounded temperature in the open field. Therefore, the aim of this study was to investigate the regulatory mechanism of flavonoid accumulation in response to different temperatures by using citrus juice sacs *in vitro*.

2. Materials and methods

2.1 Plant materials and temperature treatments

The three citrus species with different AsA, flavonoid, and carotenoid contents (Satsuma mandarin, Valencia orange, and Lisbon lemon) were randomly harvested from citrus trees.

The *in vitro* culture system was performed in accordance with a previously described in chapter 1. Citrus juice sacs were cultured at 20°C during the first two weeks. They were then exposed to different temperatures of 10°C, 20°C, and 30°C for another two weeks in the culture system. Juice sacs were sampled twice, at the second week and the fourth weeks, in culture system. They were immediately frozen in liquid nitrogen and stored at -80°C until use in the three experiments.

2.2 Flavonoid extraction and quantification

Flavonoid content and composition were measured by HPLC in the three replications for each sample. The freeze-dried samples were extracted with the mixture of MeOH/DMSO (1:1) and repeated for three times. The extracts were filtered with 13P nylon membrane filter and made up to 5 mL by MeOH. Then, 10 µL aliquots were injected to HPLC system (LC-NetII/ADC Jasco) with YMC-UltraHT Pro C18 column. The mobile phase was H_3PO_4 (85%) and CH_3CN/MeOH (1:1). The linear gradients were as follows; 78% 20mM H_3PO_4 and 22% CH_3CN/MeOH (1:1) for 47.5 mins, 16% 20mM H_3PO_4 and 84% CH_3CN/MeOH (1:1) for 78 mins, and lastly 78% 20mM H_3PO_4 and 22% CH_3CN/MeOH (1:1) for 47.5 mins. The eluent was

monitored by UV/VIS multiwavelenght detector (MD-2010/2015) from 220-450 nm measured spectra. Each flavonoid was quantified by co-chromotography with authentic standards and converted to concentration as mg g^{-1} dry weight.

2.3 RNA extraction

The total RNA extraction was extracted using phenol-chloroform, modified from the published method (38) as previously described in chapter I. Total RNA was purified by RNeasy Mini Kit (Qiagen, Hilden, Germany) with manufacturer's instructions.

2.4 cDNA synthesis and real time PCR analysis

To synthesize cDNA, the reverse transcription reactions were performed as previously described in chapter I. qRT-PCR was performed in the three replications. TaqMan MGB probes and the set of primers for *CitCHS1*, *CitCHS2*, *CitCHI*, and *CitFNS* have been previously described (42). A gene expression analysis was performed as previously described in chapter I.

2.5 Statistical analysis

Data were shown in the present study as the mean ± standard error for three replications. Statistical differences among temperature levels were evaluated with Tukey's HSD test at $P < 0.05$. Calculations were performed using JMP software (SAS Institute, Cary, NC).

3. Results

3.1 Flavonoid accumulation in citrus juice sacs at different temperatures *in vitro*

To understand the effects of temperature on flavonoid accumulation, the flavonoid quantification in citrus juice sacs cultured at different temperatures (10°C, 20°C, and 30°C) were investigated in the three citrus species. Juice sacs were culture under the same condition at 20 °C for two weeks and then cultured at 10°C, 20°C, and 30°C for another two weeks. In the present study, juice sac cultured at 20°C for four weeks were used as the control. As shown in Fig. 10-12, flavanone and flavone were observed at different levels among the three citrus species. In Satsuma mandarin, a large amount of flavonoids accumulated in juice sacs, with flavanone being identified as a major flavonoid (Fig. 10). In Lisbon lemon, the total flavonoid content was observed in a smaller amount than that of Satsuma mandarin, and flavanone predominantly accumulated in juice sacs (Fig. 11). In Valencia orange, the total flavonoid content was the lowest among the three citrus species, and flavonone was the major flavonoids accumulated in juice sacs (Fig.12).

The changes in flavonoid content in response to different temperatures showed that its accumulation was significantly induced at 10°C in juice sacs of the three citrus species (Fig. 10-12). Temperature treatment at 10°C significantly increased the total flavonoid content in Satsuma mandarin (0.8-fold), Lisbon lemon (0.8-fold), and Valencia orange (0.7-fold) by inducing the accumulation of flavanone in the juice sacs of the three citrus species. In contrast to 10°C, the temperature treatment at 30°C did not induce flavonoid accumulation in juice sacs of the three citrus species. In Satsuma mandarin, the total flavonoid content was not markedly affected, but

the slightly increased flavone content was detected at 30°C (Fig. 10). In Lisbon lemon, temperature treatment at 30°C did not induce the accumulation of flavanone and flavone, and the total flavonoid content consequently remained unchanged at 30°C (Fig. 11). In Valencia orange, the slightly decreased flavone content was detected, but the total flavonoid content remained unchanged at 30°C (Fig. 12).

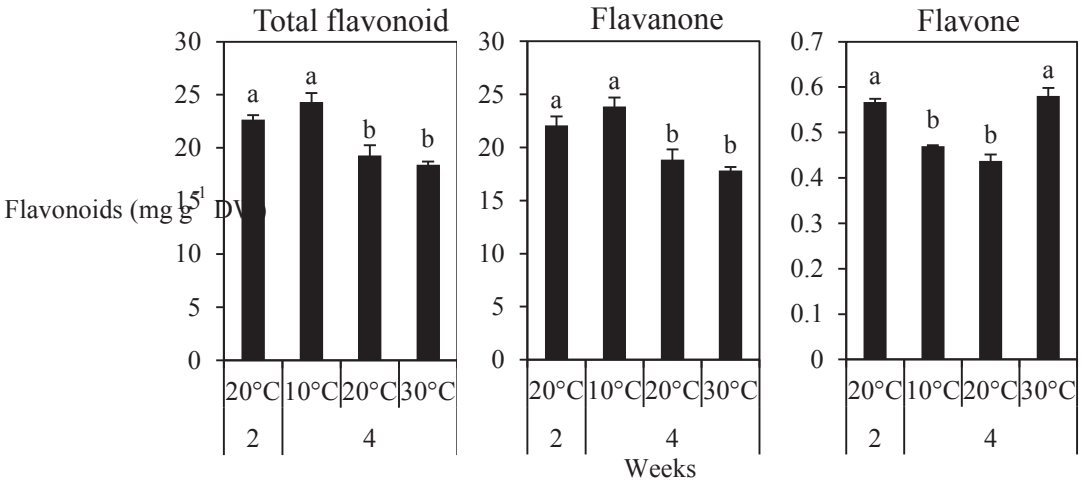

Fig. 10 flavonoid content and composition in citrus juice sacs *in vitro* of the Satsuma mandarin at different temperatures. The total carotenoid was the sum of identified carotenoid contents. The data shown are the mean ± standard error in the three replications. Tukey's HSD test was performed by JMP software (SAS Institute, Cary, NC) at $P < 0.05$. The means indicated by the same letter was not significant different.

