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Changes of Carotenoid Composition and Related gene Expression during Fruit Development in the Peel of 'Shiranuhi' Mandarin

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GRADUATE SCHOOL

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ABSTRACT

Plant carotenoids are red, orange, and yellow lipid-soluble pigments found in all chloroplasts. Their color masked by chlorophyll in photosynthetic tissues, but in late stages of plant development this pigments contribute to the bright colors of many flowers and fruit. Because color is one of the most important criteria in citrus products, understanding color development of fruits is necessary not only in quality control of products but also in breeding for improvement of fruit quality.

In this study the relationship between changes of carotenoid composition and gene expression related to carotenoid biosynthesis during fruit development was investigated in 'Shiranuhi' mandarin [(*Citrus. unshiu* Marcov x *C. sinensis* Osbeck) x *C. reticulata* Blanco]. The carotenoids in fruit peel at five different developmental stage were analyzed using HPLC and LC/MS. The genes involved in carotenoids biosynthetic pathway were cloned from fruit peel, leaf, and flower by PCR. The gene expression in the peel at each stage of fruit development was analyzed by real time quantitative RT–PCR and Northern blot.

Sixteen species of carotenoids were tentatively identified by UV absorption spectrum analysis for the fractions of fruit peel extracts separated by HPLC. Of the 16 putative carotenoids, seven compounds were identified to be lutein, -cryptoxanthin, -carotene, phytofluene, capsathin, zeaxanthin and -carotene,

respectively, by LC-MS. The composition of carotenoids in the peel was changed significantly during fruit development, especially at the stage of maturation. The number of carotenoid species identified in HPLC chromatogram was 5 in August, 5 in September, 7 in October, 14 in November and 16 in December, respectively. The levels of β-crypoxanthin, zeaxanthin, phytofulene, capsanthin and ζ-carotene in the peel were low at early stage of fruit development and increased with maturation. In contrary the levels of lutein and -carotene were high at early developmental stage and decreased with maturation.

Total number of 26 genes were cloned. They were glyceraldehyde 3 phosphate dehydrogenase, ent-copaly diphosphate synthase, geranylgeranyl pyrophosphate reductase, HMG CoA reductase, HMG CoA synthase, 1-deoxy-D-xylulose-5-phosphate synthase, 1-deoxy-D-xylulose-5-phosphate reductoisomerase, isopentenyl diphosphate isomerase, *FPP* synthase, squalene synthase, Sesquiterpene synthase 2, (+)-limonene synthase 2, (E)-beta-ocimene synthase, terpene synthase, Geranylgeranyl pyrophosphate synthase, phytoene synthase, phytoene desaturase, lycopen beat cyclase, lycopen epsilon cyclase, beta-carotene hydroxylase, epsilon-carotene hydroxylase, zeaxanthin epoxsidase, viiolaxanthin de-epoxidase, 9,10[9',10']carotenoid cleavage dioxygenase, capsanthin/capsorubin synthase, and tocopherol polyprenyltransferase genes

The expression levels of both *β-CarH* and *PSY* genes in the peel were low at early stage of fruit development and increased during maturation. The highest levels of

these genes were observed in November. In contrast, *ε-CarH* gene expression was high at early stage and decreased with fruit development. *DXPS* gene expression was higher at early stage, while *IPI* gene expression was higher at coloring stage. The simultaneous increases in the expression of *β-CarH* and *PSY* genes, which involved in β, β-xanthophyll synthesis seemed to result in the massive accumulations of β, β-xanthophylls, phytofluene and ζ-carotene in fruit peel during fruit ripening.

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Figure 29. Relationship between utein accumulation and *ε-CarH* expression.

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ABBREVIATION

ABA	abscisic acid
CarH-b	β-Carotene hydroxylase
CarH-e	ε-Carotene hydroxylase
CCS	capsanthin/capsorubin synthase
CrtISO	carotene isomerase
DDW	double distilled water
DEPC	diethylpyrocarbonate
DMAPP	Dimethylallyl diphosphate
DXP	1-deoxy-Dxylulose 5-phosphate
DXPS	1-Deoxy-D-Xylulose 5-Phosphate Synthase
DXR	1-Deoxy-D-Xylulose 5-Phosphate reductoisomerase
ESI	electrospray ionization
FPPS	farnesyl pyrophosphate synthase
G-3-P	glycerol-3-phosphate
GGPPS	geranylgeranyl pyrophosphate synthase
GPPS	geranyl pyrophosphate synthase
HMG-CoA	3-Hydroxy-3-methylgluatryl coenzyme A
HMGR	HMG-CoA reductase
HMGS	HMG-CoA synthase
HPLC	high-performance liquid chromatography
HPT	homogentisate phytyltransferase
IPI	Isopentenyl diphosphate isomerase
IPP	isopentenyl diphosphate
LC–MS	liquid chromatography–mass spectrometry
LCY-b	lycopene β-cyclase
LCY-e	lycopene ε-cyclase
MEP	C-methyl-D-erythritol 4-phosphate
MOPS	morpholinepropanesulfonic acid
MVA	acetate/mevalonate
MVK	MVA kinase
PCR	polymerase chain reaction
PDS	phytoene desaturase
PSY	phytoene synthase
RT-PCR	reverse-transcriptase-PCR
UV	ultraviolet
VDE	violaxanthin de-epoxidase
Vis	visible
ZDS	ζ-carotene desaturase
ZEP	zeaxanthin epoxidase

INTRODUCTION

In general, fruits have provided an important component of human and animal diets (Giovannoni, 2004), fruit development and ripening are unique to plants. Ripening of citrus fruit is accompanied by a series of biochemical changes including color, texture, and accumulation of sugars and reduction of acids. One of the particular features in coloration is accumulation of carotenoids and degradation of chlorophyll that leads to pigmentation of the orange color. Because color is one of the most important and complex attributes of citrus products and is largely due to the presence of diverse carotenoid pigments, considerable attention has been directed toward characterization of carotenoid pigments and their relationship with color development (Lee and Castle, 2001).

Plant carotenoids are red, orange, and yellow lipid-soluble pigments found embedded in chloroplasts and chromoplasts. Their color is masked by chlorophyll in photosynthetic tissues, but in late stages of fruit development the accumulation of these pigments contributes to the bright coloration of the tissues. Carotenoids protect photosynthetic organisms against potentially harmful photooxidative processes and are essential structural components of the photosynthetic antenna and reaction center complexes (Demmig-Adams *et al.*, 1996; Mayne, 1996; Sandmann, 2001). In plants, some of carotenoid compounds are precursors of abscisic acid (Heffron *et al.*, 2003), a phytohormone that modulates developmental and stress processes.

Carotenoids are derived from the isoprenoid pathway. All isoprenoids (terpenoids)

are synthesized through the condensation of isopentenyl diphosphate (IPP) and its allylic isomer, dimethylallyl diphosphate (DMAPP). A modular assembly process that produces compounds of 5, 10, 15, 20, or more carbons (in multiples of 5) allows the biosynthesis of the basic skeletons for the many and various isoprenoids with a relatively small number of basic reaction steps (Figure 1). In plant, terpenoids are synthesized via two IPP generating pathways, i.e. acetate-mevalonate (Ac-MVA) and non-mevalonate (non-MVA) pathways (Lichtenthaler, 1999; Rohmer, 1999).

The biosynthesis of terpenoids can be conveniently divided into four major processes. The first of which involves the conversion of acetyl-coenzyme A (CoA) to the "active isoprene unit," isopentenyl pyrophosphate (IPP). Second, the action of various transferases are generated from their precursors to form the higher order terpenoid building blocks, geranyl pyrophosphate (GPP; C_{10}), farnesyl pyrophosphate (FPP; C_{15}), and geranylgeranyl pyrophosphate GGPP; (C_{20}). The third, these branch point intermediates may then self-condense (to the C_{30} and C_{40} precursors of sterols and carotenoids, respectively), be utilized in alkylation reactions to provide prenyl side chains of a range of nonterpenoids (including proteins), or undergo internal addition (that is cyclization) to create the basic parent skeletons of the various terpenoid families. Finally, oxidation, reduction, isomerization, conjugation, or other secondary transformations elaborate the unique and manifold character of the terpenoids.

Prenylated proteins
Phytoalexins gibberellins tocopherols
Quinines taxol dolichols chlorophylls

carotenoids

Diterpenes ← GPP   GGPP   FPP → Hopnoids quinines
fragrances   Sterols triterpenes
                                        Prenylated proteins

Isoprene ← I PP → Rubber
cytokinins   quinoness

MVA

HMG

Pyruvate+G-3-P   HMG-CoA ← ← acetyl-CoA
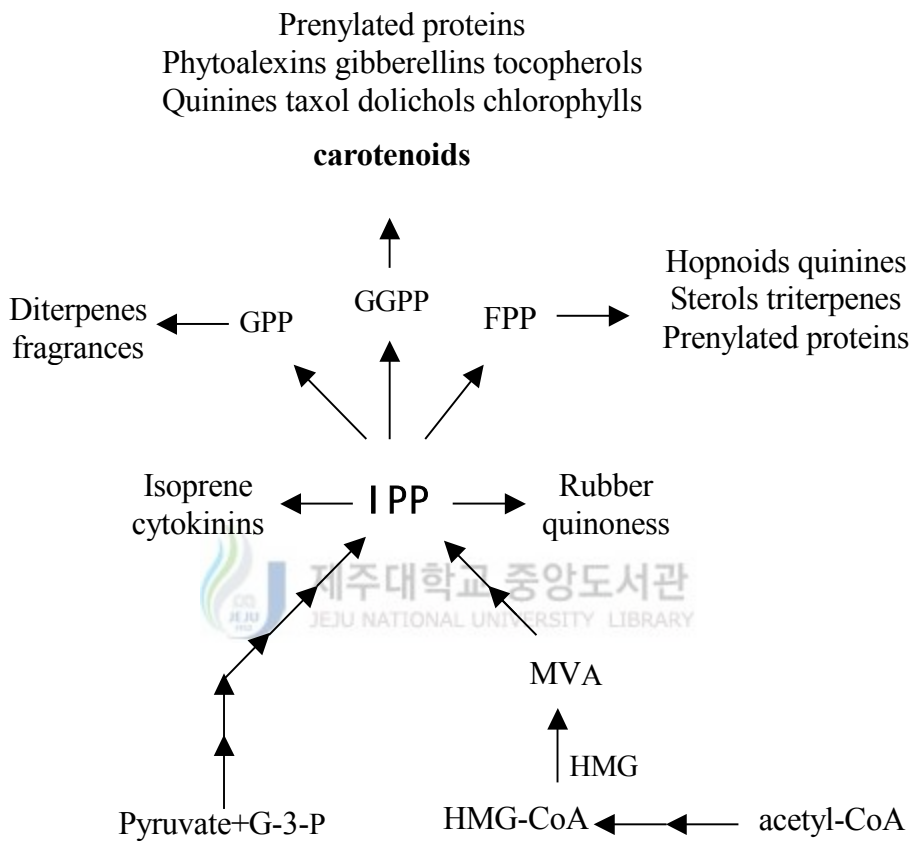
Figure 1. Isopentenyl pyrophosphate (IPP) serves the central metabolite leading to an immense variety of different isoprenoid compounds in plants.

Isoprenoid biosynthetic genes and enzyme   The classical Ac-MVA pathway involves condensation of three units of acetyl CoA to form 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA), which after reduction yields MVA. MVA is subsequently transformed to IPP via three sequential steps involving phosphorylation and decarboxylation (Figure 1)(Cunningham and Gantt, 1998).

HMG CoA synthase catalyses the condensation of acetyl-CoA and acetoacetyl-CoA to from HMG-CoA. The reduction of HMG-CoA to MVA is catalyzed by HMG-CoA reductase (HMGR), a key regulatory enzyme of this pathway that has been extensively studied (Rodwell *et al.*, 2000).

Pentyltransferase genes   There are many types of diphosphate synthaes which are classified based upon the length of the chain. The short chain prenyl diphosphate synthase consists of *GPP*-, *FP*P- and *GGPP*-synthase (Barkovich and Liao, 2001). GPP synthase gene has been cloned from pepper fruit (Kuntz *et al.*, 1992; Hugueney *et al.*, 1996a) and *Arabidopsis* (Bouvier *et al.*, 2000b). FPPS genes catalyzing next step were cloned from *Arabidopsis* (Delourme *et al.*, 1994), white lupine (Attucci *et al.*, 1995), pepper (Hugueney *et al.*, 1996b), guayule rubber (Pan *et al.*, 1996), and rubber tree (Adiwilaga and Kush, 1996). GGPP synthase gene was isolated from pepper (Kuntz *et al.*, 1992), *Arabidopsis* (Bartley *et al.*, 1994; Scolnik and Bartley, 1994), white lupine (Aitken *et al.*, 1995), and *Catharantus roseus* (Bonk *et al.*, 1997) and Brassica campestris (Lim *et al.*, 1996).

IPP biosynthesis   In mevalonate pathway key enzymes were HMG-CoA synthase (Montamat *et al.*, 1995), HMG-CoA reductase (Caelles *et al.*, 1989; Enjuto *et al.*, 1994), mevalonate kinase (Riou *et al.*, 1994), and mevalonate diphosphate

-4-

decarboxylase (Cordier *et al.*, 1999), which were cloned from *Arabidopsis*.

IPP pathway occurres in plastid which is mediated by two key step enzymes, 1-deoxy-D-xylulose 5-phosphate synthase (*DXPS*) (Estevez *et al.*, 2001b) and 1-deoxy-D-xylulose 5-phosphate reductoisomerase (Schwender *et al.*, 1999). DXPS gene was isolated from various plant species such as *M. piperita* (Lange *et al.*, 1998), *Capsicum annuum* (Bouvier *et al.*, 1998), and periwinkle (*Catharenthus roseus*) suspension cultures (Chahed K. *et al.*, 2000; Veau *et al.*, 2000).

Carotenoid and abscisic synthesis genes   Phytoene synthase, which catalyzes the first committed step in carotenoid biosynthesis, was first cloned from tomato (Bartley *et al.*, 1992). Subsequent desaturations to yield all-*trans*-lycopene are catalyzed by phytoene desaturase (Bartley *et al.*, 1991), ζ–carotene desaturase (Albrecht *et al.*, 1995), and carotenoid isomerase (Park *et al.*, 2002). Lycopene is cyclized on both ends to generate either -carotene (Pecker *et al.*, 1996) or -carotene (Cunningham *et al.*, 1996). Hydroxylation of -carotene by -carotene hydroxylase results in the formation of zeaxanthin (Sun *et al.*, 1996). Hydroxylation of -carotene, possibly by the action of the same -hydroxylase and an as yet unidentified -hydroxylase, produces lutein, the most abundant xanthophyll in plant plastids. Zeaxanthin epoxidase, which was first cloned from tobacco (Marin *et al.*, 1996) catalyzes the conversion of zeaxanthin to violaxanthin via antheraxanthin as an intermediate. Both violaxanthin and its derivative neoxanthin can be precursors for the carotenoid-derived plant hormone abscisic acid. In potato and tobacco, violaxanthin can be converted to neoxanthin by neoxanthin synthase, an enzyme with high homology to carotenoid cyclases and capsanthin/capsorubin synthase from

pepper (Al-Babili *et al.*, 2000; Bouvier *et al.*, 2000a). However, in *A. thaliana*, no additional candidate gene, besides the already characterized carotenoid cyclases, is detectable with reasonable sequence homology, indicating either that a neoxanthin synthase activity may not exist in this organism or violaxanthin is the precursor for ABA biosynthesis. Thus far, two further enzymes involved in the breakdown of xanthophylls to ABA have been cloned: genes for epoxy-carotenoid (neoxanthin) cleavage enzyme (Schwartz *et al.*, 1997) occur as a family of seven members in the *A. thaliana* genome, whereas abscisic aldehyde oxidase (Seo *et al.*, 2000) is encoded by a single-copy gene (Table 1).