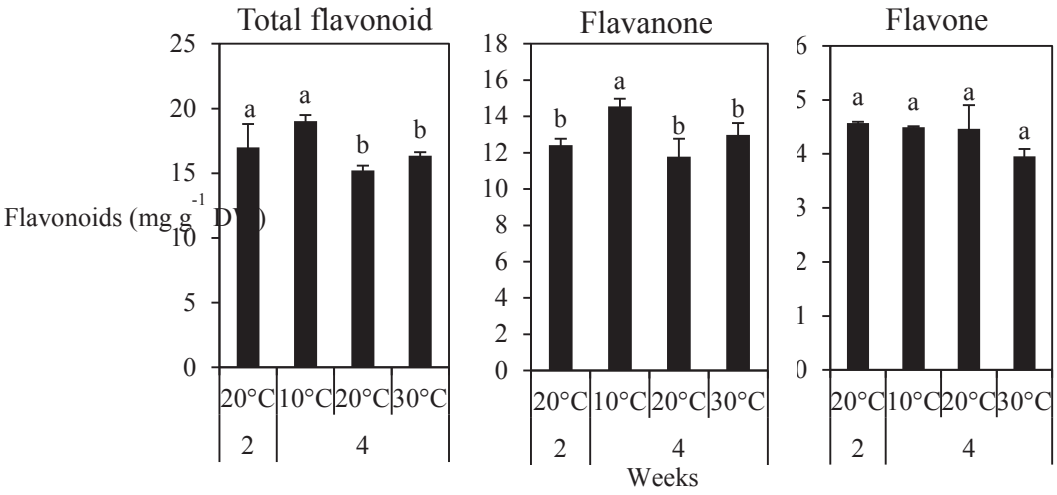

Fig. 11 flavonoid content and composition in citrus juice sacs *in vitro* of the Lisbon lemon at different temperatures. The total carotenoid was the sum of identified carotenoid contents. The data shown are the mean ± standard error in the three replications. Tukey's HSD test was performed by JMP software (SAS Institute, Cary, NC) at $P < 0.05$. The means indicated by the same letter was not significant different.

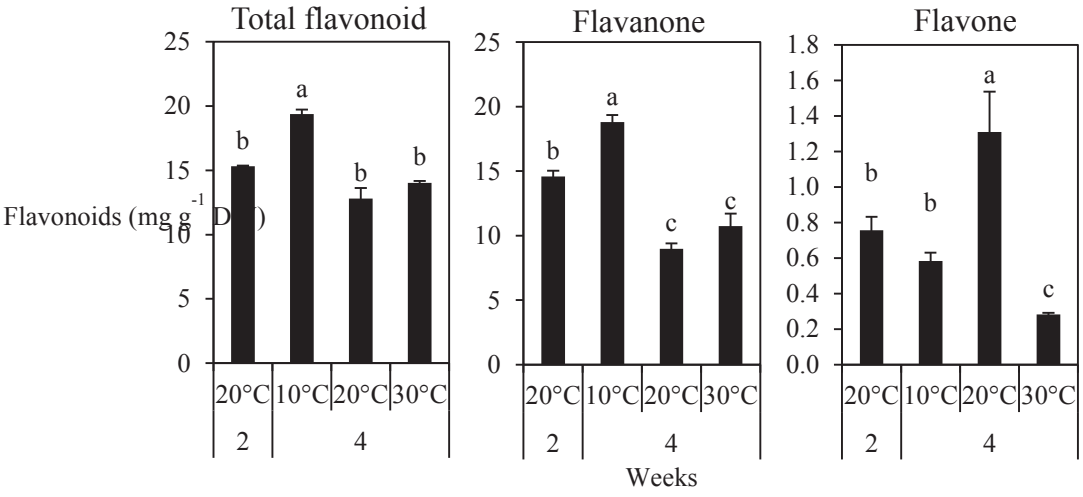

Fig. 12 flavonoid content and composition in citrus juice sacs *in vitro* of the Valencia orange at different temperatures. The total carotenoid was the sum of identified carotenoid contents. The data shown are the mean ± standard error in the three replications. Tukey's HSD test was performed by JMP software (SAS Institute, Cary, NC) at $P < 0.05$. The means indicated by the same letter was not significant different.

3.2 The expression of genes involved in flavonoid biosynthesis pathway in citrus juice sacs at different temperatures *in vitro*

To clarify the regulation of flavonoid accumulation in response to temperature, the changes in the expression of genes related to flavonoid biosynthesis pathway (*CitCHS1*, *CitCHS2*, *CitCHI*, and *CitFNS*) were investigated at different temperatures (10, 20, and 30°C) in citrus juice sacs *in vitro*. In Satsuma mandarin and Lisbon lemon, the expression of *CitCHS1*, *CitCHS2*, *CitCHI*, and *CitFNS* was obviously up-regulated at 10°C (Fig. 13-14). In Valencia orange, the expression of *CitCHS1* and *CitCHI* genes was up-regulated, while the expression of *CitCHS2* and *CitFNS* genes was unchanged at 10°C (Fig. 15). In contrast to 10°C, the expression of flavonoid biosynthetic genes was fluctuated at 30°C in the three citrus species. In Satsuma mandarin, the expression of *CitCHS2* gene was slightly increased, whereas the expression of other biosynthetic genes was unchanged or down-regulated at 30°C (Fig. 13). In Lisbon lemon, the expression of four biosynthetic genes was obviously down-regulated at 30°C (Fig. 14). In Valencia orange, the expression of *CitCHS2* was up-regulated, but the expression of other biosynthetic genes was down-regulated at 30°C (Fig. 15).

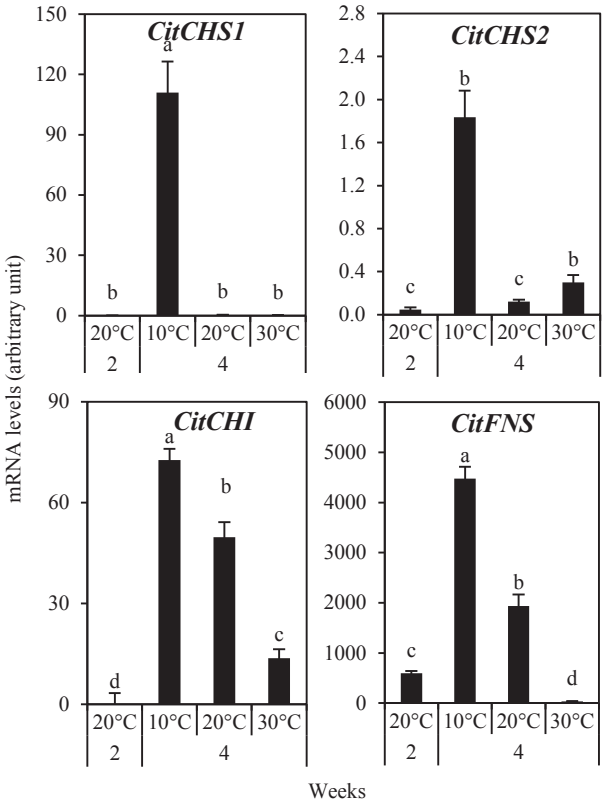

Fig. 13 The expression of genes involved in flavonoid biosynthesis pathway in Satsuma mandarin at different temperatures *in vitro*. The data shown are the mean ± standard error in the three replications. Tukey's HSD test was performed by JMP software (SAS Institute, Cary, NC) at $P < 0.05$. The means indicated by the same letter was not significant different.

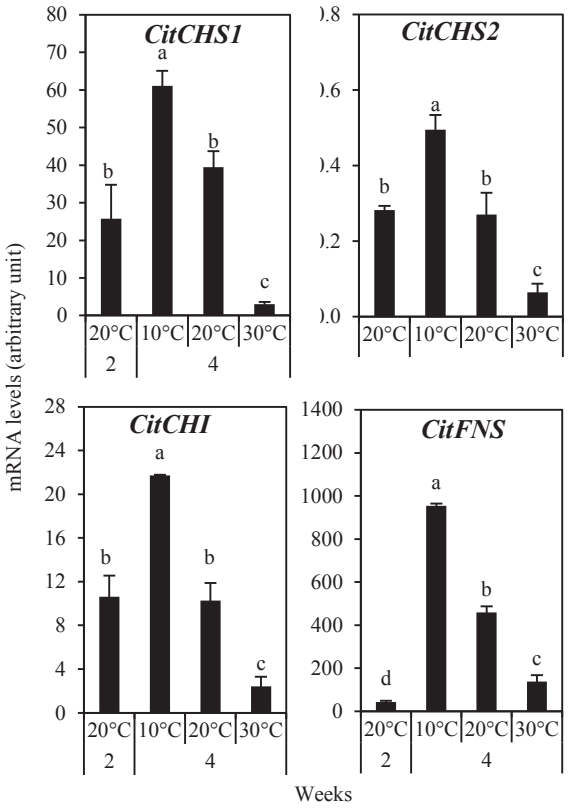

Fig. 14 The expression of genes involved in flavonoid biosynthesis pathway in Lisbon lemon at different temperatures *in vitro*. The data shown are the mean ± standard error in the three replications. Tukey's HSD test was performed by JMP software (SAS Institute, Cary, NC) at $P < 0.05$. The means indicated by the same letter was not significant different.