Table 1. Genes involved in carotenoid biosynthesis of plants

Enzyme	Plant	Gene	DNA clone Type	Accession No.	Refereneces
1-deoxy-D-xylulose-5-phosphate synthases(DXPS)	*Arabidopsis* Pepper peppermint Periwinkle Tomato	*CLa1* *Tkt2* *Tkt* *Dxs* *Dxs*	cDNA cDNA cDNA cDNA cDNA	W43562 Y15782 AF019383 AJ011840 AF143812	Mandel et al. 1996 Bouvier et al. 1998 Lange et al. 1998 Chahed et al. 2000 Lois et al. 2000
1-deoxy-D-xylulose-5-phosphate reduct isomerase(DXPR)	*Arabidopsis* peppermint Periwinkle Tomato	*Dxr(IspC)* *Dxr* *Dxr* *Dxr*	cDNA cDNA cDNA cDNA	AF148852 AF116825 AF250235 AF331705	Schwender et al. 1999 Lange and Croteau 1999[a] Veau et al. 2000 Rodriguez-Concepcion et al. 2001
4-Diphosphocytidyl-2C-methyl-D-erythritol synthase	Arabidopsis	*IspD*	cDNA	AF230737	Rohdich et al.2000[a]
4-Diphosphocytidyl-2C-methyl-D-erythritol kinase	*Arabidopsis* peppermint Tomato	*IspE* *Ipk* *IspE*	Putative orthologue cDNA cDNA	AF288615 AF179283 AF263101	Lange and Croteau 1999[b] Rohdich et al. 2000[b]
Isopentenyl pyrophosphate isomerase	*Arabidopsis* *Arabidopsis* tobacco Clarkia Clarkia C. xantiana Rice	*IPI1* *IPI2* *IPI1* *IPI2* *IPI1* *IPI2* *IPI1*	cDNA cDNA cDNA cDNA cDNA cDNA GenomicDNA	U48961 U43292 AF019383 U48963 X82627 U48962 D28222	Newman et al. 1994 Galichet et al. 2005 Blanc et al. 1996 Blanc et al. 1995 Blanc et al. 1996 Sasaki et al. 1994
Geranyl diphosphate synthase	*Arabidopsis* *V.vinifera* orange	*GPPS* *GPPS* *GPPS*	cDNA cDNA cDNA	CAC16849 AAR08151 CAC16851	Schwender et al. 1999 Oswald et al. 2003 Bouvier et al. 2000
Farnesyl pyrophosphate synthase	White lupine Pepper Rubber tree	*Fps* *Fps 1* *Fps* *Fps*	cDNA cDNA cDNA cDNA	X75789 U15777 X84695 Z49786	Delourme et al. 1994 Attucci et al. 1995 Hugueney et al 1996 Adiwilaga and Kush 1996
Geranylgeranyl pyrophosphate synthase	*Arabidopsis* *Arabidopsis* *Arabidopsis* *Arabidopsis* Pepper Periwinkle	*GGPS1* *GGPS2* *GGPS3* *GGPS4* *GGPS* *GGPS*	cDNA cDNA cDNA GenomicDNA cDNA cDNA	L25813 U44876 U44877 L22347 X80267 X92893	Scolnik and Bartley 1994[b] Scolnik and Bartley 1995[b] Scolnik and Bartley 1995[b] Bartley et al.1994 Kunzs et al. 1992 Bantignies et al. 1995[b]

Continued

Enzyme	Plant	Gene	DNA clone Type	Accession No.	Refereneces
Phytoene synthase	Peper	*Psy*	cDNA	X68017	Romer et al. 1993
	Tomato	*Psy 1*	cDNA	Y00521	Ray et al. 1987
	Tomato	*Psy 1*	cDNA	M84744	Bartley. et al. 1992
	Tomato	*Psy 1*	genomicDNA	X60441	Ray et al. 1992
	Tomato	*Psy 2*	cDNA	L23434	Bartley and Scolnik 1993
	Tomato	*Psy 2*	genomicDNA	X60440	Ray et al. 1992
	Arabidopsis	*Psy*	cDNA	L25812	Bartley and Scolnik 1994[b]
	daffodil	*Psy*	cDNA	X78814	Schledz et al. 1996
	Citrus sinensis	*Psy*	cDNA	AY669084	Tao et al. 2004
	Citrus clementina	*Psy*	cDNA	DQ109038	Distefano et al. 2005
	Citrus sinensis	*Psy*	cDNA	AY204550	Rodrigo et al. 2002c
Phytoene desaturase	Peper	*PDS*	cDNA	X68058	Hugueney et al. 1992
	Tomato	*PDS*	cDNA	M88683	Oswald,M.F.et al. 1993
	Arabidopsis	*PDS*	cDNA	L16237	Scolnik and Bartley 1993
	Maize	*PDS*	cDNA	L39266	Hable and Oishi 1995
	Soybeen	*PDS*	cDNA	M64704	Bartley 1991
	Citrus sinensis	*PDS*	cDNA	AY669082	Tao et al. 2004
	Citrus sinensis	*PDS*	cDNA	CAC85666	Marcos 2001
ζ- Carotene desaturase	*Arabidopsis*	Zds	cDNA	U38550	Scolnik and Bartley 1995[b]
	Maize	Zds	cDNA	AF047490	Luo and wurtzel 1999[b]
	Citrus sinensis	Zds	cDNA	AY675215	Hugueney,P., et al 1996
	Citrus sinensis	Zds	cDNA	AY669083	Tao et al. 2004
	Citrus sinensis	Zds	cDNA	CSI319762	Marcos et al. 2001
Lycopene-β-cyclase	Pepper	*Lcy-b*	cDNA	X86221	Hugueney et al. 1995
	Arabidopsis	*Lcy-b*	cDNA	U50739	Cunningham et al. 1996
	Tomato	*Lcy-b*	cDNA	X86452	Cunningham et al. 1996
	Tomato	*Cyc-b*	cDNA	AF254793	Ronen et al. 2000
	Tobacco	*Lcy-b*	cDNA	X81787	Cunningham et al. 1996c
	Daffodil	*Lcy-b*	cDNA	X98796	Al-Babili et al. 1996[b]
	Citrus sinensis	*Lcy-b*	cDNA	AF240787	Xu and Zhang 2001
	Citrus limon	*Lcy-b*	cDNA	AB114652	Kato et al. 2004
	Citrus unshiu	*Lcy-b*	cDNA	AB114652	Kato et al. 2004
Lcopene-ε- cyclase	*Arabidopsis*	*Lcy-e*	cDNA	U50738	Cunningham et al. 1996
	Tomato	*Lcy-e*	cDNA	Y14387	Ronen et al. 1998[b]
	Potato	*Lcy-e*	cDNA	AF321537	Cunningham and Gantt 2001
	Citrus maxima	*Lcy-e*	cDNA	AY994158	Hashimand Mat Amin 2005c
	Citrus sinensis	*Lcy-e*	cDNA	AF450280	Xu et al. 2001
	Citrus x paradisi	*Lcy-e*	cDNA	AF486650	Costa et al. 2002

Continued

Enzyme	Plant	Gene	DNA Type	clone Accession No.	Refereneces
-carotene hydroxylase	Pepper	*Bch*	cDNA	Y09225	Bouvier et al. 1998
	Pepper	*Bch 2*	cDNA	Y09722	Bouvier et al. 1998
	Arabidopsis	*Chyb1*	cDNA	U58919	Sun et al. 1996
	Tomato	*CrtR-b1*	cDNA	Y14809	Hirschberg 1998
	Tomato	*CrtR-b2*	cDNA	Y14810	Hirschberg 1998
	Citrus maxima		cDNA	DQ002893	Hashim et al. 2005[c]
	Citrus sinensis	*CHX 2*	cDNA	AY623047	Tao et al. 2004[c]
	Citrus unshiu		cDNA	AF315289	Kim et al. 2001[c]
ε-carotene hydroxylase	Pepper	*Zep*	cDNA	X91491	Bouvier et al. 1996
	Arabidopsis	*Zep*	cDNA	AF281655	Audran et al. 2000[c]
	Tobacco	*Zep*	cDNA	X95732	Marin et al 1996
	tomato	*Zep*	cDNA	Z83835	Burbidge et al. 1997
	Citrus sinensis	*Zep*	cDNA	AF437874	Xu et al. 2001
	Citrus unshiu	*Zep*	cDNA	AB114654	Kato et al. 2004
Zeaxanthin epoxidase	Pepper	*Zep*	cDNA	X91491	Bouvier et al. 1996
	Arabidopsis	*Zep*	cDNA	AF281655	Audran et al. 2000[c]
	Tobacco	*Zep*	cDNA	X95732	Marin et al 1996
	tomato	*Zep*	cDNA	Z83835	Burbidge et al. 1997
	Citrus sinensis	*Zep*	cDNA	AF437874	Xu et al. 2001
	Citrus unshiu	*Zep*	cDNA	AB114654	Kato et al. 2004
Violaxanthin de-epoxidase	Tobacco	*Vde1*	cDNA	U34817	Bugos et al. 1998
	Arabidopsis	*Vde1*	cDNA	U44133	Bugos et al. 1998
	Lettuce	*Vde1*	cDNA	U31462	Bugos and Yamamoto 1996[c]
	Citrus sinensis	*Vde1*	cDNA	AF444297	Xu et al. 2001
Capsanthin/capsorubin synthase	Pepper	*Ccs*	cDNA	X76165	Bouvier et al. 1994
	Orange	*Ccs*	cDNA	AF169241	Xu et al. 1999
neoxanthin cleavage enzyme	*Arabidopsis*	*Nc*	genomicDNA	BT002102	Nguyen et al. 2001
	Carrot	*CCD1*	cDNA	DQ192203	Just et al. 2005[c]
	Carrot	*CCD2*	cDNA	DQ192204	Just et al. 2005[c]
	Carrot	*CCD3*		DQ192205	Just et al. 2005[c]
	grape	*CCD1*		AY856353	Mathieu et al. 2005

[a]The sequences of genes cited in this table could be accessed in the GenBank database.
[b]The genes were only published on gene register of Physiology.
[c]The sequences of genes were directly submitted in the GenBank database.

Scope of this thesis   The characterization of mutants altered in the carotenoid biosynthetic pathway is a useful experimental system to identify molecular mechanisms regulating the process. This approach, however, is limited to a small number of plant species, mainly *Arabidopsis* and tomato (Hirschberg, 2001; Tian *et al.*, 2003).

In this study, composition of carotenoid compounds and the expression of carotenoid biosynthetic genes were investigated in the peel of 'Shiranuhi' mandarin during fruit development. The cDNA related to carotenoid biosynthetic pathways were cloned. Specific primers and degenerate primers were used in this study. Carotenoids were extracted from citrus peel and the compounds were analyzed by HPLC, molecular weight of these compounds was measured by LC/MS. Thus, identification of carotenoids was conducted in citrus peel of five developmental stages. Expression patterns of the carotenoid biosynthesis genes, such as *DXS*, IPP isomerase (*IPI*), phytoene synthase (*PSY*), -carotene hydroxylase, -carotene hydroxylase, neoxanthin cleavage dioxygenase (*CCD*) and violaxanthin de-epoxidase (*VDE*) were investigated by real time RT-PCR. This study was conducted to investigate to understand how these changes are related to the evolution of carotenoids composition in the peel of citrus 'Shiranuhi' mandarin during fruit ripening.

MATERIALS AND METHODS

Plant materials

'Shiranuhi' mandarins [(*Citrus unshiu* Marcov x *C. sinensis* Osbeck) x *C. reticulata* Blanco] grown under protected system at the Jeju Agriculture Research & Extension Service were used as materials. The fruit had been harvested periodically from August 2004 to January 2005 at intervals of one month. The leaves and flower of citrus were collected from flowering time and immature fruits were sampled from 20 days after flowering for gene cloning materials. Fruit peel was striped off from sample fruits, immediately frozen in liquid nitrogen. All samples were kept at -80 until use.

HPLC analysis

Extraction of crude carotenoids from fruit peel of 'Shiranuhi' mandarin was conducted as follows (Figure 2). Approximately 2 g of peel was ground using mortar and pestle with liquid nitrogen. The peel powder was extracted with 50 ml extraction solvent (Methanol: Ethyl acetate=1:1 containing 0.1% BHT). The mixture was stirred for 3 hours and filtered through a fitted glass funnel. Extract was filtered under suction. The residue was extracted two times with same extraction solvent indicated above.

The liquid chromatograph apparatus is consisted of a Thermo Separation Products (Table 2).

Table 2. Operation condition of HPLC for carotenoids analysis

Instrument:	P4000 gradient pump, UV6000LP detector, SCM1000 vacuum degasser, As1000 auto sampler with column oven
Column:	Waters PAH C_{18} 5μm 4.6x250mm Column
Mobile phase #A:	ACN:MeOH: Ethyl acetate(80:15:5, v/v)
Mobile phase #B :	ACN:MeOH: Ethyl acetate(60:25:15, v/v)
Gradient:	1% to 99% #A and 99% to 1% #B mobile phase with a linear for 0 to 60 min
Flow rate:	1mℓ/min
Wavelength:	450 nm
Other condition	- Sample inject : 20 μℓ
	- Equilibration time : 3.0 min
	- Run time : 60.0 min.
	- Column temperature: ambient

Peel 2g

⬇ Grinding on liquid nitrogen

Extraction

50ml of MeOH:EtOAC 1:1,v/v
containing 0.2% BHT,3h 3 time

Filteration

⬇

Evaporation

⬇ At 40

Disssolving in MeOH:EtOAC 1:1,v/v

⬇

Crude carotenoid solution

⬇

PTFE syringe filter

⬇

HPLC

⬇

Fractionation

⬇

Confirm fraction by HPLC
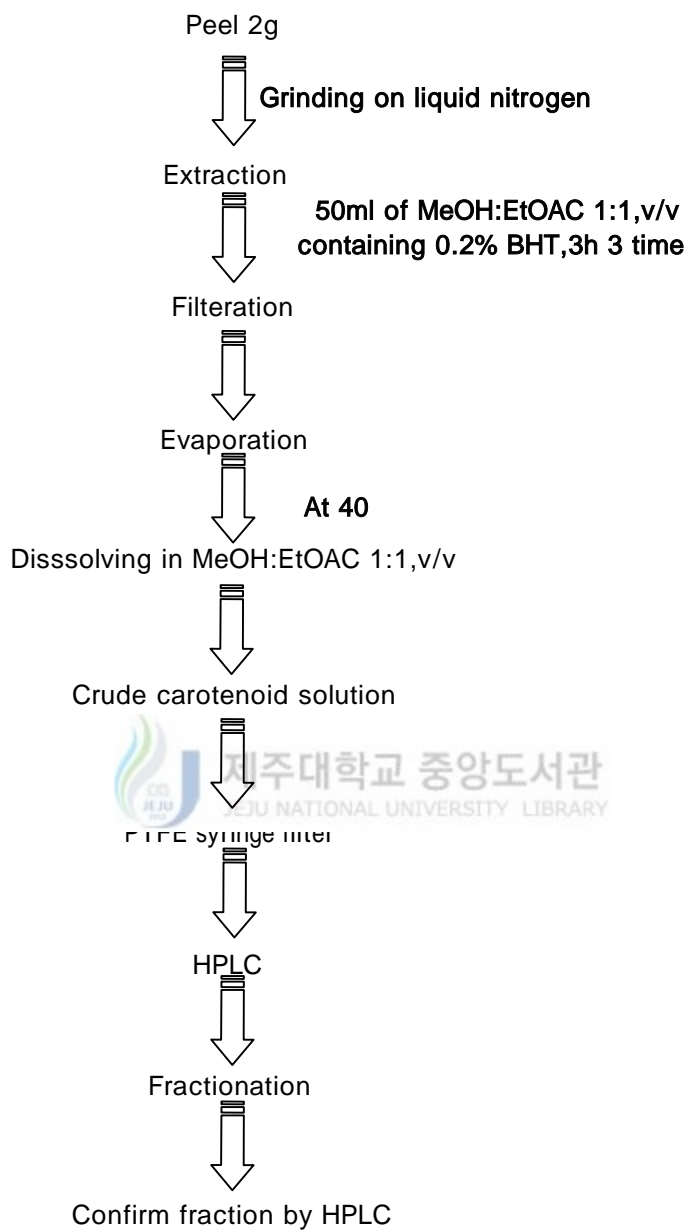
Figure 2. Experimental procedures for extraction and analysis scheme of carotenoids from fruit peel of 'Shiranuhi' mandarin.

Solvent run at a flow rate of 3 mℓ/min and fraction was collected at intervals of one minute. 2 mℓ of concentrated carotenoids solvent sample was injected. PRO DIGY ODS3 (4) 250 x 10 mm column was used and another condition was same designated above (Table 2).

The diode array detector was programmed to collect absorbance spectra from 300 to 600 nm and to monitor at 450nm for plotting the chromatograms. The wavelength, 450 nm was chosen because it is near the wavelength of maximum absorption for several carotenoids and chlorophyll.

LC/MS analysis

The molecular mass was determined using electrospray ionization mass spectrometry on a Quattro micro^{TM} API mass spectrometer (Waters, America). The analytical column was a MSC$_{18}$ column (4.67 x 50 mm, 5 µℓ) (XTerra, Waters, Ireland). The optimized mobile phase was methanol–ethyl acetate (50/50;v/v), and the flow rate was kept at 0.25 ml/min. The fraction of carotenoids collected from microbore HPLC was analyzed by injection of 10 µℓ. The spectral analysis was done in a positive ion mode at a capillary voltage of +3.0 kV, a cone voltage of 30 V and at a source temperature of 120°C. The molecular weight of the carotenoid was determined using the maximum entropy deconsolution algorithm (MaxEnt) to transform the range of 500/700 m/z to give a true mass scale spectrum.

Gene cloning

First strand cDNA preparation   Total RNA was extracted from the leaves and the green flavedos of fruits. Trizol, chloroform and isopropyl alcohol were used respectively to homogenize the sample, and separate the aqueous phase of RNA and sediment RNA. The RNA pellet was washed once with 75% ethanol and briefly vacuum-dried. In the end, the RNA pellet was dissolved in sterile H_2O treated with diethylpyrocarbonate (DEPC) and stored at –80°C. The concentration of total RNA was measured with UV/VIS spectrometer (PerkinElmer, USA) and the A260/A280 ratio of RNA was approximately 1.8 to 2.0.

The first strand cDNA was synthesized from 5μg of the total RNA with the 20μL of a mixture containing 4 μℓ of 5× reaction buffer, 1 μℓ of M-MuLV reverse transcriptase (MBI), 2 μℓ of dNTP, 1 μℓ of RNAsin (20 U/μℓ), 1 μℓ of Oligod(T)$_{18}$ (100 pico mol/μℓ), and DEPC-treated H_2O. The reaction step follows: heating at 70°C for 5 min, incubate at 37°C for 5 min, at 42 °C for 60 min, at 70 °C for 10 min. The first strand cDNA was stored at -20 °C until use.

On the basis of the conserved amino acid sequences among plant species in isoprenoid biosynthetic genes, five sets of degenerated primers were designed for each of *HMGS, HMGR, DXS, DXR, CCD* and *CPS* (Table 6) and primers were also used in this study (Table 7).

PCR were performed in 50 μℓ of reaction mixture with 20 pmol of degenerate primers sets (Table 6) or specific primers sets (Table 7) and conditions were conducted to enhance the expected band. The amplified DNAs were cloned into a

pGEM®- T Easy Vector (Promega, USA). The entire nucleotide sequences of the cloned cDNA were determined by dideoxychain termination method (Sanger *et al.*, 1977) using BigDye™ Terminator v3.0 Sequencing Kit (Amersham Pharmacia Biotech, USA). Nucleotide and amino acid sequences analyzed with NCBI (http://www.ncbi.nlm.nih.gov/BLAST/), ExPASy (http://us.expasy.org/tools), and CAP3 Sequence Assembly Program (http://pbil.univ-lyon1.fr/cap3.php).

Basis on ESTs searching and gene cloning   The BLAST (Madden *et al.*, 2003) and HarvEST:Citrus version 0.51 were used to search for ESTs (Qian *et al.*, 2002) in the citrus library, and these ESTs were electrically elongated by using the CAP3 Sequence Assembly program (http://pbil.univ-lyon1.fr/cap3.php).

Sequence divergence values among species were calculated using the DNADIST program of PHYLIP 3.65 (http://evolution.genetics.washington.edu/phylip.html) and the numbers of nucleotide substitutions were estimated using Kimura's two-parameter method (Kimura, 1980).

Genes expression assay

In this study, gene expression analyses were performed by Northern blot and real time semi-quantitative RT-PCR.

Northern blot   Fifteen microgram of total RNA were separated on 1.2% agarose gel containing 5 mM iodoacetamide in MOPS buffer (pH 7.0). The RNA transferred to a Nytran-Plus membrane (Schleicher & Schuell, USA) by using capillary transfer in 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) buffer. The RNA

was cross-linked to the membrane by using a Stratalinker UV cross-linker (Stratagene, USA). Hybridization was carried out with α^{32}P labeled DNA probes that was made by PCR. Hybridization overnight at 65°C in hybridization buffer (BD Bioscience, USA). The membranes were exposed to X-ray film (Kodak, USA).

Real time semi-quantitative RT-PCR The frozen material was immediately processed for RNA extraction by Trizol reagent and DNase treatment and first-strand cDNA synthesis followed by M-Mulv reverse transcriptase. The cDNA material was stored at -20 .

Real- time quantitative PCR was performed with a MJ-PTC 200 apparatus. For each combination of primer pairs and cDNA sample the following master mix was prepared fresh on ice: 10 µℓ of 2× master mix, 1 µℓ of cDNA sample, 4 µℓ of DDW and 1 µℓ of primer pairs. A master mix for each primer set was prepared such that each well contained the following: 2× SYBR Green master mix (FINNZYMES Finland) that primer and sample combination were run in triplicate in each real time PCR test. The qPCR cycling regimen was 15 min at 95 , 45 cyhcles of a three step temperature series (30 second at 95 , 30 second at the optimal annealing temperature for each pair of primer, 30 second at 72). Fluorescence measurements were done and recorded at each 30 second extension step. An optimal annealing temperature was determined for each pair of primer (Table 3). For each reaction tube, a PCR cycle threshold (Ct) was defined as the cycle value at which the second derivatives of the growth function of Sybr green fluorescence was maximal. For relative quantification of expression levels, the value of Ct for each of the target - amplified products in each experimental condition were determined using 2^{-Ct}

method. The $2^{-\triangle\triangle Ct}$ method is a convenient way to analyze the relative changes in gene expression from real time quantitative RT-PCR

Table 3. Primer sequences for real time RT-PCR

Primer	Forward/reverse	Primer sequence	At ( )	Amplication length (bp)
Ci-IPP	F	GGGGGAGCATGAACTTGACTAC	60	183
	R	TGTCCACAACCAGTCTGAATCC		
Ci-CCD	F	TACCACTGGTTTGATGGAGATG	58	158
	R	AATAGCCCCTTAAGGTCTCCAA		
Ci-PSY	F	CATCTGTCCTCCTACACCACAC	58	186
	R	GTGATCTTGATGTGAACCCAGA		
Ci-VDE	F	CAAAGACTTCAATGGGAAGTGG	58	176
	R	CAAATCTCTGCATAGCTGATCG		
Ci-DXPS	F	ACACCATTGTTGGACACGATTA	55	155
	R	AGCTCCACCACTCCTAAGTTTG		
Ci-eCarH	F	TCAATGCGTCTCTACCCACATCC	60	192
	R	CATGGGGCCTTCCAAGTCAAAC		
Ci-bCarH	F	CGATGTGTTTGCCATAATCAAC	55	165
	R	AACCTTTTGTGAACGAGACCAT		
Ci-GAPDH	F	CTGTCACTGTTTTCGGCGTTAG	58	156
	R	TGGGGGCTGAGATGATAACTTT		

Following amplification, samples were slowly heated in order to detect the loss in fluorescence that occurs at the melting temperature, which is characterized by a specific melting peak for each PCR product (Figure 3). The sharp and fully overlapping melting peak provides the specific sequence confirmation for the amplified PCR product with each dsDNA product.
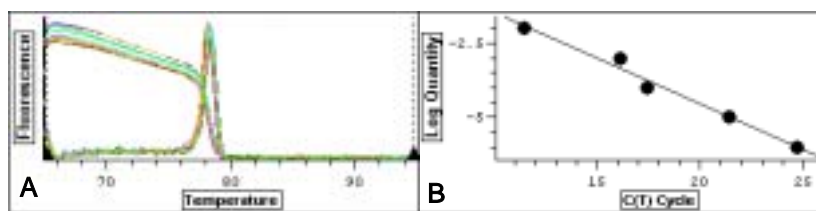
Figure 3. Melting curve and standard curve for quantitative analysis by real time PCR. A. Fluorescence versus temperature for GAPDH. Following amplification, samples were slowly heated in order to detect the loss in fluorescence that occurs at the melting temperature (Trentmann and Kende) the melting profile showed a (Trentmann and Kende) 79   for GAPDH. B. The curve derives from plotting Ct against log fluorescence. B. Standard curve for the PCR efficiency to quantitation, linearity and reproducibility**.**

RESULTS

Analysis of carotenoid composition

Carotenoids analysis was performed by HPLC. Analysis condition showed in Table 2. Figure 4 shows chromatograms of 'Shiranuhi' mandarin peel samples using UV absorbance at 450 nm. A wavelength of 450 nm was chosen to maximize absorbance in the 300-600 nm region of the visible spectrum, which is an optimal area for studying carotenoids of 'Shiranuhi' mandarin peel.

HPLC spectral characteristics from the peaks labeled 1-16 in Figure 4 are shown in Table 6. Composition of carotenoid was increased during maturation and ripening (October, November and December). Carotenoid peaks from August to December were detected 5, 7, 11, 14 and 14 peaks, respectively. Carotenoid of relative quantity also changed during development and maturation of citrus fruit. For example, the relative amount of peaks 1 and 11 were higher in early stage than in later stage. By contrast, peaks 3 and 5 were higher in later stage than in early stage.
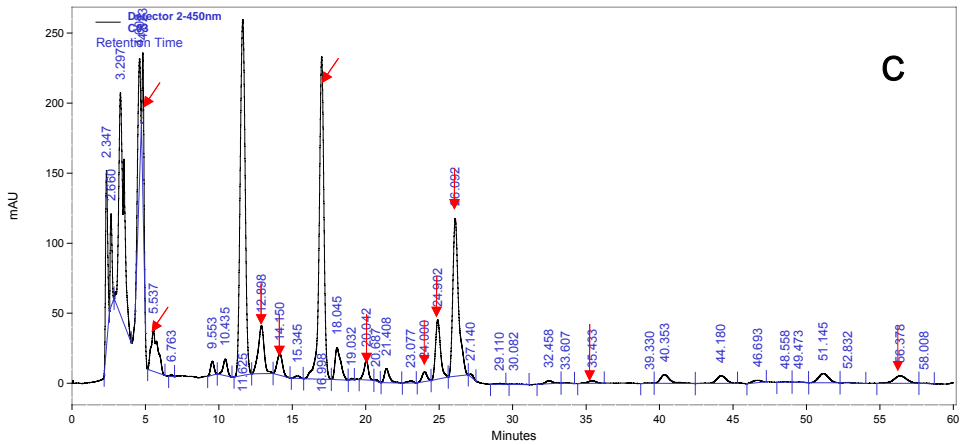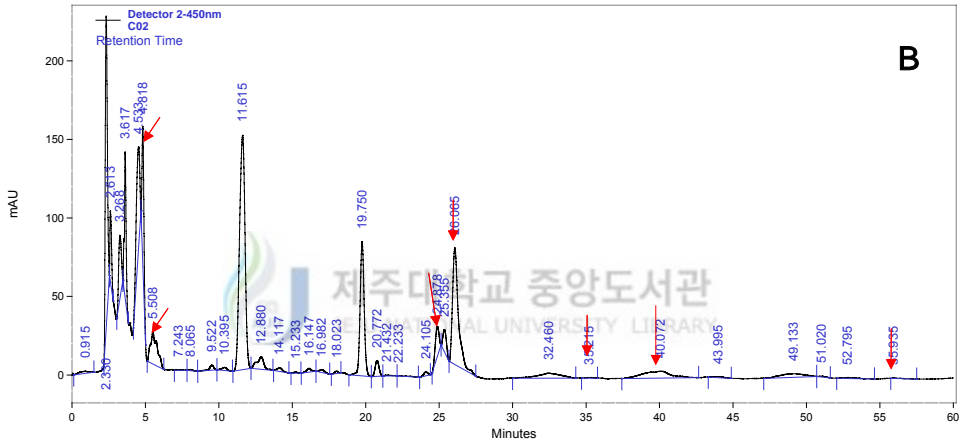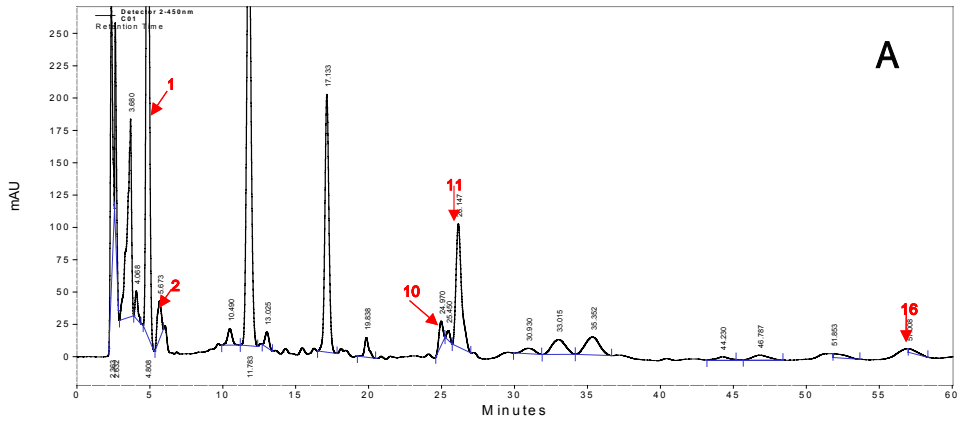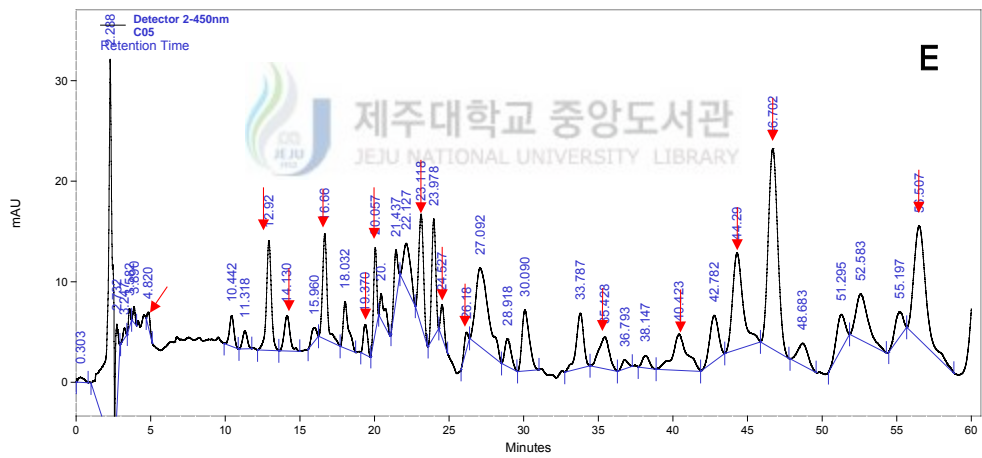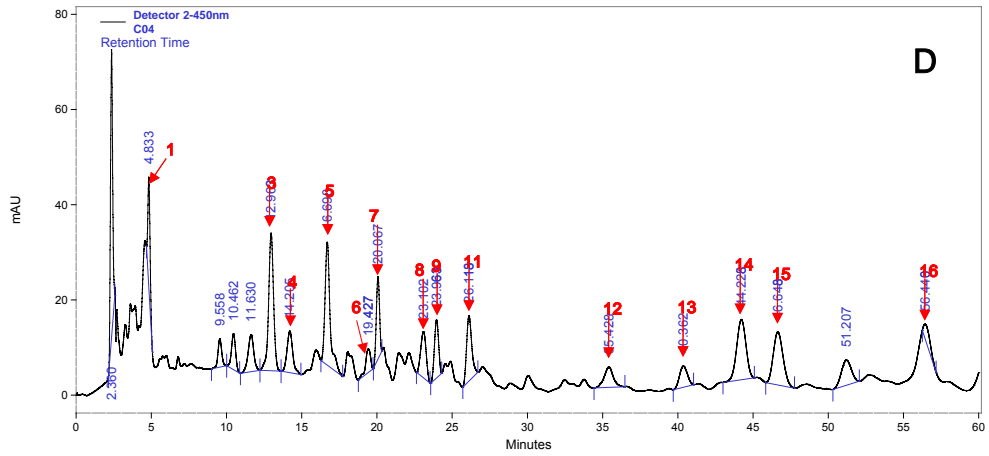
Figure 4. HPLC chromatogram of carotenoids extracted from fruit peel of 'Shiranuhi' mandarin at different development stages. A, August; B, September; C, October; D, November; E, December. Numbers above the arrows indicate carotenoids peak number.

Table 4. Spectroscopic characteristics of carotenoids in fruit peel of 'Shiranuhi' mandarin

Peak No.	Tentative identification	Retention time	Observed Max ABS wavelength	Peak ratio
1	Lutein	4.808	417.440.471	73
2	Unknown	5.673	420.444.470	37
3	z-carotene	12.923	428.457.484	
4	Unknown	14.15	420.443.471	62
5	β-cryptoxanthin	16.698	413.439.467	72
6	phytofluene	19.43	403.424.450	86
7	capsanthin	20.042	414.439.467	75
8	zeaxanthin	23.092	413.437.446	61
9	Unknown	24	413.437.468	72
10	Unknown	24.878	425.449.455	52
11	β-carotene	26.118	428.454.480	5
12	Unknown	35	410.439.468	65
13	Unknown	40.353	421.447.471	88
14	Unknown	44.23	413.438.466	81
15	Unknown	46.787	418.454.469	21
16	Unknown	56.958	417.433.468	79

Identification of carotenoids

Spectral characteristics are compared with those reported in the literature showing that the chosen peaks matched reported (Table 5) and confirmed the peaks with molecular mass species of carotenoid. The fraction was examined with LC/MS. The peak 1 elutes and the mass spectrum of the elution showed the molecular ion at mass-to-charge ratio (m/z) 569($[M+H^+]$). The absorption maximum (422,448 and 476nm) were close to those of lutein reported previously (Tai and Chen, 2000). The

Peak 3 elutes and the mass spectrum of the elute showed the molecular ion at mass-to-charge ratio (m/z) 541([M+H+]) (Figure 5). It was close to those of ζ-carotene reported previously (Tai and Chen, 2000). Composition of carotenoid was increased during ripening of citrus such as capsanthin, β-cryptoxanthin and zeaxanthin.

Table 5. Identification of carotenoids found in 'Shiranuhi' mandarin

Peak	Carotenoid compound	Max ABS wavelength	/ x100%	M.W.	Max ABS wavelength / Rodrigo,2003	x100%	Max ABS wavelength / Britton, 1995	x100%
1	Lutein	422.448.476	54	568.88	418.444.472	65	421.445.474	60
2	-Cryptoxanthin	428.457.484	30	552.85			435.459.485	27
3	-Carotene	428.454.480	5	540			378.400.425	
4	Phytofluene	399.420.444	67	542	329.346.364	71	331.348.367	90
5	Capsanthin	420.442.471	64	584.85			450.475.505	
6	Zeaxanthin	400.420.446	72	568.88	430.450.478	35	428.450.478	26
7	-carotene	424.456.480		536.88	426.451.473	31	425.450.477	25

Peak ratio is %   /   for carotenoids (Britton, 1995)