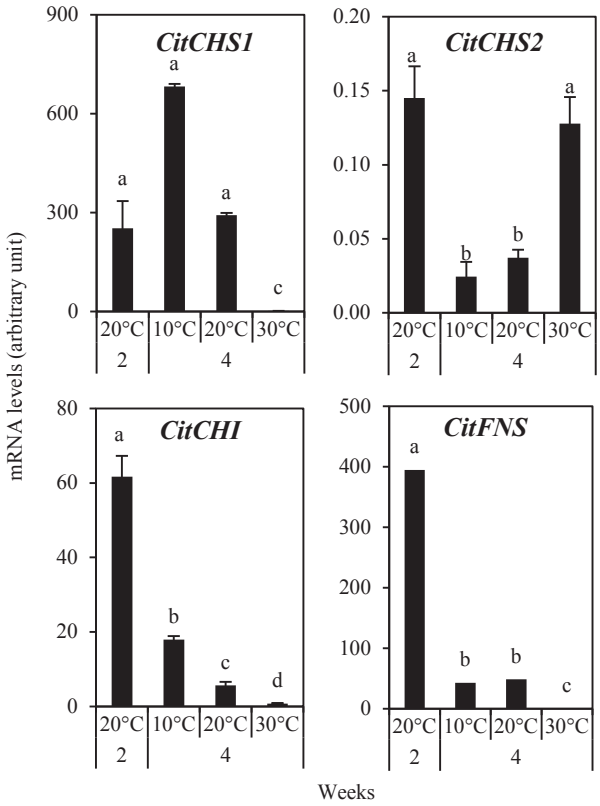

Fig. 15 The expression of genes involved in flavonoid biosynthesis pathway in Valencia orange at different temperatures *in vitro*. The data shown are the mean ± standard error in the three replications. Tukey's HSD test was performed by JMP software (SAS Institute, Cary, NC) at $P < 0.05$. The means indicated by the same letter was not significant different.

4. Discussion

The accumulation of flavonoid in response to environmental factors, especially temperature, has been received much attention for a long time. The sensitivity of flavonoid accumulation to temperature changes was observed in several kinds of fruits, vegetables, and flowers (16, 23, 40, 83). The study of citrus flavonoid is a great attraction because citrus fruits contain an abundance of flavonoid, especially flavanone. However, a little is known about the regulation of flavonoid in response to temperature. In the present study, flavonoid accumulation and the expression of flavonoid biosynthetic genes were examined at different temperatures in the citrus juice sacs *in vitro*. It was observed that the accumulation of flavonoid was induced at 10°C, but its accumulation remained unchanged at 30°C in juice sacs of the three citrus species. The previous finding suggested that 10°C was an optimal temperature for inducing the biosynthesis of phenolic acid, flavonoid, and anthocyanin. their contents were decreased with the higher temperature (116). High temperature influenced the biosynthesis, accumulation, and degradation which determined the quantity of flavonoid in plants (46). The high temperature at 30°C reduced the biosynthesis of flavonoid to less than half of that in low temperature (8). In grape berry, it was reported that the coloration of grape skin was delayed at high temperature (17). The mechanism of grape skin coloration was inhibited at temperature range from 30°C to 35°C by reduced the expression of flavonoid biosynthetic genes (45). In floriculture plants, a poor coloration under high temperature at 30-40°C was also wildly described. The decrease in flavonoid content was caused by a reduction in gene expression and enzyme activity in flavonoid biosynthesis pathway (23, 83). The previous finding reported that plants grown in the cold

weather can maintain higher photosynthetic rate than plant grown in the warm weather, and they can produce more energy for secondary metabolites synthesis (60).

The molecular study indicated that the accumulation of flavonoid in plants was transcriptionally regulated by flavonoid biosynthetic genes and its accumulation could be induced by a number of environmental stimulus (112). In citrus fruits, some of genes encoded enzymes related to flavonoid biosynthesis was previously isolated. It was found that the accumulation of flavonoid during citrus fruit development was highly regulated by the gene expression and enzyme activity involved in flavonoid biosynthesis pathway (76, 109). In the present study, to elucidate flavonoid regulation in response to temperature, the expression of four structural genes encoding enzymes that directly involved in flavonoid biosynthesis (*CitCHS1*, *CitCHS2*, *CitCHI*, and *CitFNS*) was investigated in citrus juice sacs *in vitro*. The results found that the changes in the expression of flavonoid biosynthetic genes were correlated with the flavonoid accumulation at 10°C in juice sacs of the three citrus species. The up-regulated expression of *CitCHS1, CitCHS2, CitCHI,* and *CitFNS* genes in Satsuma mandarin and Lisbon lemon and the up-regulated expression of *CitCHS1* and *CitCHI* in Valencia orange were observed at 10°C. The up-regulated expression of those biosynthetic genes was correlated with an increase in flavonoid content at 10°C in juice sacs of the three citrus species. The present results were similar to those in the previous studies in grape that the temperature affected flavonoid and anthocyanin accumulation in grape skin through the regulation of flavonoid biosynthetic genes (4, 74, 75, 117). It revealed that low temperature during grape berry maturation increased anthocyanin content by induced the transcript level of flavonoid biosynthetic genes, but high temperature decreased anthocyanin content by suppressed the transcript level of those biosynthetic genes and increased flavonoid degradation. In the present

study, it might be indicated that the increase in flavonoid content in citrus juice sacs at 10°C was transcriptionally regulated by the expression of flavonoid biosynthetic genes. In several plants, CHS and CHI was proposed as key enzymes that directly participated in the formation of flavonoid (77). It was reported that CHS and CHI were the entry point of flavonoid pathway, and they could be made up the regulating factor of flavonoid biosynthesis (109). The genetic engineering of those two structural genes has been extensively used to modify flavonoid content in plants. In many kinds of flowers, CHS was one of interesting targets for genetic engineering in modifying flower color (58, 81, 119). In petunia, the overexpression of Freesia CHS1 gene in petunia increased the total amount of flavonoid, and induced the alteration of flower color from white to pink (101). In tomato, the over-expression of petunia CHI gene or onion CHI gene in tomatoes resulted in an increased in total flavonoid content in tomato fruit (59, 80). In the present study, the expression of *CitCHS1* and *CitCHI* was simultaneously increased along with the flavonoid content at 10ºC in the three citrus species. The expression of *CitCHS1* and *CitCHI* genes appeared to be critical to regulate flavonoid accumulation in response to low temperature in the three citrus species. It might be suggested that the multi-enzyme complexes were involved in the synthesis of flavonoid. The increase in the expression level of a specific gene related to flavonoid biosynthesis pathway could led to increase the level of specific flavonoid, but it was probably not enable to increase total flavonoid content(113). By the up-regulated expression of several flavonoid biosynthetic genes at low temperature, the flux through the biosynthetic pathway was induced, and led to increase total flavonoid content in citrus.

In contrast to 10°C, flavonoid accumulation was not significantly affected, but the marked changes in flavonoid biosynthetic gene expressions were observed at 30°C in the three citrus species. In Satsuma mandarin and Valencia orange, the expression of *CitCHS2* genes was

up-regulated, but the expression of other biosynthetic genes was down-regulated at 30°C. In Lisbon lemon, the down-regulated expression of four flavonoid biosynthetic genes was observed at 30°C. The expression levels of those flavonoid biosynthetic genes were not corresponding to the unchanged flavonoid level in the three citrus species. These results might be indicated that other mechanisms might be participated in regulating flavonoid accumulation in response to high temperature, such as the involvement of phenylpropanoid pathway that supply a precursor to flavonoid pathway, or the enzymatic activities that varied among different temperatures. The further study will be required to fully clarify the regulatory mechanism of flavonoid accumulation in response to high temperature.

In conclusion, the temperature treatment at 10°C effectively induced the accumulation of flavonoid by the up-regulated expression of flavonoid biosynthetic genes in juice sacs of the three citrus species. The transcriptional regulation by flavonoid biosynthetic genes was a major mechanism responsible for the enhancement of flavonoid accumulation at 10°C in the three citrus species. In contrast to 10°C, the changes in the expression of the flavonoid biosynthetic genes were observed, but the flavonoid accumulation was not significantly affected at 30°C in the three citrus species. The understanding in flavonoid regulation in response to different temperatures during the fruit ripening process will be helpful for enhancing flavonoid content and make an approach to molecular breeding of flavonoid biosynthetic pathway in citrus fruits in future research.

CHAPTER 3

Effect of temperature on carotenoid metabolism in citrus juice sac *in vitro*

1. Introduction

The diversities of carotenoid content and composition were observed among different citrus species (3, 28, 71). Beside genetic factors, a number of evidences indicated that environment was one of the most important factors to regulate carotenoid accumulation in higher plants (48, 52, 68). Our previous study found that the accumulation of carotenoid was induced by blue LED light in citrus juice sacs *in vitro* of Satsuma mandarin, Valencia orange, and Lisbon lemon (123).

Temperature is known to be one of the most important environmental factors affecting the accumulation of bioactive compounds in higher plants (48, 52, 68, 123). The previous studies suggested that temperature showed a great effect on carotenoid accumulation in plants, such as pepper, tomato, and including citrus (9, 68, 85). In citrus fruits, the effects of temperature on carotenoid accumulation have attracted attention for a long time. However, the underlying regulatory mechanisms have not yet been clearly understood (51, 72, 100). The previous finding found that the biosynthesis of carotenoid during fruit development was found to be strongly influenced by temperature both in the peel and pulp (3, 37, 85). In lemon, it was reported that the color change in the peel of lemon fruits was induced by low field temperature (67, 68). In contrast to low temperature, citrus fruits did not normally accumulate carotenoid and produced

greenish-pale color fruits under high field temperature (33, 37, 41, 102). In addition, variations in the content of carotenoids in citrus pulp were also observed among different cultivated regions. The relatively low day/night temperature in Mediterranean was found to be optimum for carotenoid synthesis, whereas the higher temperature in tropical regions inhibited carotenoid synthesis in citrus fruits (24, 78, 79). These finding suggested that the mechanism underlying the biosynthesis and accumulation of carotenoid in citrus fruits are sensitive to temperature.

2. Materials and methods

2.1 Plant materials and temperature treatments

The three citrus species with different AsA, flavonoid, and carotenoid contents (Satsuma mandarin, Valencia orange, and Lisbon lemon) were randomly harvested from citrus trees.

The *in vitro* culture system was performed in accordance with a previously described in chapter 1. Citrus juice sacs were cultured at 20°C during the first two weeks. They were then exposed to different temperatures of 10°C, 20°C, and 30°C for another two weeks in the culture system. Juice sacs were sampled twice, at the second week and the fourth weeks, in culture system. They were immediately frozen in liquid nitrogen and stored at -80°C until use in the three experiments.

2.2 Carotenoid extraction and quantification

Carotenoid content was measured by HPLC in the three replications with the method described in the previous study (43). The content of β-carotene, β-cryptoxanthin, all-*trans*-violaxanthin, 9-*cis*-violaxanthin, and lutein were investigated in the juice sacs *in vitro* of the three citrus species. Juice sac samples were homogenized with extraction solvent (hexane-acetone-ethanol, 50:25:25, v/v/v) containing magnesium carbonate basic and centrifuged at 4,000 rpm for 20 mins. The supernatant containing hexane and the pigment was evaporated to dryness. The dry sample was re-suspended in diethyl ether containing 0.1% (w/v) butylated hydroxytoluene (BHT) and sponified overnight using 20% (w/v) methanolic KOH. After

sponification, NaCl-saturated water was added to eliminate water soluble compounds. Anhydrous Na_2SO_4 was added to eliminate residual water from the extract. Retained carotenoids were eluted from anhydrous Na_2SO_4 by diethyl ether, and diethyl ether was subsequently evaporated to dryness. Carotenoid residues were then dissolved in TBME : methanol (1:1, v/v) containing 0.5% (w/v) BHT.

An aliquot (20 µL) was injected into HPLC with a reverse-phase HPLC system (Jasco) fit with a YMC Carotenoid S-5 column of 250 × 4.6-mm-i.d. (Waters, Milford, MA) at a flow rate of 1 mL min^{-1}. The eluent was monitored by a photodiode array detector (MD-910, Jasco). In order to assess carotenoids in the samples, three different gradient elution schedules were used according to a previously described method (Kato *et al*., 2004). Peaks were identified by comparing their retention times and absorption spectra with authentic standards and converted to concentration as µg g^{-1} fresh weight.

2.3 RNA extraction

The total RNA extraction was extracted using phenol-chloroform, modified from the published method (38) as previously described in chapter I. Total RNA was purified by RNeasy Mini Kit (Qiagen, Hilden, Germany) with manufacturer's instructions.

2.4 Real time PCR analysis

To synthesize cDNA, the reverse transcription reactions were performed as previously described in chapter I. qRT-PCR was performed in the three replications. TaqMan MGB probes

and the set of primers for *CitPSY*, *CitPDS*, *CitZDS*, *CitLCYb1*, *CitLCYb2*, *CitLCYe*, *CitHYb*, *CitCYP97C* (*CitHYe*), *CitcZEP*, *CitVDE*, *CitNCED2*, and *CitNCED3* have already been descried previously (43, 44, 63, 66). A gene expression analysis was performed as previously described in chapter I.

2.5 Statistical analysis

Data were shown in the present study as the mean ± standard error for three replications. Statistical differences among temperature levels were evaluated with Tukey's HSD test at $P < 0.05$. Calculations were performed using JMP software (SAS Institute, Cary, NC).

3. Results

3.1 Carotenoid accumulation in citrus juice sacs at different temperatures *in vitro*

In order to evaluate the effects of temperature on carotenoid accumulation, an *in vitro* culture system of citrus juice sacs were performed. Juice sacs were cultured under the same condition at 20°C for two weeks and then cultured at different temperatures (10, 20, and 30°C) for another two weeks. Juice sacs cultured at 20°C for four weeks were used as the control in the present study. After juice sacs had been treated by different temperatures for two weeks, they were collected, and changes in carotenoid contents and compositions were examined by HPLC. As shown in Fig.16-18, carotenoid profiles differed among the three citrus species. In the Satsuma mandarin, a large amount of carotenoids accumulated in juice sacs, with β–cryptoxanthin being identified as a major carotenoid. In the Valencia orange, violaxanthin, particularly 9-*cis*-violaxanthin, predominantly accumulated in juice sacs. In Lisbon lemon, the total carotenoid content was markedly lower than that of Satsuma mandarin and Valencia orange. β–Cryptoxanthin and lutein were the major carotenoids accumulated in the juice sacs of Lisbon lemon.