capsanthin m/z 585([M +H$^+$]) **A**

β-cryptoxanthin m/z 552([M +H$^+$]) **B**

ζ-carotene m/z 541([M +H$^+$]) **C**

β-carotene   m/z 536([M +H⁺])   **D**

phytofluene   m/z 543([M +H⁺])   **E**
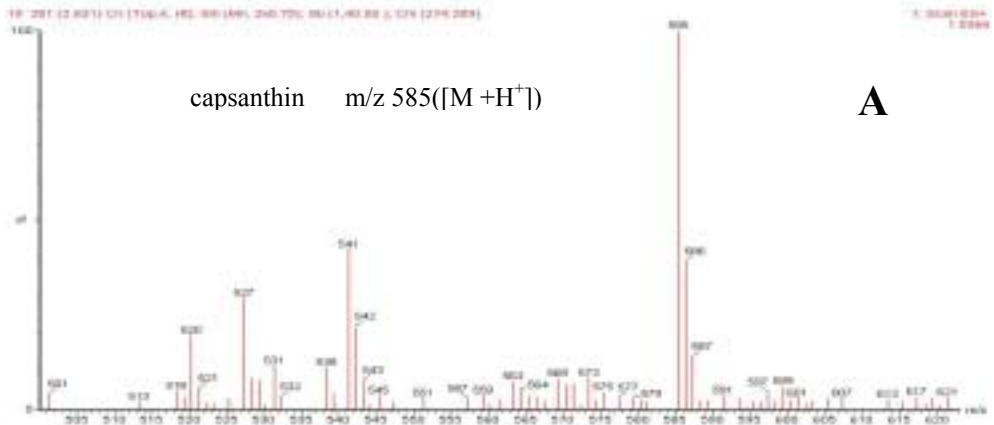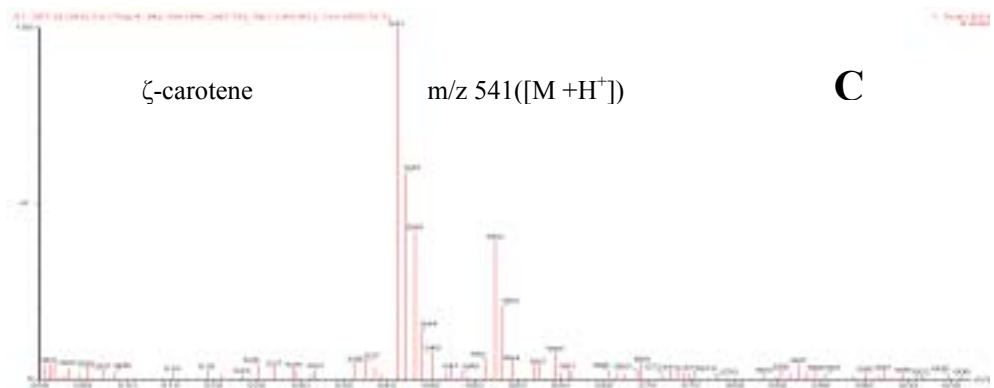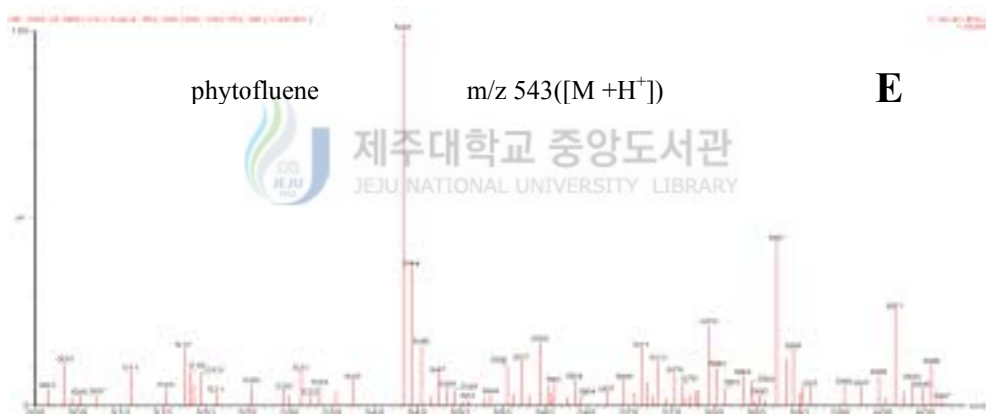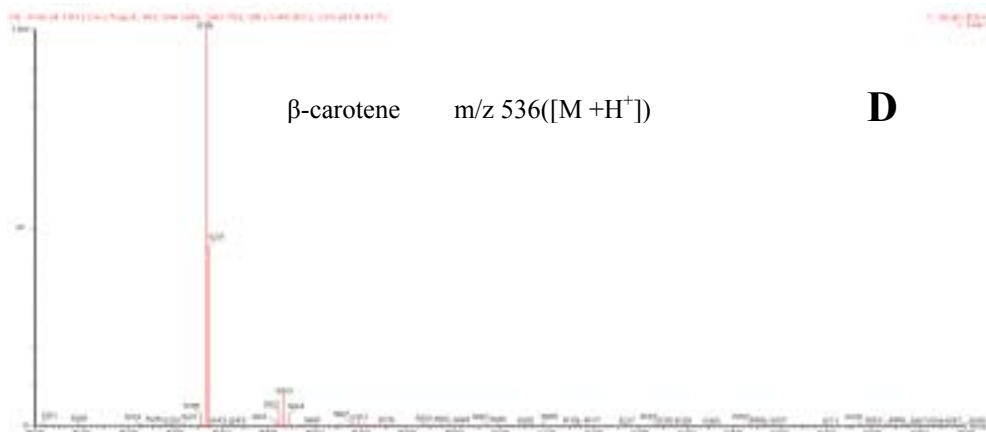
Figure 5. LC/MS profiles of carotenoid compounds in fruit peel of 'Shiranuhi' mandarin. Positive ion elecrospray mass spectra of the biosynthetic product (A) capsanthin, (B) β-cryptoxanthin, (C) ζ-carotene (D) β-carotene (E) phytofluene recoded during the LC/MS analysis shown in the chromatogram.
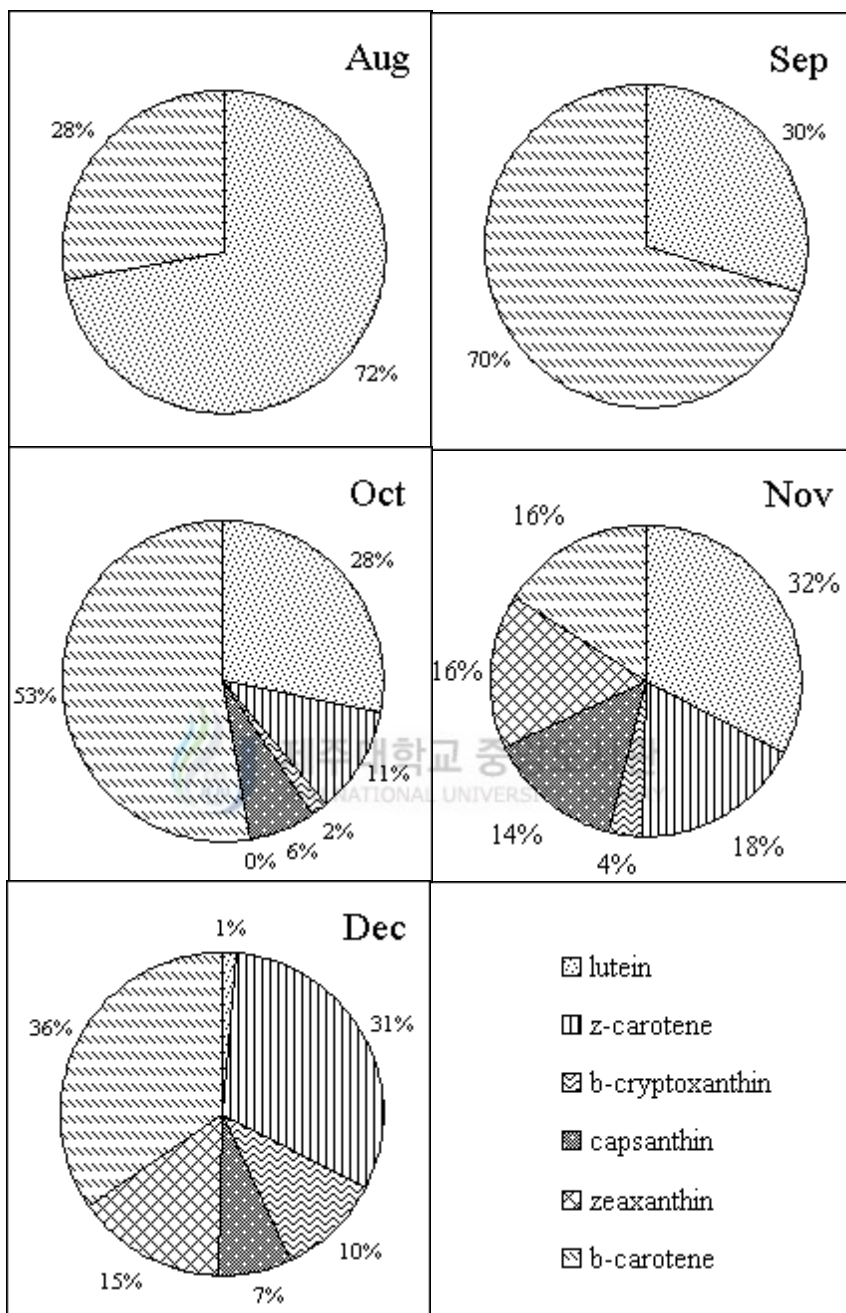
Figure 6. Change of carotenoids composition during maturation in fruit peel of 'Shiranuhi'mandarin.

Lutein, β-carotene, β-crypoxanthin, zeaxanthin, phytofulene, capsanthin, and ζ-carotene were identified by LC/MS analysis and spectrum characterization. During ripening, content of lutein and β-carotene decreased and content of β-crypoxanthin, zeaxanthin, phytofulene, capsanthin and ζ-carotene increased (Figure 5).

Cloning of genes involved in carotenoid biosynthesis

On the basis of the conserved amino acid sequences among plant species in carotenoids biosynthetic genes, degenerate primers were designed for each of *HMGS, HMG-R, CPS, GGPP, CCD* and *GAPDH* (Table 6). Reverse transcription PCR was performed using total RNA from leaves, flowers and fruit peel of citrus 'Shiranuhi' mandarin. Also IPP isomerase gene was cloned on the basis of EST database of NCBI.

Table 6. Primer sequences for degenerate PCR

Primer	Primer sequence (5'-3')	At( )
GAPDH DE-UP(SP26)	GAGCTGGTGGCCGTGAAYGAYCCNTT	
-DN(EP265)	CGGACTCCTCCTTGATGGCNGCYTTDAT	
-DN(SP42)	CACCGACTACATGACCTACATGTTYAARTA	62
-DN(EP265)	GGGCGATGCCGGCYTTNGCRTC	
HMGS DE-UP(SP8)	TCCTGGCCATGGACRTNTAYTTYCC	
UP(SP122)	GCCGCCCTGTTCAACTGYGTNAAYTGGG	
DN(EP260)	CGAAGGACTTCTGCACCARYTTRTTRTA	52
DN(EP445)	CATCAGCTTCATGGTCTCCACRAANTYYTC	
HMGR -DEUP(SP100)	CTTCATCTACCTGCTGGGCTTYTTYGGNATHG	
-DEUP(SP246)	CGAGATGCCCATCGGCTAYGTNCARNT	
-DEDN(EP380)	GACACCATGTTCATGCCCATNGCRTC	52
-DEDN(EP507)	GCCGTCGTTCACGGCYTCNADCAT	
CPS DE-UP	CCCGTGGACCTGTTCGARCAYATNTGG	
-DN	AGGGTCTTGCCGATCCANACRTC	
GGDR DE-UP1	GCGGCGGCCCNGCNGGNGG	58
DE-UP2	TGAAGCCACACGAGTACATHGGNATGGT	
DE-DN1	TGCCGGTGCCCACNGCNACRTG	
DE-DN2	TCGTCGGCGCACATYTCNACRAA	52
Ci-CCD-DEUP(SP70)	GCCTGAGTGCCTGAATGGNGARTTYGT	
-DEUP(SP170)	CACTGCTAATACTGCTATGGTTTACCAYCAYSRNAA	
-DEDN(EP490)	GAACAAAGAAAATCAGGTAGCCATCRTCYTCYTC	

Table 7. Primer sequences for carotenoid biosynthetic related genes

Primer		Primer sequence (5'-3')	At( )
Ci-PDS	F	ATTGCTTTCAAACGCGAAAT	52
	R	ATAAACCCTGCCTCCAGCTT	
Ci-VDE	F	TGCGGTCTCAAGAAAGAAGTG	49
	R	ATTGCTGCCCCGATAGTACA	
Ci-ZEP	F	ATGGTTTCATCTATGTTCTACAATTCA	50
	R	TTACACTGCCTGAAGAATTTCAC	
Ci-FPS	F	ATGAGTGATCTGAAGTCAAGATTCA	51
	R	TCACTTCTGTCTCTTGTATATCTTTG	
Ci-LYC-e	F	AAAAACACTGGCAAATGCTC	51
	R	TCACGTATGGCAATCCAAAA	
Ci-LYC-b	F	GCCATGGATACTGTACTCAAAACTC	51
	R	GGTCACCTTAATCTGTATCTTGTACC	
Ci-GPPS	F	ATGGTTATTGCTGAGGTTCCTA	51
	R	TTAAACAAAAACTCTGGCACAATG	
Ci-SQUAN	F	ATGGCTCTTAATCTGCTATCTTCA	52
	R	GGACAAAACACGAGCTTTAGTAAT	
Ci-PSY	F	TTTCTTTACCAACATCAAACCC	55
	R	GAGCTCATCTGTCCTCCTACAC	
Ci-CarH-b	F	ATGGCGGTCGGACTATTGGC	51
	R	TATTTTTTTCATTAATAACACTATTG	
Ci-carH-e	F	AAAAACACTGGCAAATGCTC	51
	R	TCACGTATGGCAATCCAAAA	
Ci-IPI	F	GCCTCTGTCCTTGAGAGGTG	51
	R	CAGCAAATGAGGCTTCACAA	

Ci-HMGR (3-hydroxy-3-methylglutaryl-Coenzyme A reductase) was 524 bp of length. This partial gene showed that sequence identity was 82% with HMGR1 from *Hevea brasiliensis*. The deduced amino acid sequences of clone *Ci-HMGR* was compared with other plant *HMGR* and showed high sequence similarly those of *Cucumis melo* (86%, BAA36291), *Arabidopsis thaliana* (82%, NP_177775), *Raphanus sativus* (83%, CAA48611), *Hevea brasiliensis* (86%, CAA38469), *Gossypium hirsutum* (83%, O64967), and *Camptotheca acuminata* (83%, AAB69726) (Table 6).
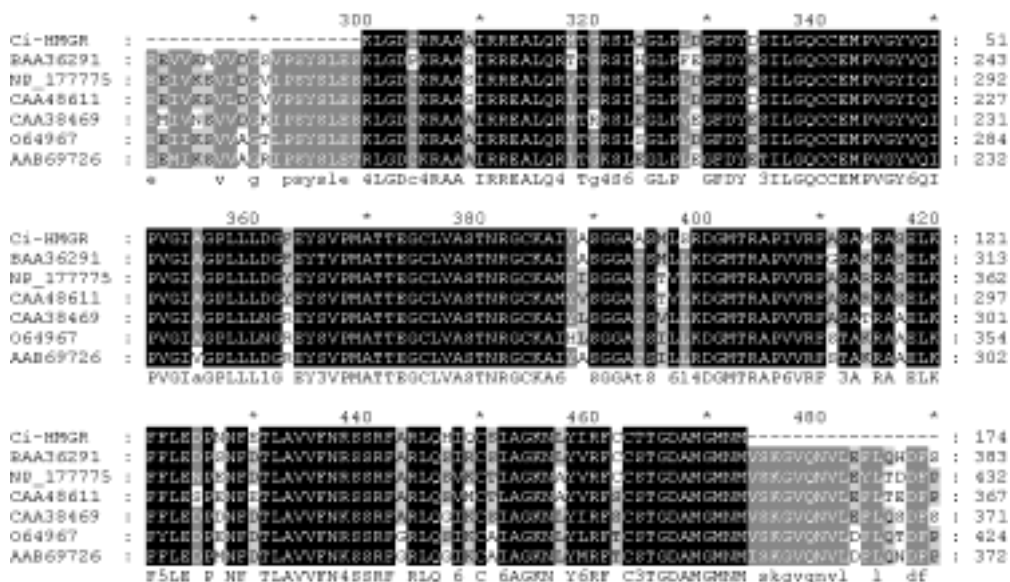
Figure 7. Alignment of deduced amino acid sequences of Ci-HMGR with other plant *HMGR* genes. The amino acid sequences of HMGR from *Cucumis melo* (BAA36291), *Arabidopsis thaliana* (NP_177775), *Raphanus sativus* (CAA48611), *Hevea brasiliensis* (CAA38469), *Gossypium hirsutum* (O64967), *Camptotheca acuminata* (AAB69726) and *Gossypium hirsutum* (AAF69804 and AAG32923) are shown. The alignment was created with GeneDoc program. Gaps in the sequences are indicated by dashes. Numbers of nucleotides are indicated on the right margin.

The full length cDNA was cloned from 'Shiranuhi' mandarin flower. It was identical to the full-length cDNA based on assembling ESTs. The 705 bp of PCR product was sequenced and the result was identical to this gene. The deduced amino acid sequences were alignment with IPI genes from *Camptothecine*, tobacco and tea tree (Figure 8 and Figure 9).

Figure 8. Alignment of the Ci-IPI amino acid sequences with other plant IPI. The alignment was made using GeneDoc program. The nucleotides sequences of *IPI* from accession no. AAB94132 (*Camptotheca acuminata*), BAB40974 (*Nicotiana tabacum*), AAL91979 (*Melaleuca alternifolia*) are shown. Gaps in the sequences are indicated by dashes. Numbers of nucleotides are indicated on the right margin.

A phylogenetic tree of the *IPI* isomerase genes was generated on the basis of nucleotides sequence. Pylogenetic analysis indicates that the *Ci-IPI* in citrus and the IPI of *Arachis stenosperma* and *Pueraria nontana* share a common origin (Figure 9).
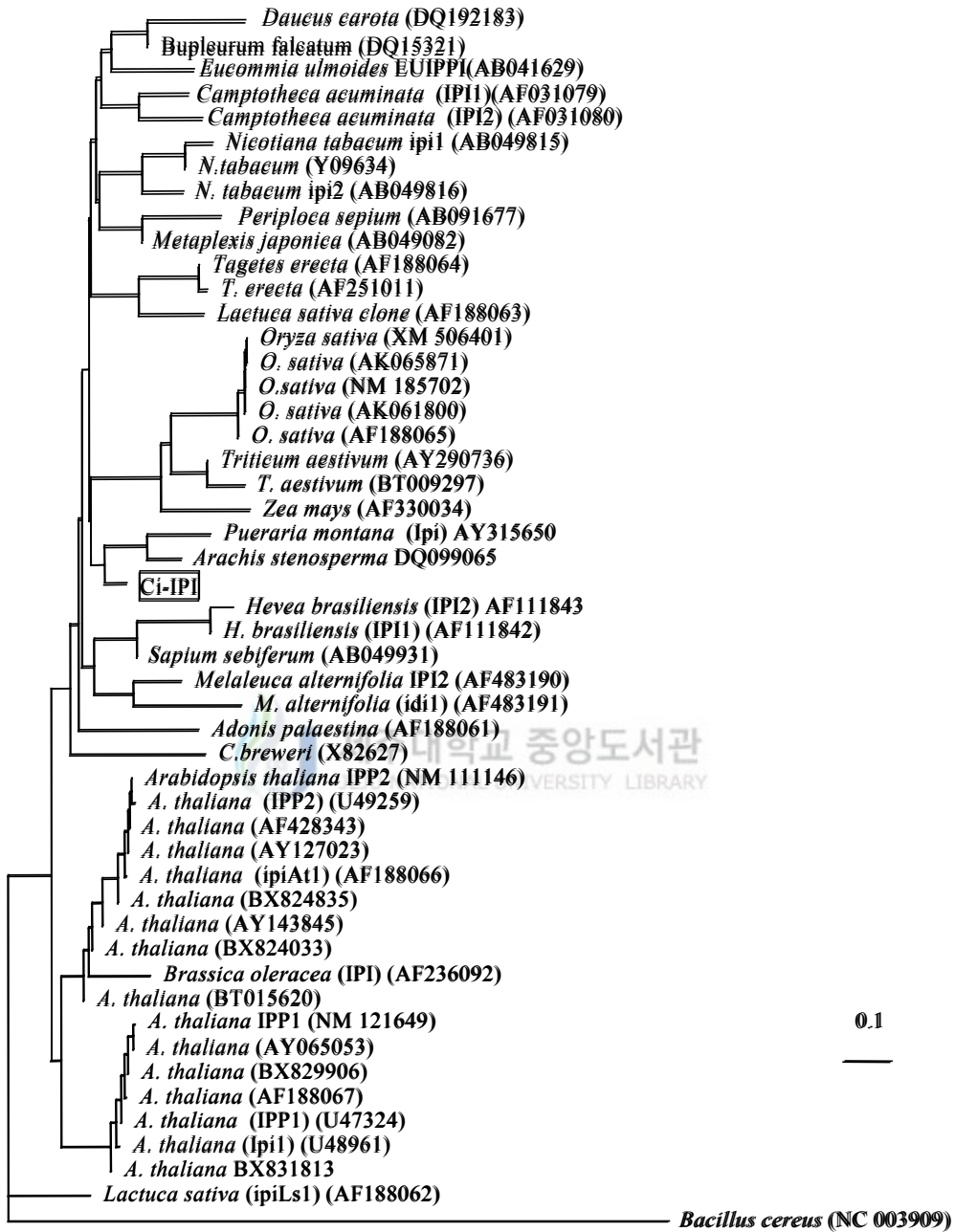
Figure 9. Neighbor-joining dendrogram based on the combined IPI sequences.