An investigation of the effects of temperature on carotenoid accumulation revealed that its accumulation was induced at 10°C in the three citrus species examined (Fig. 16-18). In the Satsuma mandarin, the temperature treatment at 10°C induced the accumulation of carotenoids in the following compositions; all-*trans*-violaxanthin, 9-*cis*-violaxanthin, lutein, β-cryptoxanthin, and β-carotene, and as a result, the total carotenoid content was significantly increased (1.7-fold) at 10°C (Fig. 16). In the Valencia orange, the temperature treatment at 10°C significantly

increased the total carotenoid content (3-fold) by inducing the accumulation of 9-*cis*-violaxanthin and lutein (Fig. 17). Carotenoid contents at 10°C were lower in the Lisbon lemon than in the Satsuma mandarin and Valencia orange. The temperature treatment at 10°C slightly induced the accumulation of all-*trans*-violaxanthin, 9-*cis*-violaxanthin, and β-carotene, and, as a result, a slight increase in the total carotenoid content (1.5-fold) was observed at 10°C in the Lisbon lemon (Fig. 18).

In contrast to 10°C, the temperature treatment at 30°C did not promote carotenoid accumulation in the juice sacs of the three citrus species. In the Satsuma mandarin and Valencia orange, 30°C did not induce the accumulation of each carotenoid composition, consequently, the total carotenoid content was not affected at 30°C (Fig. 16 and Fig. 17). In the Lisbon lemon, only 9-*cis*-violaxanthin content was slightly increased, and a slight increase was observed in the total carotenoid content at 30°C (Fig. 18).

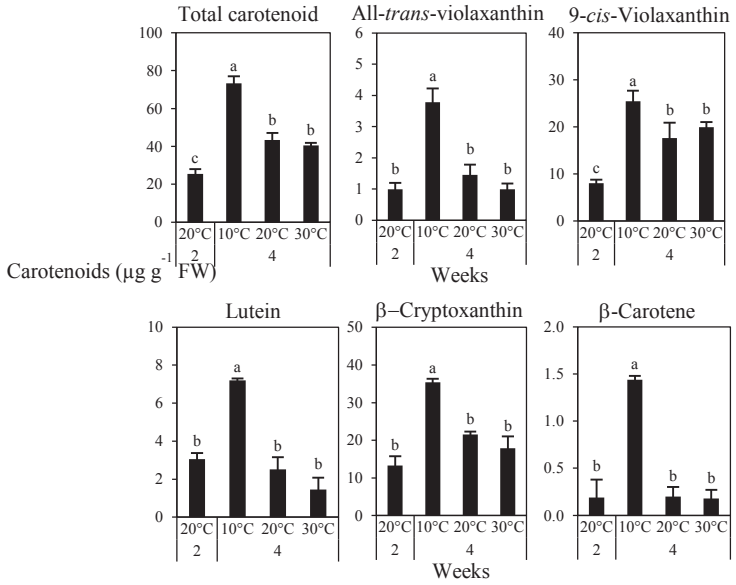

Fig. 16 Carotenoid content and composition in citrus juice sacs *in vitro* of the Satsuma mandarin at different temperatures. The total carotenoid was the sum of identified carotenoid contents. The data shown are the mean ± standard error in the three replications. Tukey's HSD test was performed by JMP software (SAS Institute, Cary, NC) at $P < 0.05$. The means indicated by the same letter was not significant different.

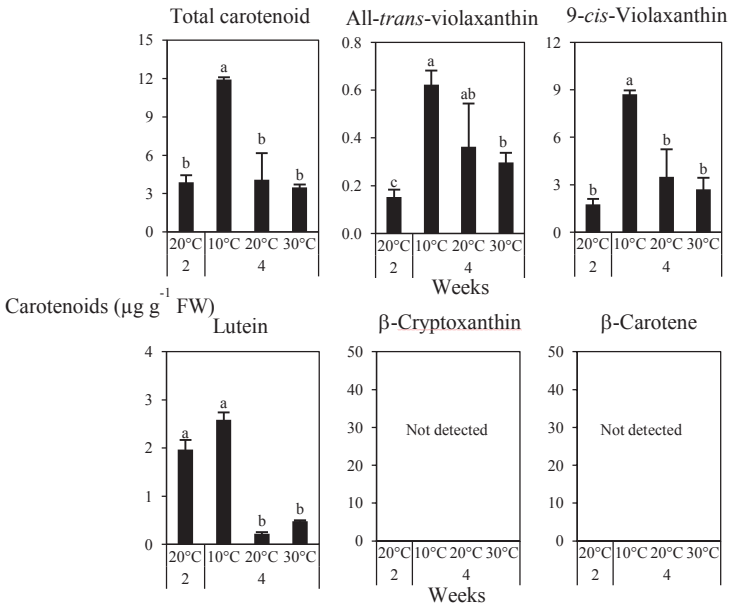

Fig. 17 Carotenoid content and composition in citrus juice sacs *in vitro* of the Valencia orange at different temperatures. The total carotenoid was the sum of identified carotenoid contents. The data shown are the mean ± standard error in the three replications. Tukey's HSD test was performed by JMP software (SAS Institute, Cary, NC) at $P < 0.05$. The means indicated by the same letter was not significant different.

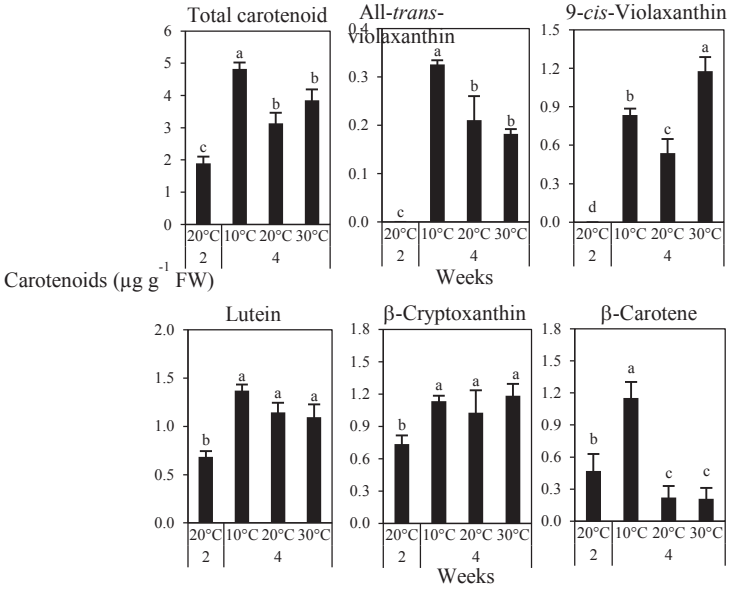

Fig. 18 Carotenoid content and composition in citrus juice sacs *in vitro* of the Lisbon lemon at different temperatures. The total carotenoid was the sum of identified carotenoid contents. The data shown are the mean ± standard error in the three replications. Tukey's HSD test was performed by JMP software (SAS Institute, Cary, NC) at $P < 0.05$. The means indicated by the same letter was not significant different.

3.2. The expression of genes involved in carotenoid metabolism pathway in citrus juice sacs at different temperatures *in vitro*

In order to characterize the regulatory mechanisms underlying carotenoid accumulation in response to temperature, the expression of genes involved in carotenoid metabolic pathway were investigated at different temperatures in citrus juice sacs *in vitro*. In the Satsuma mandarin, the expression of carotenoid biosynthetic genes (*CitPSY*, *CitPDS*, *CitZDS*, *CitLCYb2*, *CitLCYe*, *CitHYe*, *CitHYb*, *CitZEP*, and *CitVDE*) and carotenoid catabolic genes (*CitNCED2* and *CitNCED3*) was significantly increased at 10°C (Fig. 19). In the Valencia orange, the expression of carotenoid metabolic genes in response to low temperature was similar to that in the Satsuma mandarin. The temperature treatment at 10°C induced the expression of carotenoid biosynthetic genes (*CitPSY*, *CitPDS*, *CitZDS*, *CitLCYb1*, *CitLCYb2*, *CitZEP*, and *CitVDE*) and carotenoid catabolic genes (*CitNCED2* and *CitNCED3*) (Fig. 20). In Lisbon lemon, the expression of genes investigated in the present study was lower than in the Satsuma mandarin and Valencia orange. At 10°C, the expression levels of two carotenoid biosynthetic genes (*CitPSY* and *CitVDE*) were slightly higher than the control, whereas those of two catabolic genes (*CitNCED2* and *CitNCED3*) were markedly lower in the juice sacs of Lisbon lemon (Fig. 21).

In contrast to 10°C, the expression of carotenoid biosynthetic and carotenoid catabolic genes remained unchanged or was slightly decreased at 30°C in the juice sacs of the Satsuma mandarin (Fig. 19). Similarly, no marked increase was observed in the expression of carotenoid biosynthetic and catabolic genes at 30°C, except *CitLCYb1* in the juice sacs of the Valencia orange (Fig. 20). In Lisbon lemon, the expression of most carotenoid metabolic genes was markedly increased at 30°C, except *CitLCYe*, *CitHYe*, *CitZEP*, and *CitNCED3* (Fig. 21).

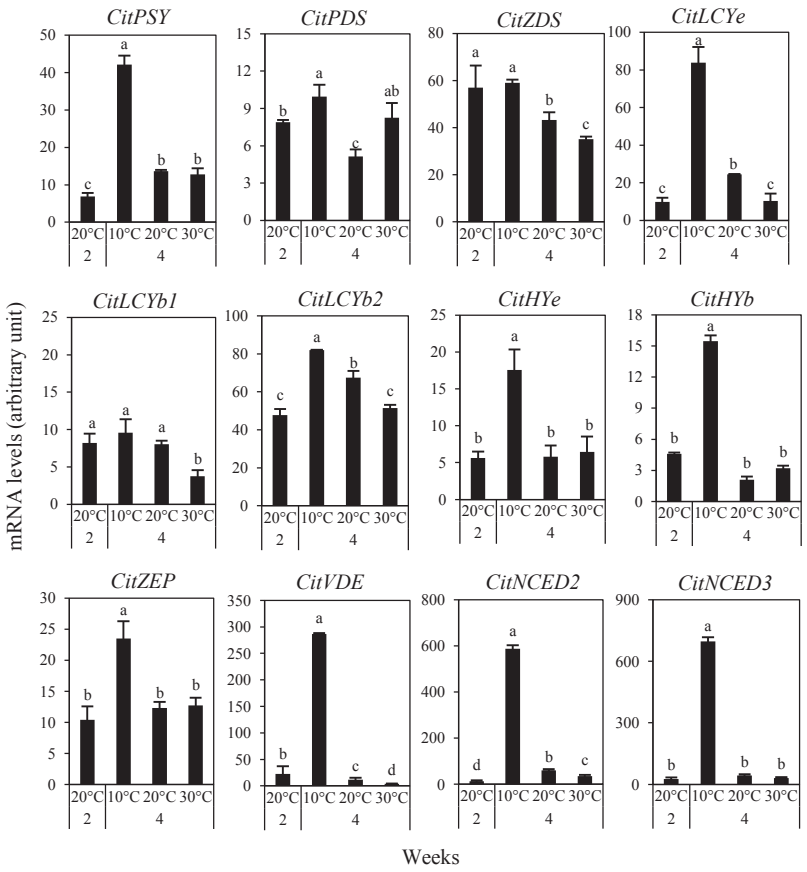

Fig. 19 The expression of carotenoid metabolic genes in citrus juice sacs *in vitro* of the Satsuma mandarin at different temperatures. The data shown are the mean ± standard error in the three replications. Tukey's HSD test was performed by JMP software (SAS Institute, Cary, NC) at $P < 0.05$. The means indicated by the same letter was not significant different.

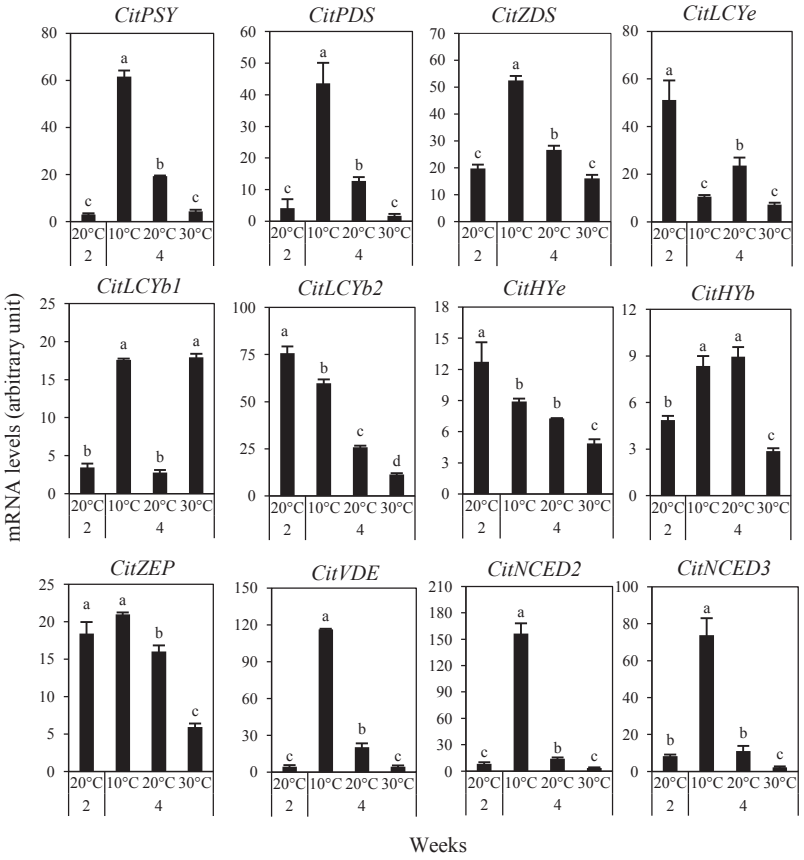

Fig. 20 The expression of carotenoid metabolic genes in citrus juice sacs *in vitro* of the Valencia orange at different temperatures. The data shown are the mean ± standard error in the three replications. Tukey's HSD test was performed by JMP software (SAS Institute, Cary, NC) at $P < 0.05$. The means indicated by the same letter was not significant different.

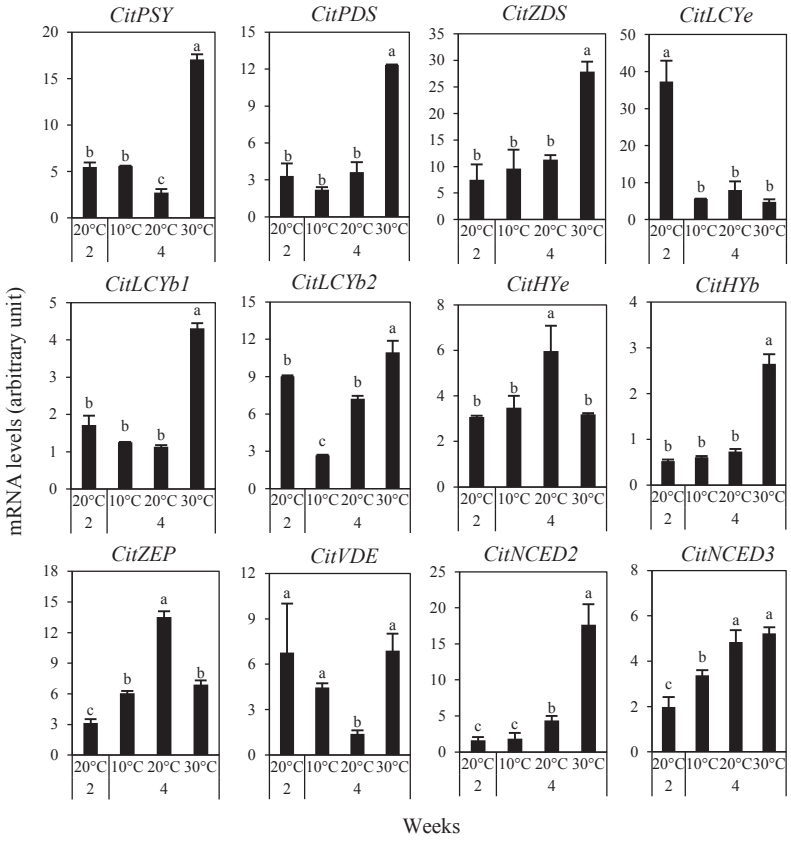

Fig. 21 The expression of carotenoid metabolic genes in citrus juice sacs *in vitro* of Lisbon lemon at different temperatures. The data shown are the mean ± standard error in the three replications. Tukey's HSD test was performed by JMP software (SAS Institute, Cary, NC) at $P < 0.05$. The means indicated by the same letter was not significant different.