Bacillus cereus was used as an outgroup. The tree was calculated on the basis of a

CLUSTALW 1.8 alignment by the program PHYLIP.

We isolated 1181 bp length of *HMGS* (3-hydroxy-3-methylglutaryl-Coenzyme A synthase) gene, it showed 85% identity on nucleotides sequence level with HMG-CoA synthase 2 gene from *Hevea brasiliensis*. The deduced amino acid sequence of the *Ci-HMGS* has high similarity to those of *H. brasiliensis* (89%, accession No. AAS46245), *Brassica juncea* (85%, accession No. AAF69804, 83%, accession No. AAG32923), *Arabidopsis thaliana* (82%, accession No. CAA58763), and *Oryza sativa* (81%, accession no. BAD46696l) (Figure 10).
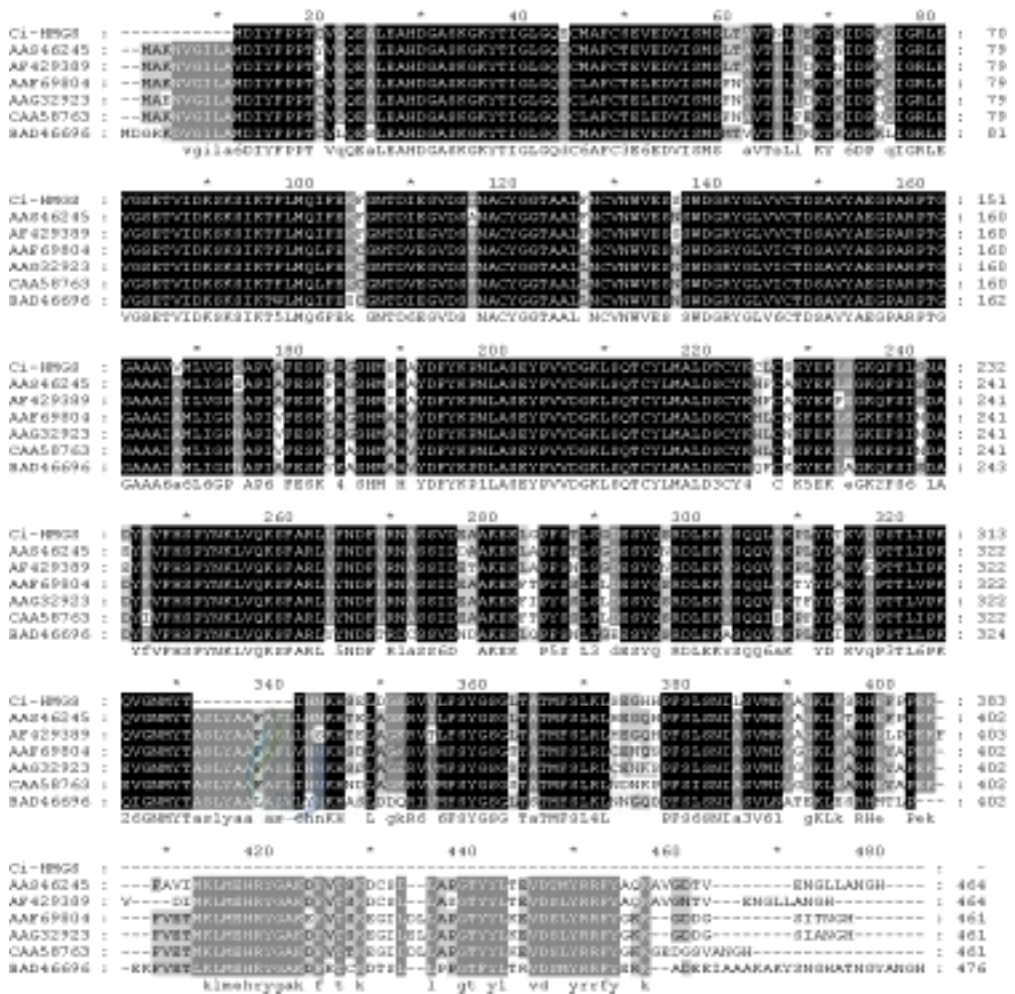
Figure 10. Comparison of the deduced amino acid sequences of *Ci-HMGR* with other plant *HMGR* genes. The amino acid sequences of HMGS from *Hevea brasiliensis* (AAS46245 and AF429389), *Brassica juncea* (AAF69804and AAG32923), *Arabidopsis thaliana* (CAA58763), and *Oryza sativa* (BAD46696) were alignmented. The alignment was made using GeneDoc program. Gaps in the sequences are indicated by dashes. Numbers of nucleotides are indicated on the right margin**.**
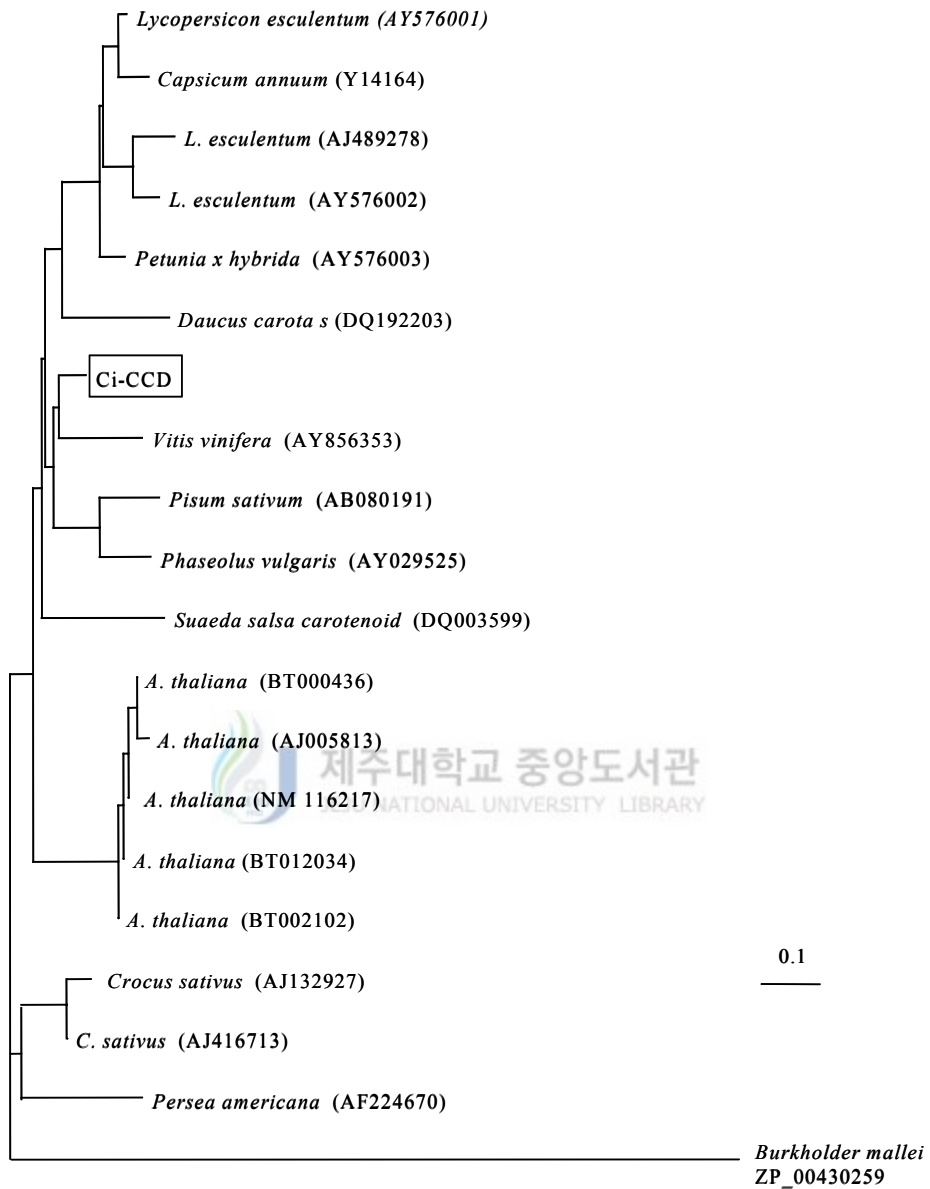
Figure 11. Phylogenetic analysis of *Ci-CCD* and *CCD* from various plant species.

A phylogenetic tree of the *CCD* genes was generated on the basis of nucleotide sequences alignment.

A citrus cDNA clone *Ci-CCD* showing 81% nucleotide identity to *Vitis vinifera* 9,10 [9',10'] carotenoid cleavage dioxygenase (*CCD1*) (Accession No. AY856353) was found to contain an 1197 bp of insert and the deduced amino acid sequences showed 91% with that of *Vitis vinifera*. The phylogenetic dendrogram indicated that *Ci-CCD* isolated from citrus could be clustered with a group including *Vitis vinifera*, *Pisum sativum* and *Phaseous vulgaris* (Accession No. AB080191, AY856353, and AY029525) (Figure 11).

Analysis of the cDNA of *Ci-DXPS* revealed that it has similarity with 1-deoxy-D-xylulose 5-phosphate synthase gene of *Medicago truncatula* (*dxs*2 gene, Accession No. AJ430048; 80% identity to *M. truncatula dxs2* gene). The phylogenetic dendrogram showed that *Ci-DXPS* groups with those from *Medicago truncatula* (AJ011840) and *Catharanthus roseus* (AJ430048) (Figure 12).

A cDNA clone *Ci-b-CarH* showed 99% nucleotide identity with citrus *CHX1* and *CHX2*, 98% nucleotide identity with citrus beta-carotene hydoxylase gene of *Citrus maxima*. Phylogeneic analysis of *Ci-b-CarH* gene indicated that it belongs to β-carotene of citrus hydroxylase gene group.
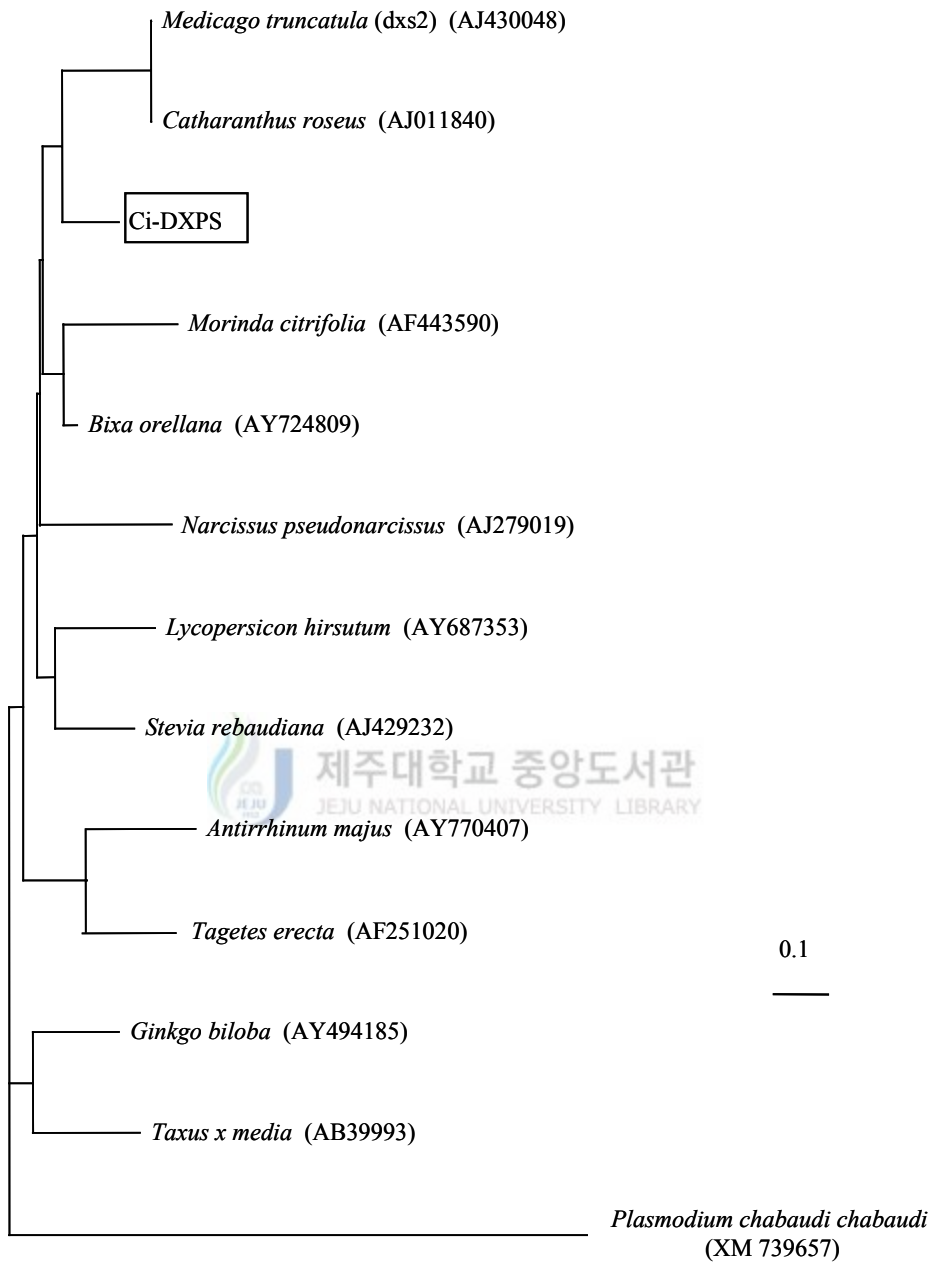
Figure 12. Phylogenetic tree of *DXPS* nucleotide sequences from plants and *Plasmodium chabaudi chabaudi.* The dendrogram was created using the PHYLIP program.
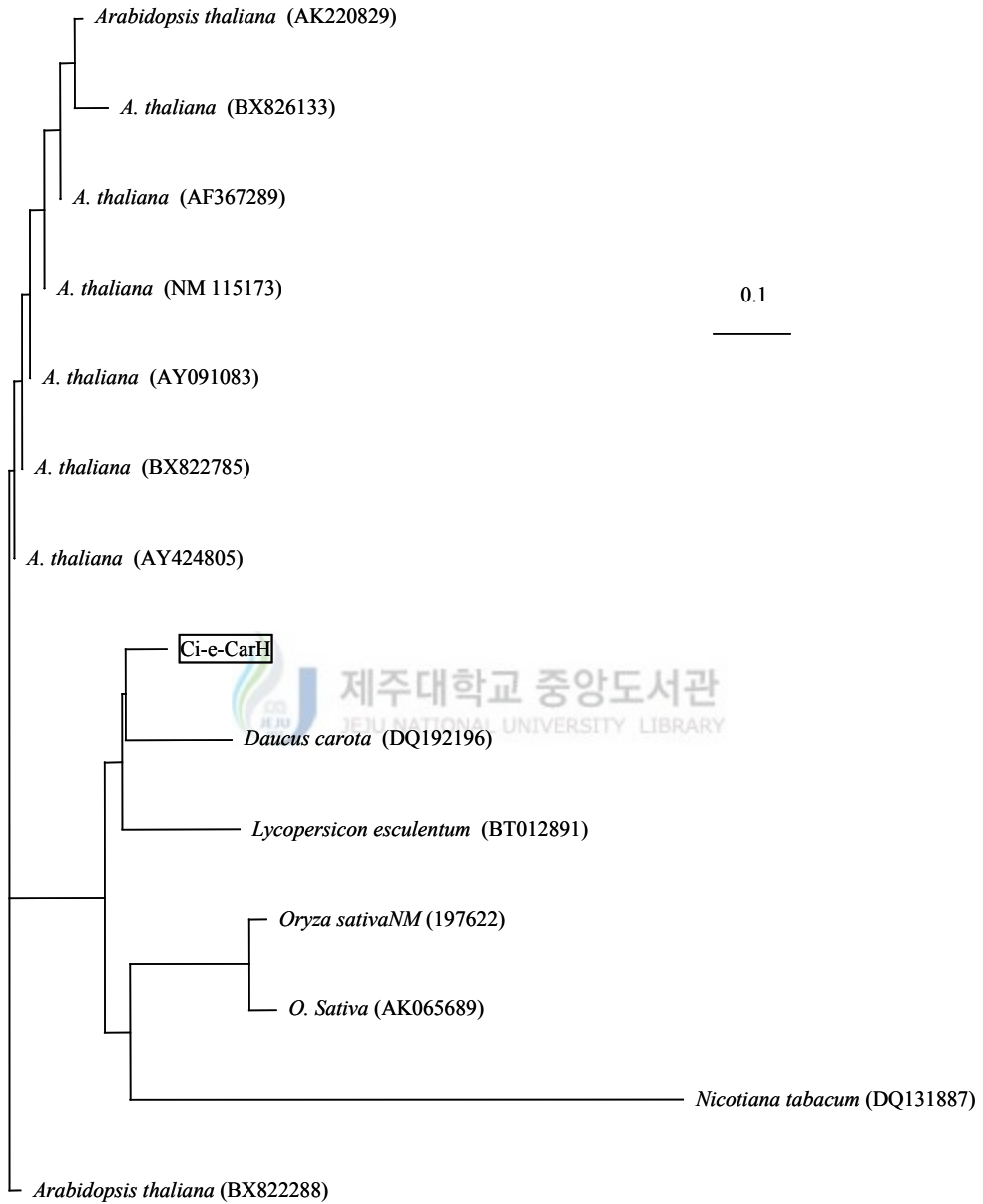
Figure 13. Phylogenitic tree of *Ci-e-CarH* gene and related genes from plants.

The tree includes the following taxa:

- *Arabidopsis thaliana* (AK220829)
- *A. thaliana* (BX826133)
- *A. thaliana* (AF367289)
- *A. thaliana* (NM 115173)
- *A. thaliana* (AY091083)
- *A. thaliana* (BX822785)
- *A. thaliana* (AY424805)
- Ci-e-CarH
- *Daucus carota* (DQ192196)
- *Lycopersicon esculentum* (BT012891)
- *Oryza sativaNM* (197622)
- *O. Sativa* (AK065689)
- *Nicotiana tabacum* (DQ131887)
- *Arabidopsis thaliana* (BX822288)

Scale bar: 0.1

It was found that nucleotide sequences of the *Ci-e-CarH* gene grouped with epsilon ring hydroxylase genes from carrot and tomato (Figure 13).

Phylogenetic analysis of *Ci-b-CarH* gene and two CHX genes (*CHX1* (AF296158), *CHX2* (AF315289)) of *Citrus. unshiu* indicated that *Ci-b-CarH* is all most similar to the *CHX1* and *CHX2* (Figure 15).