4. Discussion

Temperature has been proposed as one of the major factors responsible for variations in carotenoid accumulation in fruits and vegetables, including citrus (37, 46, 104). Previous studies investigated the effects of postharvest temperatures on carotenoid accumulation in citrus fruits during storage in order to enhance fruit coloration (13, 71, 103). However, few studies have examined the effects of field temperatures on carotenoid accumulation during citrus fruit ripening on the tree. In the present study, an *in vitro* culture system was used to elucidate the regulation of carotenoid accumulation in response to different temperatures. The carotenoid content and expression of carotenoid metabolic genes were investigated at different temperatures (10, 20, and 30°C) in the three species of citrus juice sacs *in vitro*.

The results obtained revealed that low temperature at 10°C induced carotenoid accumulation in the juice sacs of the Satsuma mandarin, Valencia orange, and Lisbon lemon, whereas a high temperature at 30°C did not. Citrus fruits generally require relatively low temperature to synthesize carotenoid, and carotenoid biosynthesis was found to be inhibited at high temperature (24, 67, 68, 78). Temperature ranges between 8-15°C are considered to be optimal for synthesizing a number of carotenoid compositions, such as, β-carotene, β-cryptoxanthin, all-*trans*-violaxanthin, and 9-*cis*-violaxanthin (3).

In citrus fruits, the transcriptional regulation of carotenoid metabolic genes is considered as a major mechanism for regulating carotenoid accumulation during the fruit ripening process (3, 28, 43, 62, 65, 92). In order to elucidate the mechanisms responsible for the induction of carotenoid accumulation by temperature, the expression of carotenoid metabolic genes was investigated. The expression of nine carotenoid biosynthetic genes (*CitPSY*, *CitPDS*, *CitZDS*,

CitLCYe, *CitLCYb2*, *CitHYe*, *CitHYb*, *CitZEP*, and *CitVDE*) in the Satsuma mandarin and seven carotenoid biosynthetic genes (*CitPSY*, *CitPDS*, *CitZDS*, *CitLCYb1*, *CitLCYb2*, *CitZEP*, and *CitVDE*) in the Valencia orange was markedly up-regulated at 10°C. The transcriptional increase observed in these genes was in accordance with the higher level of each carotenoid composition and total carotenoid in the juice sacs of the Satsuma mandarin and Valencia orange at 10°C. The similar findings were previously found that the overexpression of carotenoid desaturase, isomerase, and cyclase genes induced the accumulation of each specific carotenoid and total carotenoid in tomato (93, 32). The stimulation of carotenoid biosynthetic gene expression by a low temperature at 10°C may have been critical for enhancing carotenoid content in the Satsuma mandarin and Valencia orange. Regarding carotenoid catabolic genes, the expression of *CitNCED2* and *CitNCED3* genes did not strongly correlate with carotenoid accumulation at 10°C in the Satsuma mandarin and Valencia orange. The group of carotenoid cleavage dioxygenases, particularly NCEDs, was identified as one of the important steps influencing the regulation carotenoid accumulation (95, 96). In citrus fruits, NCEDs enzymatically catalyze the oxidative cleavage of 9-*cis*-violaxanthin to xanthoxin and play an important role in regulating carotenoids catabolism and ABA synthesis at the transcriptional level (44). In the present study, the results obtained showed that 10°C markedly induced the up-regulation of *CitNCED2* and *CitNCED3*, but did not affect carotenoid levels in the Satsuma mandarin and Valencia orange. Variations in the enzymatic activity of carotenoid cleavage dioxygenase among different temperatures were previously studied. It was reported that carotenoid cleavage activity was constantly low at 10°C and catalytic activity was subsequently enhanced when temperature increased (6, 29). According to these findings, the differences between transcript level of *CitNCEDs* and carotenoid content in the present study indicated that other mechanisms, such as the temperature dependence of

enzymatic activity, were involved in the regulation of carotenoid catabolism at 10°C in these two citrus species. Therefore, in the Satsuma mandarin and Valencia orange, the mechanisms responsible for the increase in carotenoid content at 10°C was attributed to the up-regulated expression of several genes encoding key enzymes in the carotenoid biosynthetic pathway, which might induce the metabolic flux through the entire biosynthetic pathway.

Although the stimulation of carotenoid biosynthetic gene expression by a low temperature was observed in the Lisbon lemon, its gene expression level was still lower than those in the Satsuma mandarin and Valencia orange. Consequently, the juice sacs of the Lisbon lemon contained the lowest carotenoid content among the three citrus species. Regarding carotenoid biosynthetic genes, only the expression of *CitPSY* and *CitVDE* genes was slightly up-regulated in parallel with the higher carotenoid content at 10°C. The role of PSY as a rate-limiting step in the carotenoid biosynthetic pathway has been elucidated in many plants, including citrus fruits (14, 109, 123). PSY was proposed to transcriptionally control the metabolic flux of the carotenoid pathway, which is one of the important mechanisms influencing carotenoid synthesis and accumulation (54). Besides PSY, the expression of the VDE gene also affected the abundance of carotenoids in plants. Previous studies demonstrated that the transcript level of the VDE gene may be increased by biotic and abiotic stresses, which is an adaptive mechanism to protect plants against stress conditions (22, 50). These findings suggested that the expression of the *CitPSY* and *CitVDE* genes was transcriptionally responsive to low temperature, and the biosynthesis of carotenoid was consequently enhanced at 10°C in Lisbon lemon. Regarding carotenoid catabolic genes, the effects of a low temperature on the expression of *CitNCEDs* genes in the Lisbon lemon differed from those in other species. A significantly decrease in the expression of *CitNCED2* and *CitNCED3* genes was observed at 10°C. The

reduction in the oxidative cleavage of carotenoid by *CitNCEDs* contributed to the greater accumulation of carotenoid compositions and total carotenoid content at 10°C. The present results suggested that the transcriptional regulation between the up-regulated expression of biosynthetic gene (*CitPSY* and *CitVDE*) and the down-regulated expression of catabolic genes (*CitNCED2* and *CitNCED3*) was responsible for the induction of carotenoid accumulation at 10°C in the juice sacs of the Lisbon lemon.