Other isolated genes listed in Table 8 and the nucleotide sequences in Appendix.
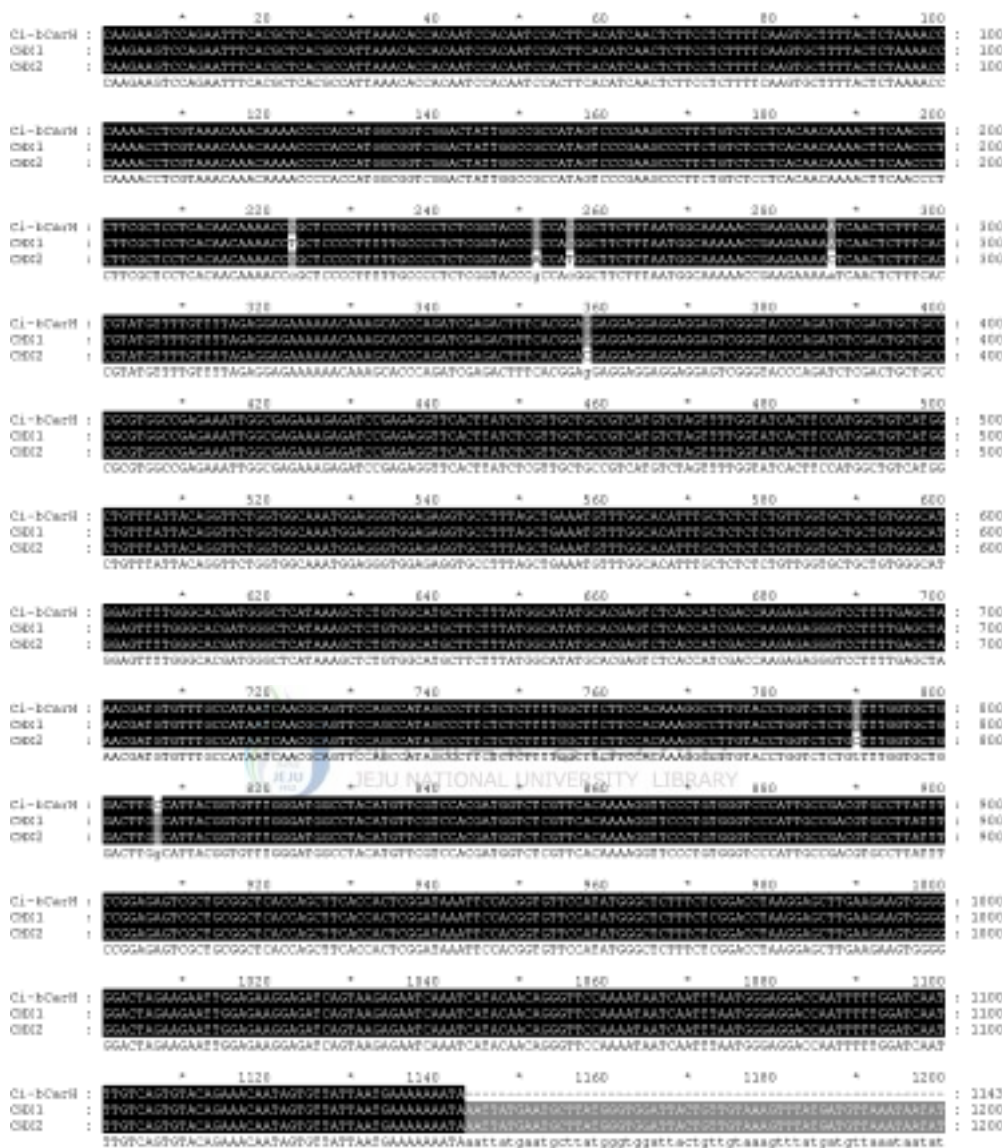
Figure 14. Comparison of the nucleotide sequences of *Ci-CarH* with *CHX1* and *CHX2*'Satsuma mandarin (*Citrus unshiu*). *CHX1* (AF296158) and *CHX2* (AF315289) is carotene hydroxylase gene from *Citrus unshuu*.
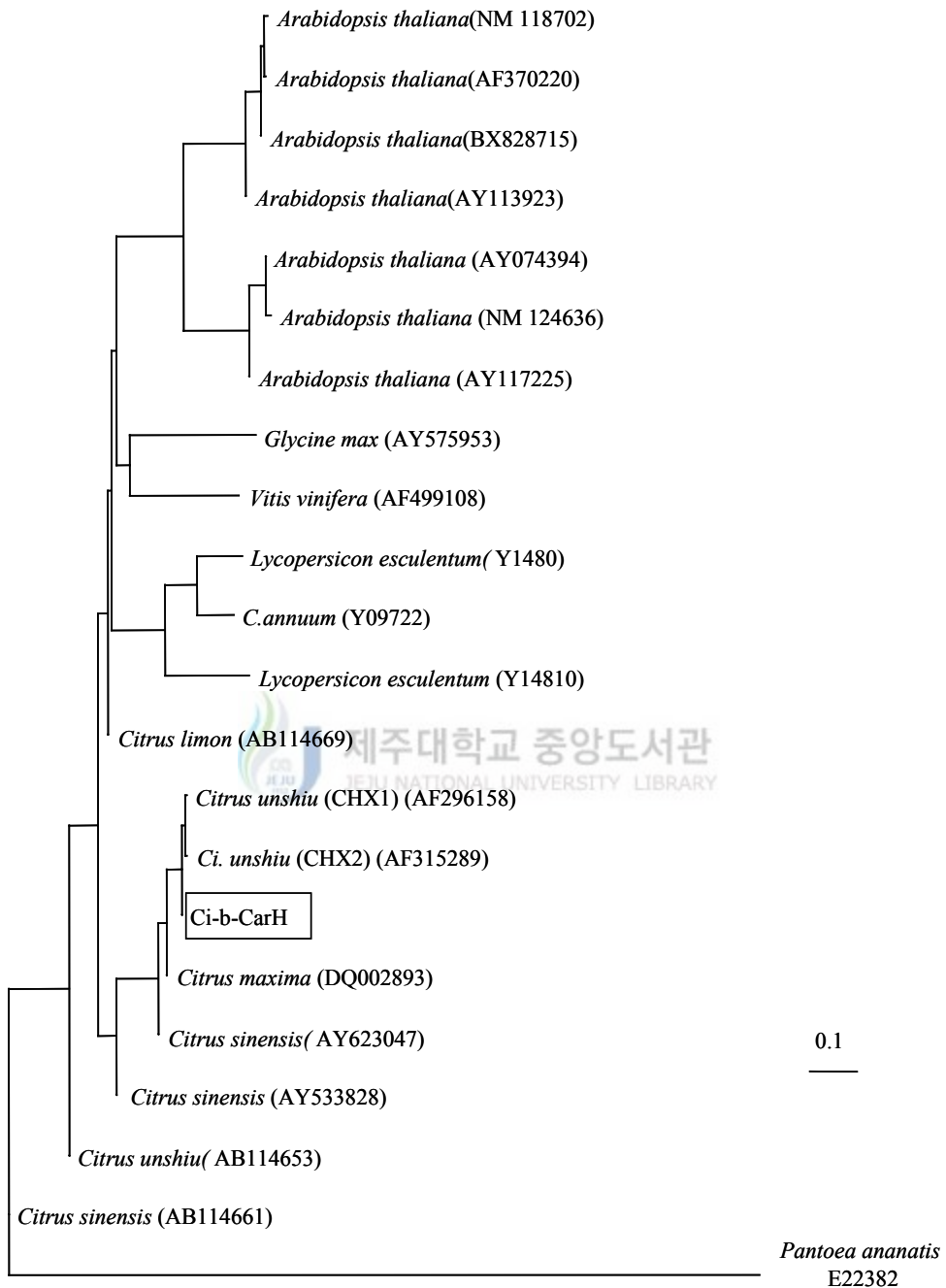
Figure 15. Phylogenitic tree of *Ci-b-CarH* gene and related genes from plant.

Pantoea ananatis was used as outgroup.

Table 8. List of carotenoid biosynthetic genes in 'Shiranuhi' mandarin

Abbreviation	Gene name	Length (bp)	Position of Start and End	Gene source	Expected Full length (bp)
GAPDH	Glyceraldehyde 3 phosphate dehydrogenase	684	170-853	Leaf cDNA	1-1339
CPS	ent-copaly diphosphate synthase	554	1005-1566	Leaf cDNA	1-2478
GGDR	geranylgeranyl pyrophosphate reductase	640	645-1284	Leaf cDNA	41-1441
HMGR	HMG CoA reductase	524	582-1105	Flower cDNA	42-1769
HMGS	HMG CoA synthase	1181	334-1524	Flower cDNA	317-1711
DXPS	1-deoxy-D-xylulose-5-phosphate synthase	1899	37-1926	GeneBankCNU	37-2187
DXPR	1-deoxy-D-xylulose-5-phosphate reductoisomerase	1054	420-867,1089-1473	GeneBankCNU	162-1622
IPI	isopentenyl diphosphate isomerase	669	1-669	Flower cDNA	1-669
FPS	FPP synthase	1126	103-1228	Flower cDNA	103-1228
Squa	squalene synthase	420	9-428	Flower cDNA	partial
SEQS	Sesquiterpene synthase 2	1005	669-1671	GeneBankCNU	1-1674
LIMS	(+)-limonene synthase 2	1827	1-424,1418-1827	GeneBankCNU	1-1827
β-OCIS	(E)-beta-ocimene synthase	334	1517-1850	GeneBankCNU	1-1854
TPS	terpene synthase	1846	22-704,1127-1867	GeneBankCNU	64-1701
GGPPS	Geranylgeranyl pyrophosphate synthase	181	1-181	GeneBankCNU	1-1113
PSY	phytoene synthase	452	220-671	Leaf cDNA	112-1422
PDS	phytoene desaturase	1761	47-747,1109 -1808	Flower cDNA	151-1821
LYC-β	lycopen beat cyclase	1525	403-1088,1284-1928	Flower cDNA	406-1920
LYC-ε	lycopen epsilon cyclase	1273	13-655,718-1285	Leaf cDNA	1-1314
β-CarH	beta-carotene hydroxylase	1115	1-354,374-1134	Leaf cDNA	131-1066
ε-CarH	epsilon-carotene hydroxylase	512	1007-1620	Leaf cDNA	1-1620
ZEP	zeaxanthin epoxsidase	1988	6-708,1290-1993	Flower cDNA	1-1995
VDE	viiolaxanthin de-epoxidase	409	1-409	Flower cDNA	1-409
CCD	9,10[9',10']carotenoid cleavage dioxygenase (CCD1)	1244	392-1638	Leaf cDNA	1-1963
CCS	Capsanthin/capsorubin synthase	1655	1702-2407,2689-3356	GeneBankCNU	1722-3233
HPT	tocopherol polyprenyltransferase	447	495-777,824-997	GeneBankCNU	1-1221

Expression profiles of genes involved in carotenoid biosynthesis

Northern blot Analysis Expression pattern of genes involved in carotenoid biosynthesis was investigated by Northern blot. During ripening of citrus (Novermber), expression of β-carotene hydroxylase was increased (Figure 16 A). The transcripts level of ε-carotene hydroxylase and isopentenyl diphosphate isomerase showed not change in all investigated stages (Figure 16 B, C).
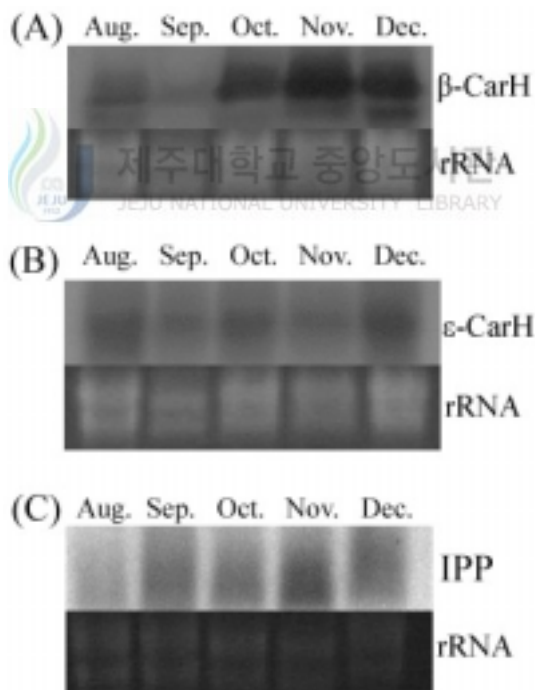
Figure 16. Northern blot analysis of carotenoid biosynthetic genes.

Real time RT-PCR For each reaction tube a PCR cycle threshold (Ct) was defined as the cycle value at which the second derivatives of the growth function of Sybr green fluorescence was maximal. For relative quantitative of expression levels, the value of Ct for each of the target-amplified products in each experimental condition was determined. The $2^{-\Delta Ct}$ —method is a convenient way to analyze the relative changes in gene expression from real time quantities PCR experiments. The method for copy number determination by real time PCR is the comparative Ct ($2^{-\Delta\Delta Ct}$) method. While requiring an endogenous control and a calibrator, it differs from the relative standard method by relying on equal PCR efficiencies with the sample gene and the endogenous control (reference) genes (Livak and Schmittgen, 2001). If all amplicons amplify with the same efficiency, the difference ΔCt between the Ct for the sample (Ct_s) and the Ct for the reference control (Ct_r) is constant, independent of the amount of initial cDNA: $\Delta Ct = Ct_s - Ct_r$

As for quantification with relative standards is homozygous a stage sample in this experiment. Then, all samples with same ΔCt as the calibrator (one sample) calculate $\Delta Ct = Ct_s - Ct_c$. We carried out real time RT-PCR for seven genes, β-carotene hydroxylase, -carotene hydroxylase, isopentenyl pyrophosphate isomerase, DXS, phytoene synthase, violaxanthin diepoxidase and neoxanthin dioxygenase. Relative transcription level of these genes was compared with Ct ($2^{-\Delta\Delta Ct}$) method.

β-carotene hydroxylase gene expression   The gene expression was increased during fruit ripening as shown in Figure 17. This result is closely similar to Northern blot analysis.
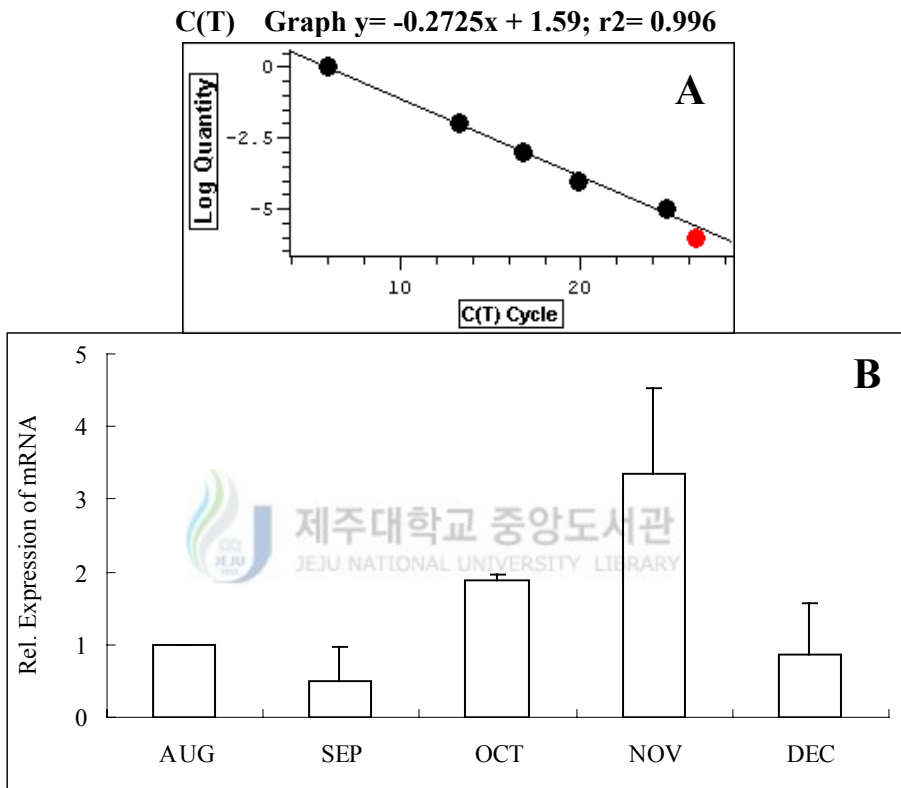
Figure 17. Real time RT-PCR analysis for β-carotene hydroxylase gene expression.

(A) Standard curve for β-carotene hydroxylase gene expression. The curve derives from plotting Ct against log quantity of fluorescence. The result shows $R^2 >0.99$ with 97% efficiency of the PCR, with a slope of –0.2725 (B) Real time RT-PCR of the β- carotene hydroxylase gene expression for in citrus peel. The level of the gene expression was calculated after normalizing against GAPDH in each sample and is presented as relative mRNA expression units (n=3 per time point).

-carotene hydroxylase gene expression by real time RT-PCR   Real time

PCR analysis revealed that expression of -carotene hydroxylase gene was

decreased during development and ripening of citrus fruit (Figure 18).
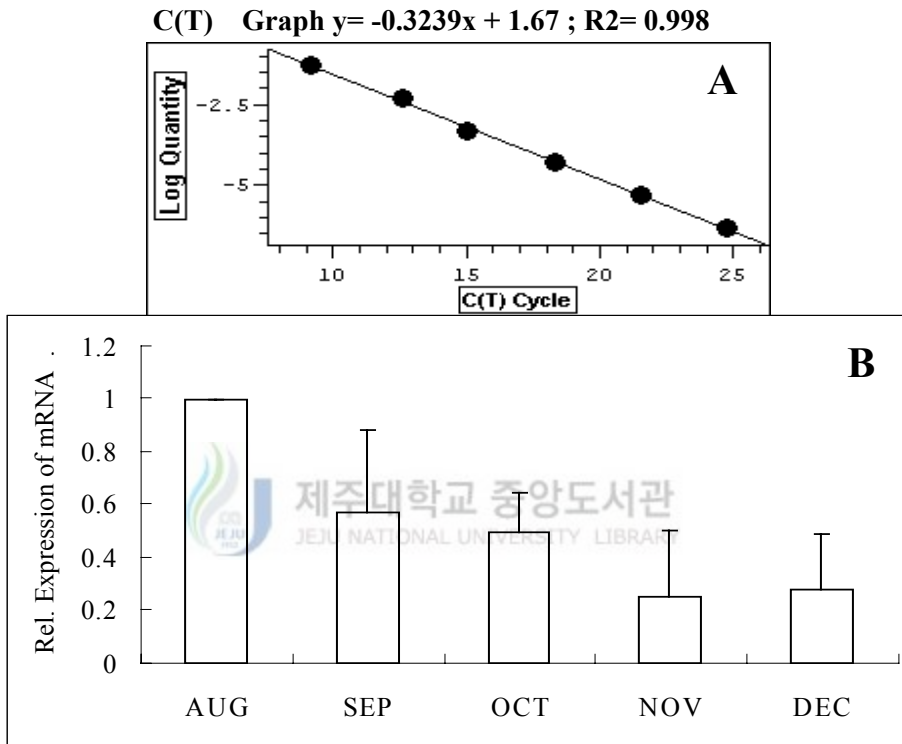
Figure 18. Real time RT-PCR analysis for -carotene hydroxylase gene expression.

(A) Standard curve for -carotene hydroxylase gene expression. The curve derives from plotting Ct against log quantity of fluorescence. The result shows R2 >0.99 with 97% efficiency of the PCR, with a slope of –0.3239 (B) Real time RT-PCR of the -carotene hydroxylase gene expression for in citrus peel. The level of the gene expression was calculated after normalizing against GAPDH in each sample and is presented as relative mRNA expression units (n=3).

Isopentenyl pyrophosphate isomerase (IPP) gene expression by real time RT-PCR Gene expression of Isopentenyl pyrophosphate isomerase (*IPP*) was increased in coloration stage of citrus fruit.

C(T) Graph y= -0.2725x + 1.59; R2= 0.996
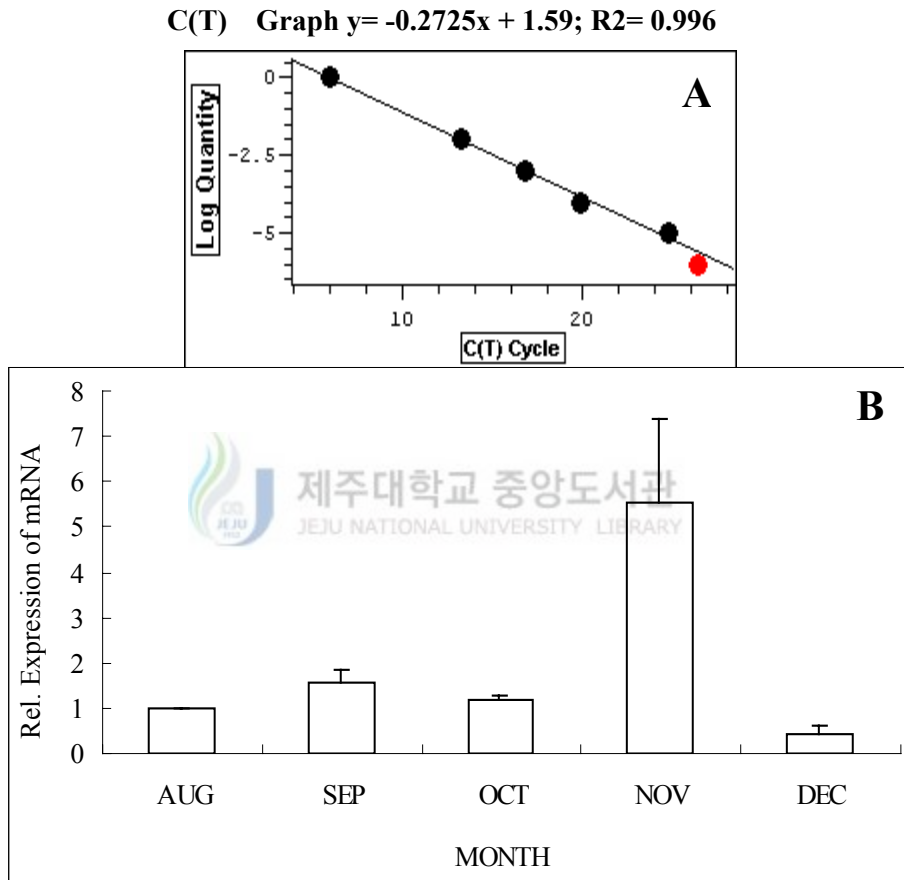
Figure 19. Real time RT-PCR analysis for IPI gene expression.

(A) Standard curve for IPP gene expression. The curve derives from plotting Ct against log quantity of fluorescence. The result shows R2 >0.99 with 97% efficiency of the PCR, with a slope of –0.2725 (B) Real time RT-PCR of the IPP gene expression for in citrus peel fruit during development and maturation. The level of the gene expression was calculated after normalizing against GAPDH in each sample and is presented as relative mRNA expression units (n=3).

DXP synthase gene expression by real time RT-PCR Expression of DXP synthase gene showed no difference in peel of citrus during development and maturation.

C(T)   Graph y= -0.3239x + 1.67 ; R2= 0.998
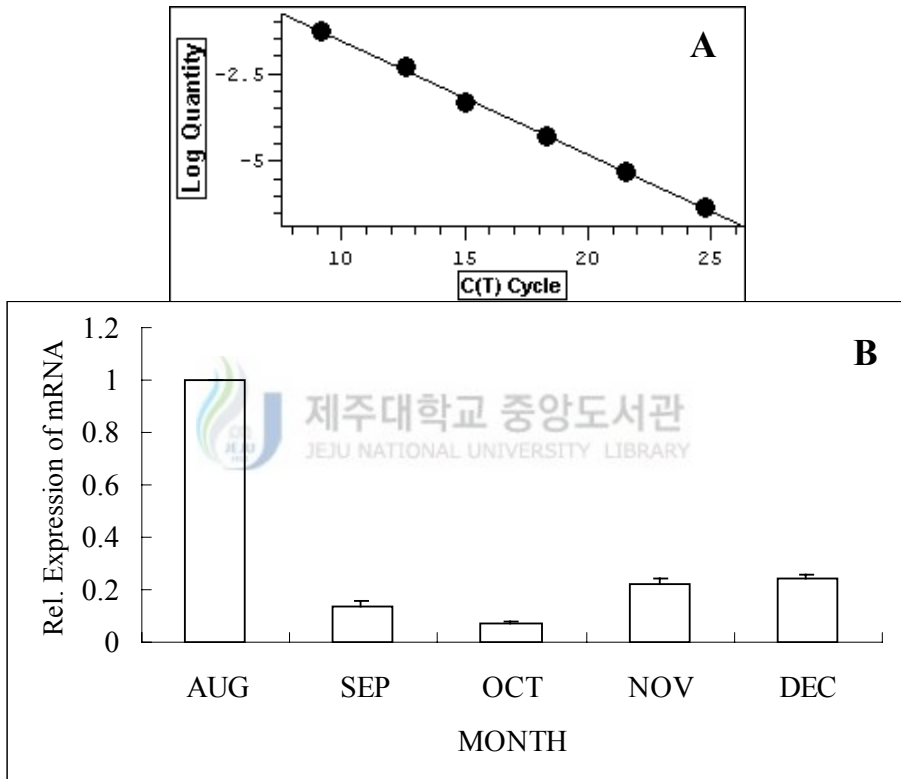
Figure 20. Real time RT-PCR analysis for *DXPS* gene expression.

(A) Standard curve for DXPS gene expression. The curve derives from plotting Ct against log quantity of fluorescence. The result shows R2 >0.99 with 97% efficiency of the PCR, with a slope of –0.3239. (B) Real time RT-PCR of the DXPS gene expression for in citrus peel fruit during development and maturation. The level of the gene expression was calculated after normalizing against GAPDH in each sample and is presented as relative mRNA expression units (n=3).

Phytoene synthase gene expression by real time RT- PCR   Phytoene synthase

gene expression was increased in coloration stage (November).

C(T)   Graph y= -0.2583x + 1.47 ; R2= 0.982
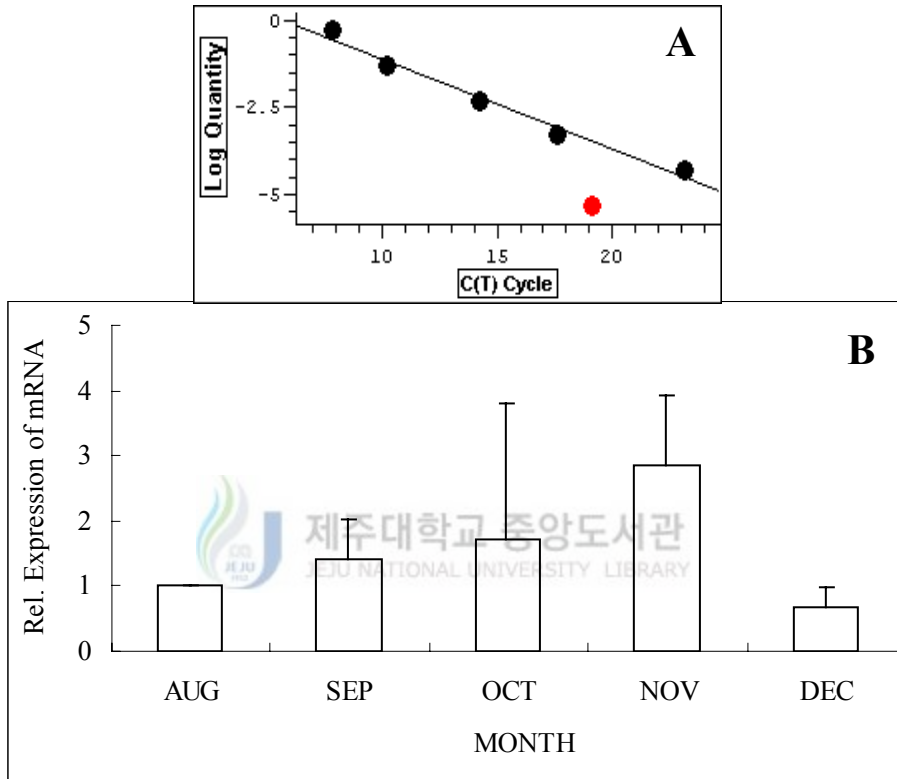
Figure 21. Real time RT-PCR analysis for phytoene synthase (*PSY*) gene expression.

(A) Standard curve for PSY gene expression. The curve derives from plotting Ct against log quantity of fluorescence. The result shows with a slope of –0.2583 (B) Real time RT-PCR of the PSY gene expression for in citrus peel fruit during development and maturation. The level of the gene expression was calculated after normalizing against GAPDH in each sample and is presented as relative mRNA expression units (n=3).

**Violaxanthin diepoxidase gene expression by real time RT-PCR**　Expression of VDE gene showed differences in citrus peel during development. Expression of this gene was higher at early fruit stage compared to mature green (October) and ripening (November and December) stages in fruit peel.

C(T)　Graph y= -0.2583x + 1.47 ; R2= 0.982
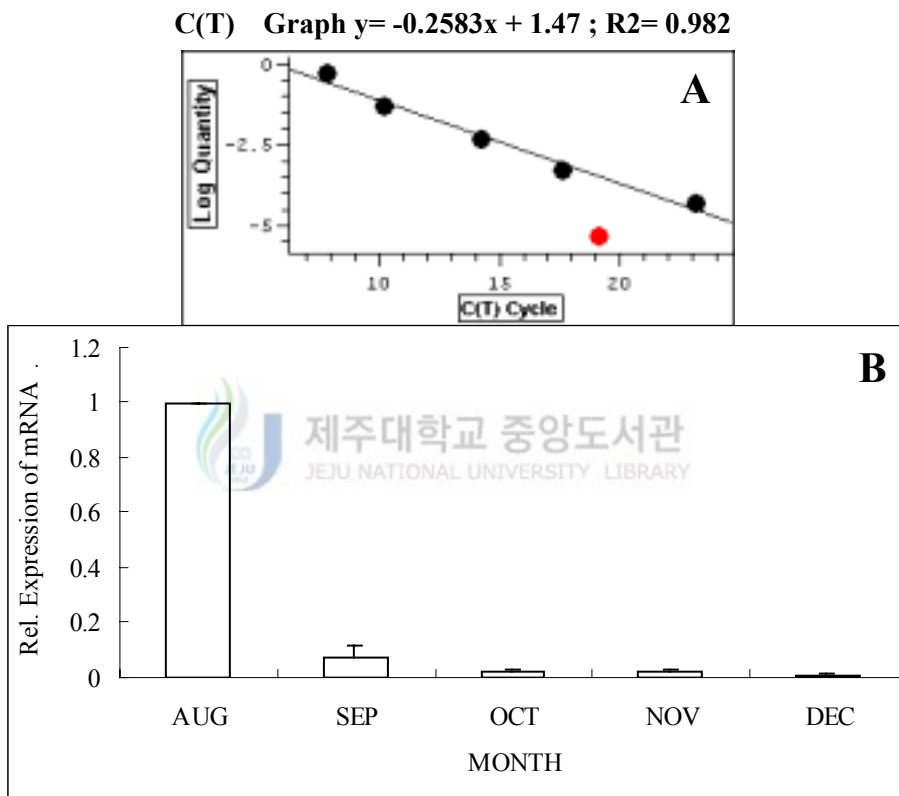
Figure 22. Real time RT-PCR analysis for violaxanthin diepoxidase (*VDE*) gene expression.

(A) Standard curve for DXPS gene. The curve derives from plotting Ct against log quantity of fluorescence. The result shows R^2 =0.982 with a slope of –0.2583 (B) Real time RT-PCR of the VDE gene expression for in citrus peel fruit during development and maturation. The level of the gene expression was calculated after normalizing against GAPDH in each sample and is presented as relative mRNA expression units (n=3).

Carotenoid cleavage dioxygenase gene expression by real time RT-PCR

Expression of carotenoid cleavage dioxygenase gene showed no difference in fruit development stages.

C(T)   Graph y = -0.2583x + 1.47 ; R2= 0.982
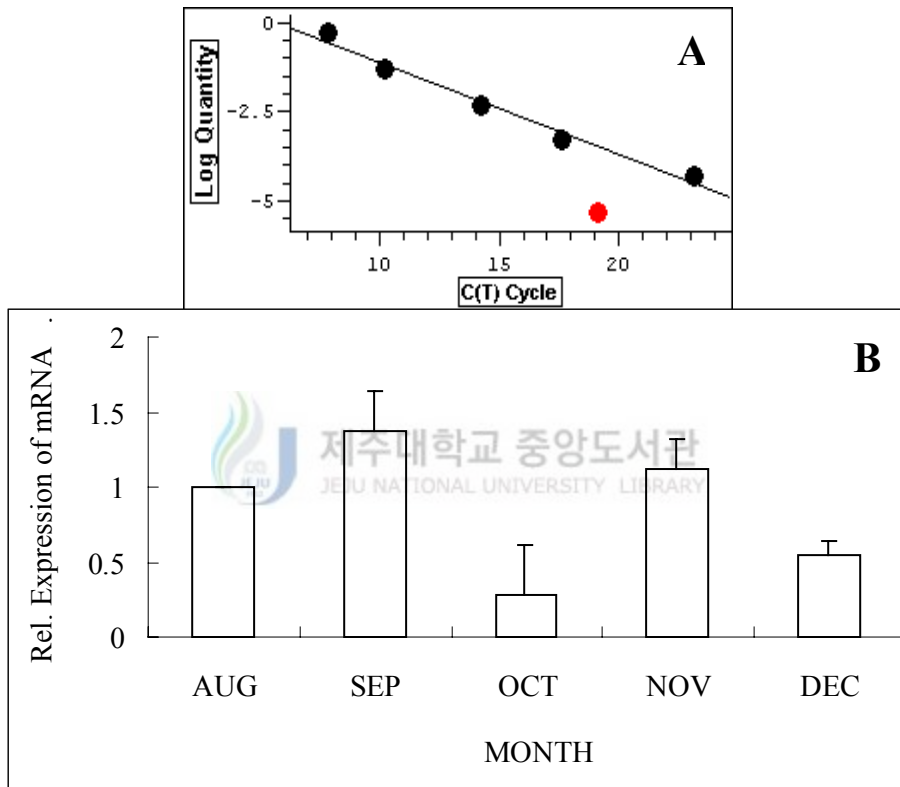
Figure 23. Real time RT-PCR analysis for carotenoid cleavage dioxygenase gene expression.

(A) Standard curve for DXPS gene. The curve derives from plotting Ct against log quantity of fluorescence. The result shows R^2 =0.982 with a slope of –0.2583 (B) Real time RT-PCR of the CCD gene expression for in citrus peel fruit during development and maturation. The level of the gene expression was calculated after normalizing against GAPDH in each sample and is presented as relative mRNA expression units nt (n=3).

DISCUSSION

Biosynthetic pathway of carotenoids in plants

The initial steps of the pathway involve condensation of three molecules of acetyl-CoA to produce the C6 compound 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA), which after reduction yields MVA. In the next two steps, mevalonate kinase and mevalonate 5-diphosphate kinase catalyze MVA to form mevalonate 5-diphosphate, which is subsequently decarboxylated to yield IPP. The mevalonate pathway provides IPP for the synthesis of some sesquiterpenes, sterols, and triterpenes and is localized in the cytosol (Figure 24).