In contrast to low temperature, the expression of carotenoid biosynthetic and catabolic genes was unchanged or slightly down-regulated, which was consistent with the constantly low carotenoid content at 30°C in the juice sacs of the Satsuma mandarin and Valencia orange. The regulation of carotenoid accumulation by high temperature was reported in a previous study on citrus fruits during storage at different temperatures. It was demonstrated that a post-harvest temperature at 30°C gradually down-regulated the expression of carotenoid biosynthetic genes along with the carotenoid content during storage, thereby contributing to the low carotenoid content in citrus fruits after storage at 30°C for three weeks (71). In the present study, the expression of most carotenoid biosynthetic genes was slightly down-regulated at 30°C in the Satsuma mandarin and Valencia orange. These results indicated that high temperature affected carotenoid accumulation in the Satsuma mandarin and Valencia orange at the transcriptional level by down-regulating or maintaining the expression of biosynthetic genes at low level at several steps of the carotenoid biosynthesis pathway. In the Lisbon lemon, the expression of carotenoid biosynthetic genes at 30°C did not correlate well with the accumulation of each carotenoid composition and total carotenoid. The expression of most carotenoid biosynthetic genes was up-regulated, whereas the carotenoid content remained unchanged at 30°C. Meanwhile, the expression of carotenoid catabolic genes (*CitNCED2*) was strongly up-regulated

at 30°C in Lisbon lemon. It was previously reported that the expression of the *CitNCED2* gene was primarily responsible for the oxidative cleavage of carotenoids to synthesize xanthoxin, a substrate for ABA, in lemon fruits (44). In the present study, the up-regulated expression of *CitNCED2* gene suggested an enhancement in the carotenoid catabolism rate that was responsible for the low carotenoid content at 30°C in the juice sac of the Lisbon lemon.

In conclusion, the present results indicated that the regulation of carotenoid accumulation in response to temperature differed among the three citrus species. In the Satsuma mandarin and Valencia orange, the induction of the expression of most carotenoid biosynthetic gene at 10°C contributed to increase the carotenoid content. In contrast, the expression of carotenoid biosynthetic and catabolic genes was unchanged or slightly decreased, as a result, the carotenoid content remained unchanged at 30°C in the juice sacs of the Satsuma mandarin and Valencia orange. In the Lisbon lemon, the expression of carotenoid biosynthetic and catabolic gene was found to be involved in the regulatory mechanisms underlying carotenoid accumulation in response to temperature. At 10°C, the up-regulated expression of carotenoid biosynthetic genes and down-regulated expression of carotenoid catabolic genes promoted a higher carotenoid content in the Lisbon lemon. In contrast to 10°C, the expression of carotenoid metabolic genes did not correlate well with carotenoid accumulation at 30°C in the Lisbon lemon. The simultaneously up-regulated expression of carotenoid biosynthetic and catabolic genes might be responsible for the unchanged carotenoid content at a low level at 30°C in the Lisbon lemon. The present results have provided further insights into the regulatory mechanisms of carotenoid accumulation in response to temperature, which might enable the improvement of carotenoid content in citrus fruit production.

CONCLUSION

Citrus is one of the most popular fruits worldwide due to the perfect combination of sweet and sour flavors. In addition to the delicious taste, the diverse health benefits of citrus consumption attract consumer's attention. The present of ascorbic acid, flavonoid, and carotenoid in citrus fruits is responsible for protective benefits against oxidative stress related illnesses. Therefore, the modification of those compounds has been actively researched since the past few years. In citrus fruits, the diversity of bioactive compounds was observed among different species and environmental conditions. Temperature was proposed as one of important factors affecting the accumulation of ascorbic acid, flavonoid, and carotenoid in citrus. In the present study, the accumulation of ascorbic acid, flavonoid, and carotenoid in response to different temperatures (10°C, 20°C, and 30°C) was investigated in citrus juice sacs *in vitro* (Valencia orange, Lisbon lemon, and Satsuma mandarin).

1. The effect of temperature on ascorbic acid accumulation in citrus juice sacs *in vitro*

Firstly, to elucidate the regulation of AsA in response to temperature, the three species of citrus juice sacs were cultured at different temperatures. The AsA accumulation was induced at 10°C in juice sacs of the three citrus varieties. The accumulation of AsA in response to temperature was regulated by the expression of genes involved in AsA biosynthetic, oxidation, and regeneration pathway. In Valencia orange and Satsuma mandarin, the higher expression of *CitVTC4* gene in biosynthetic pathway, and the higher expression of *CitMDAR1* and *CitMDAR2*

genes in regeneration pathway, together with the lower expression of *CitAO* gene in oxidation pathway contributed to the higher AsA level at 10°C. In Lisbon lemon, the higher expressions of *CitVTC1*, *CitVTC2*, and *CitVTC4* genes in biosynthetic pathway and the lower expression of *CitAO* gene in oxidation pathway contributed to the higher AsA level at 10°C. In contrast to 10°C, AsA accumulation was not significantly affected at 30°C as compared with the control at 20°C. In Valencia orange, an unchanged in most of AsA metabolic genes was responsible for maintaining AsA content at 30°C. In Lisbon lemon and Satsuma mandarin, the simultaneously increase in the biosynthetic and oxidation gene expressions might be attributed to maintain AsA content at 30°C.

2. The effect of temperature on flavonoid accumulation in citrus juice sacs *in vitro*

Secondly, in order to investigate the effect of temperature on flavonoid metabolism, the three species of citrus juice sacs were cultured at different temperatures. Flavonoid accumulation was induced at 10°C, but it was not significantly affected at 30°C in the juice sacs of the three citrus species. In Satsuma mandarin and Lisbon lemon, the increase in the expression of *CitCHS1, CitCHS2, CitCHI,* and *CitFNS* genes contributed to the higher flavonoid content at 10°C. In Valencia orange, the higher expression of *CitCHS1* and *CitCHI* genes contributed to the higher flavonoid content at 10°C. In contrast to 10°C, the changes in the expression of flavonoid biosynthetic genes were found, but the flavonoid accumulation was not significantly affected at 30°C in the three citrus species.

3. The effect of temperature on carotenoid metabolism in citrus juice sacs *in vitro*

Lastly, to clarify the regulation of carotenoid in response to temperature, the three species of citrus juice sacs were cultured at different temperatures. Carotenoid accumulation was induced at 10°C, but it was not affected at 30°C in the three citrus species. In the Satsuma mandarin and Valencia orange, carotenoid accumulation at 10°C was transcriptionally regulated by carotenoid biosynthetic genes. The up-regulated expression of nine carotenoid biosynthetic genes (*CitPSY*, *CitPDS*, *CitZDS*, *CitLCYe*, *CitHYb*, *CitHYe*, *CitLCYb2*, *CitZEP* and *CitVDE*) in the Satsuma mandarin and seven carotenoid biosynthetic genes (*CitPSY*, *CitPDS*, *CitZDS*, *CitLCYb1*, *CitLCYb2*, *CitZEP* and *CitVDE*) in the Valencia orange was responsible for enhancing the content of carotenoid at 10°C. In contrast to 10°C, no marked changes were observed in carotenoid metabolic gene expression, and carotenoid accumulation was not significantly affected at 30°C in the Satsuma mandarin and Valencia orange. In the Lisbon lemon, the expression of carotenoid biosynthetic and catabolic genes was involved in the regulation of carotenoid accumulation in response to temperature. The up-regulated expression of biosynthetic genes (*CitPSY* and *CitVDE*) and down-regulated expression of catabolic genes (*CitNCED2* and *CitNCED3*) increased the content of carotenoid at 10°C in the Lisbon lemon. In contrast to 10°C, the expression of most carotenoid biosynthetic and catabolic genes was simultaneously up-regulated, whereas carotenoid levels remained unchanged and were constantly low at 30°C in the Lisbon lemon.

In conclusion, the present results indicated that temperature treatment at 10°C effectively induced the accumulation of AsA, flavonoid, and carotenoid, but temperature treatment at 30°C

did not significantly affect the accumulation of those compounds in juice sacs of the three citrus species. The gene expression results indicated that the induction of those compounds by low temperature was transcriptionally regulated by the expression of genes involved in their metabolism pathway. The changes in the expression of those genes varied among the three citrus species in response to temperature. The present study provided more insight into the molecular basis of AsA, flavonoid, and carotenoid regulation in response to temperature in citrus fruits. This information in this doctoral thesis will contribute to improve effective strategies for producing high value-added citrus in the fruit production and storage technology in the future.

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