Higher plants contain plastidic DXP/MEP pathway for isoprenoid biosynthesis. In this pathway, the initial step is condensation of pyruvate and glyceral- dehyde-3-phosphate which yields DXP; the latter product can be converted to IPP. Subsequent reactions of this pathway involve transformation of DXP to MEP, which after condensation with CTP forms CDP-ME. Thereafter, CDP-ME is phosphorylated to CDP-ME2P and to ME-2, 4CPP. hydroxymethylbutenyl 4-diphosphate synthase catalyzes the formation of HMBPP from ME-2,4cPP, which is subsequently converted to IPP and DMAPP in a certain ratio (Cunningham *et al.*, 2000; Rohdich *et al.*, 2002).
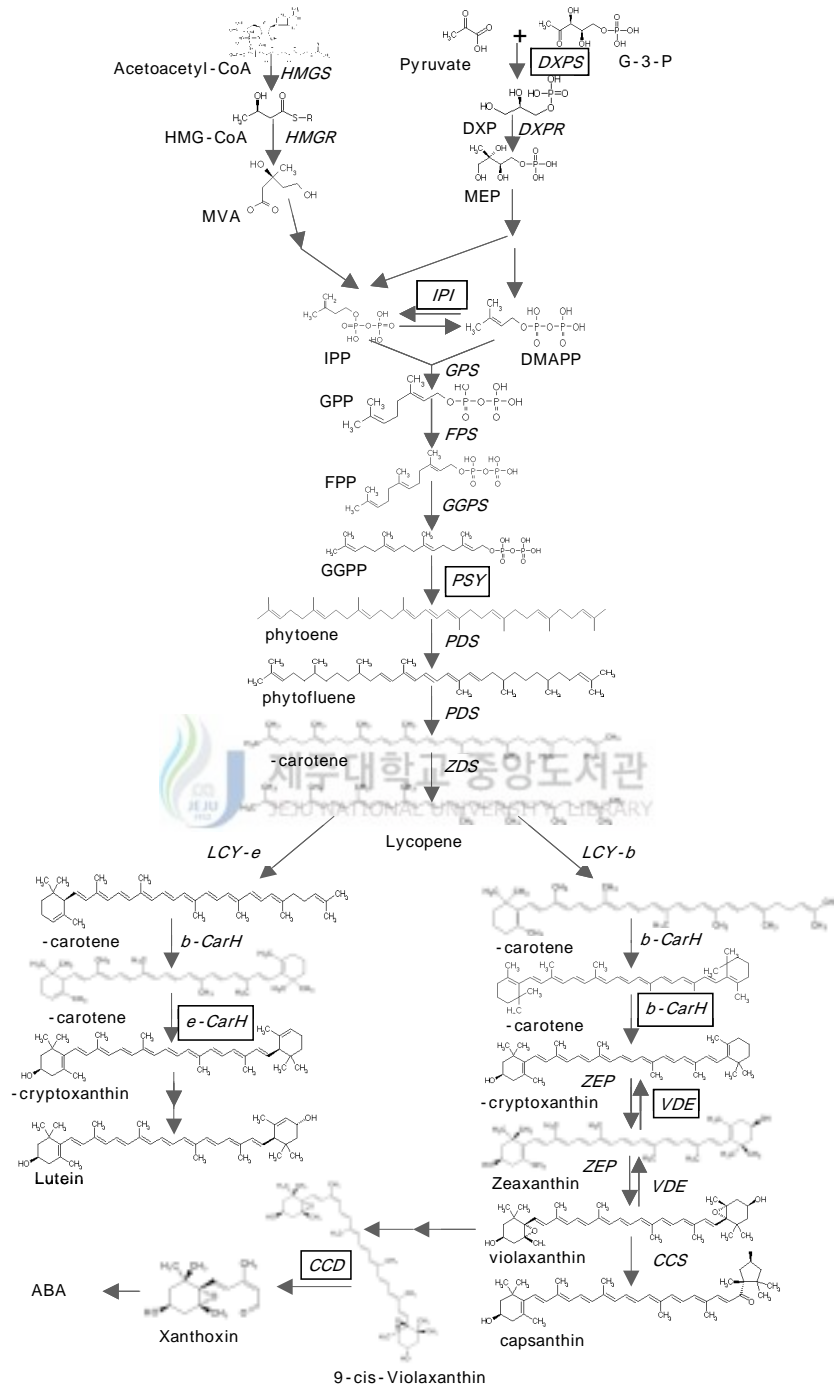
Figure 24. Carotenoid biosynthetic pathway in plants.

IPP is the basic C5 building block that is added to prenyl pyophosphate cosubstrates to form longer chains. Condensation of dimethylallyl diphosphate with one IPP in a head-to tail fashion generates GPP; addition of a second IPP unit generates FPP; a third IPP generates GGPP.

Isoprene, the simplest of the terpenoids, is synthesized directly from DMAPP by diphosphate elimination. The reaction is catalyzed by the enzyme isoprene synthase (Silver and Fall, 1991; Kuzma and Fall, 1993) Higher terpenoids are generated via the action of prenyl transferases, which perform multistep reactions beginning with DMAPP (or a longer allylic pyrophosphate) and IPP to form higher isoprenologs, generally with all *frans* geometry. GPP synthase forms the C_{10} intermediate (GPP) from DMAPP and IPP. This synthase has been characterized in a number of plant species and is clearly separable from FPP synthase (Endo and Suga, 1992). FPP synthase forms the C15 intermediate (FPP) in two discrete steps: first DMAPP and IPP form GPP, which remains bound to the enzyme; then another IPP is added to yield FPP. GGPP synthase operates in a similar manner, via three condensation steps, to form the C20 intermediate. Rubber (*cis*-l, 4-polyisoprene) is a linear polyprenoid consisting of 400 to more than 100,000 isoprene units. Rubber biosynthesis is primed by a trans-allylic diphosphate initiator that is then extended by a cis-prenyltransferase, the rubber transferase (Cornish, 1993). In contrast to the linear polymer rubber, most terpenoids are cyclic and the various classes are formed from the branch point C_{10}, C_{15}, C_{20}, and C_{30} intermediates by enzymes called cyclases or

synthases. The reactions of the monoterpene cyclases GPP is first ionized and isomerized to enzyme-bound linalyl pyrophosphate (LPP), the tertiary allylic isomer. This preliminary isomerization step is necessary because the trans-2, 3-double bond of the geranyl precursor prevents direct cyclization. Ionization of LPP promotes cyclization to the terminal double bond to yield the enzyme-bound α-terpinyl cation, a universal intermediate of these cyclization reactions.

Changes of carotenoid composition during fruit development

The composition of carotenoids was analyzed in the peel of 'Shiranuhi' mandarin at five developmental stages: August September, October, November and December fruit. With an HPLC analysis using a C_{18} column and by comparison of spectra with those spectroscopic characteristics of peak identify 16 individual carotenoids. Also, -Cryptoxanthin, ζ -Carotene, Phytofluene, Capsanthin, β-carotene were confirmed by LC/MS. The chromatographic and spectroscopic features of more relevant carotenoids found in the peel of citrus 'Shiranuhi' mandarin are summarized (Table 4).

The characters of carotenoid composition in peel during development and ripening of the 'Shiranuhi' mandarin were variety of carotenoids (Figure 4). The main carotenoids identified in the peel of early stage (August) were lutein and β-carotene.

Satsuma mandarin (*Citrus unshiu*) accumulates a large amount of carotenoids that lead to an orange pigmentation (Ikoma *et al.*, 2001). The main carotenoid was lutein and violaxanthin in flavedo from immature green citrus fruits (*C. sinensis*) (Rodrigo *et al.*, 2004) and the fruit assumed yellow color, but the ripe tomato fruit accumulated large amounts of lycopene (Bramley, 2002) and the fruit color was red.

Changes of carotenoid content during fruit development

HPLC profiles of the carotenoids from citrus peel were analyzed at five developmental stages. Six carotenoid pigments were identified. In green stages fruit (August, September and October), main carotenoids were β-carotene and lutein. However, in ripening stages (November and December), several types of carotenoid were detected. The number of carotenoid forms was increased during coloration and ripening stages of 'Shiranuhi' mandarin. The β-crypoxanthin, zeaxanthin, phytofulene, capsanthin and ζ-carotene were newly detected and relative contents of lutein and β-carotene were decreased during this stage (Figure 25 A, B). It was reported that lutein and α-and β-carotene were main carotenoids while β-cryptoxanthin and zeaxanthin were not detectable in peel of immature green fruits. In peel of mature fruit, α-and β-carotene were hardly detectable, but lutein, zeaxanthin and especially β-cryptoxanthin were the major carotenoids (Ikoma *et al.*, 2001).

From peak area percentages indicated that lutein was a predominant carotenoid form at early stage (August), approximately 50%. In contrast, There was no predominant carotenoids in coloration stages (November and December), but could detect several kinds of carotenoid form. The results agree with the previous report in orange (*C.sinensis*) (Rodrigo *et al.*, 2003; Kato *et al.*, 2004; Rodrigo *et al.*, 2004) and in Satsuma mandarin (*C.unshiu*) (Kato *et al.*, 2004).
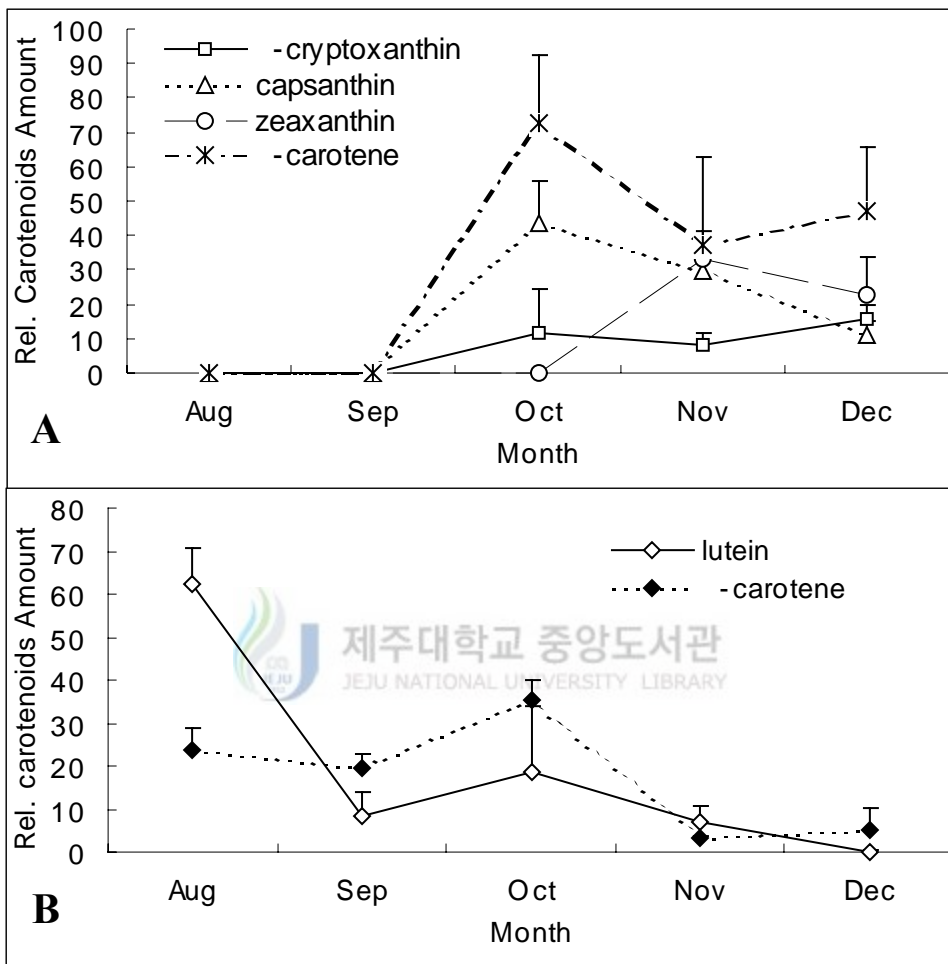
Figure 25. Changes of carotenoid levels in the fruit peel during fruit development.

Changes of expression of related genes

Expression of both *β-CarH* and *PSY* gene increased during maturation, the highest transcript level was showed in November. After that time decreased expression level of these genes. On the other hand, *ε-CarH* gene expression was showed a tendency to reduce along with the fruit development and maturation (Figure 26). During the orange stage, *β-CarH* and *PSY* gene controlling β, β-xanthophylls synthesis were increased simultaneously in the expression of genes to participate, led to the massive accumulation of β, β-xanthophylls in the flavedo of satsuma mandarin and Valencia orange (Kato *et al.*, 2004). This result agrees with our experiments but according to another report the gene expression of *PSY* increased in the peel with the onset of coloration, whereas the genes expression of *β-CarH* was constant in all stages during fruit development (Kim *et al.*, 2001).
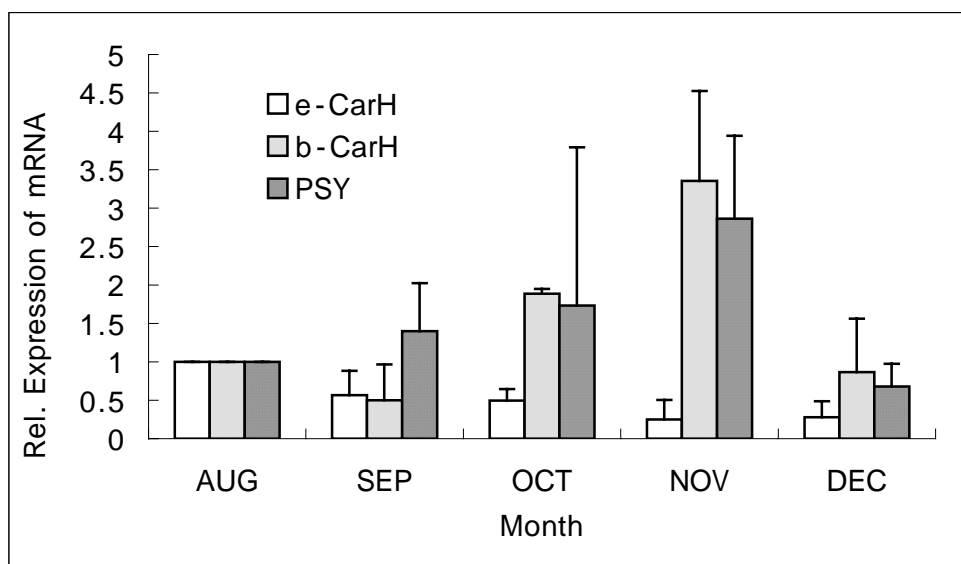
Figure 26. Expression pattern of PSY, β-CarH and ε-CarH during development and maturation in citrus fruit peel.

In plants, the five-carbon compound isopentenyl diphosphate (IPP) and its allylic isomer and dimethylallyl pyrophosphate (DMAPP) are served as substrates for the synthesis of carotenoids and other isoprenoids. DXP catalyzed by DXPS is utilized in plastidic IPP biosynthesis (Estevez *et al.*, 2001a).

DXPS gene expression was the highest in August than other fruit developmental stages, while *IPI* gene expression was the highest in November (Figure 26). This result suggests that expression of *DXPS* gene was not changed in coloration stage but *IPI* gene was highly expressed. This expression pattern was similar to that of -*CarH* gene. In case of *Arabidopsis*, DXPS has been known as key enzyme at an

early stage of leaf development (Araki N. *et al.*, 2000). It also has been suggested that DXPS catalyses the first potentially regulatory step in carotenoid biosynthesis during early fruit ripening in tomato(Lois *et al.*, 2000). These showed same tendency as our results.

Expression level of *VDE* was lower during ripening stage (Figure 22). We couldn't also detect the violaxanthin by HPLC. In the future, the correlation between violaxanthin synthesis and *VDE* gene expression will be proved 'Shiranuhi' mandarin. At the stage corresponds to the onset of ripening to mature, significant induction of the gene expression approaching was observed in grapes.

A significant induction of carotenoid Cleavage Dioxygenase (*VvCCD1*) expression in the grape berries (*Vitis vinifera*) during the week preceding the onset of ripening was observed, after this stage, the expression of the gene remained almost stable throughout the ripening stages (Mathieu *et al.*, 2005). Expression of *CCD* gene was not significantly changed during citrus fruit developmental stages in this study.

Carotenoid relative content and genes expression

The ß-carotene hydroxylase catalyzes the hydroxylation reaction from ß-carotene to zeaxanthin via ß–cryptoxanthin. Transcript level of ß-carotene hydroxylase was increased during maturation and at the same time downstream

products such as ß–cryptoxanthin and capsanthin were increased. However, ß-carotene content, the substrate of ß-carotene hydroxylase, was decreased during this stage (Figure 27A, B).
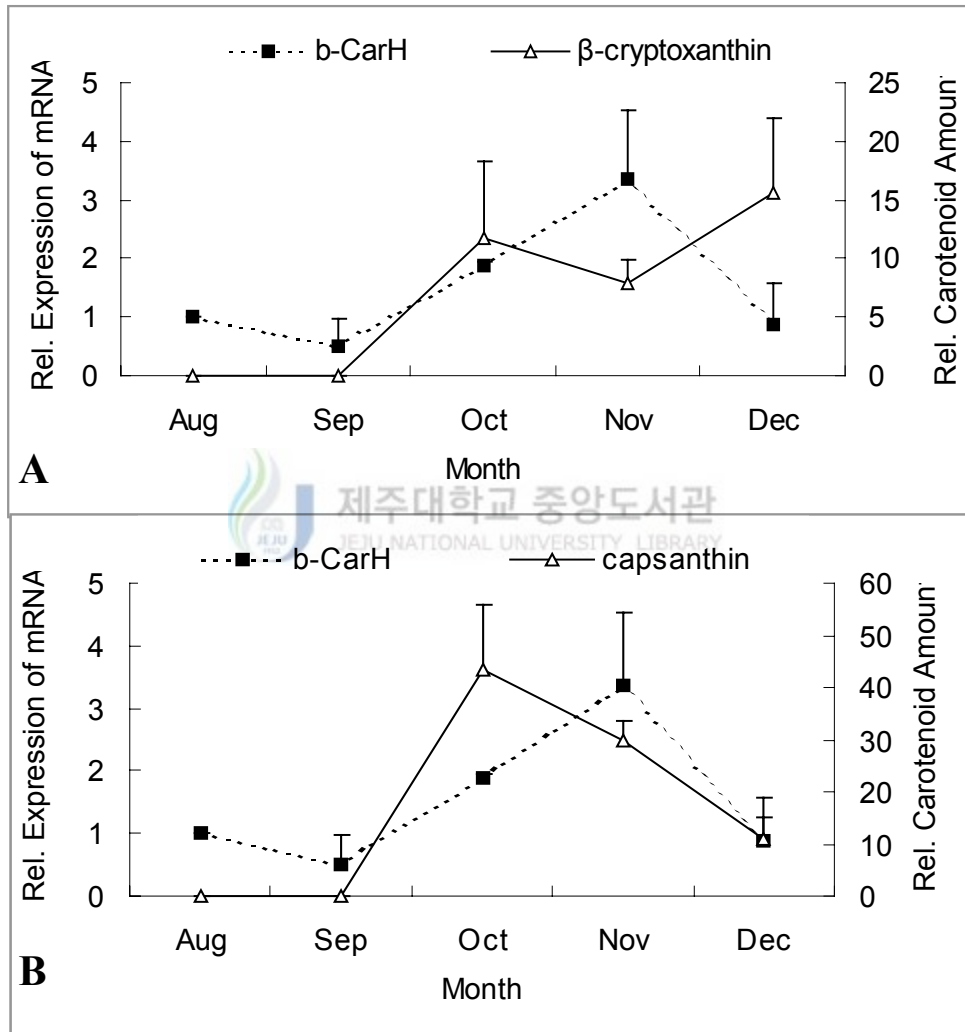
Figure 27 Relationship between xanthophylls accumulation and *b-CarH* expression.
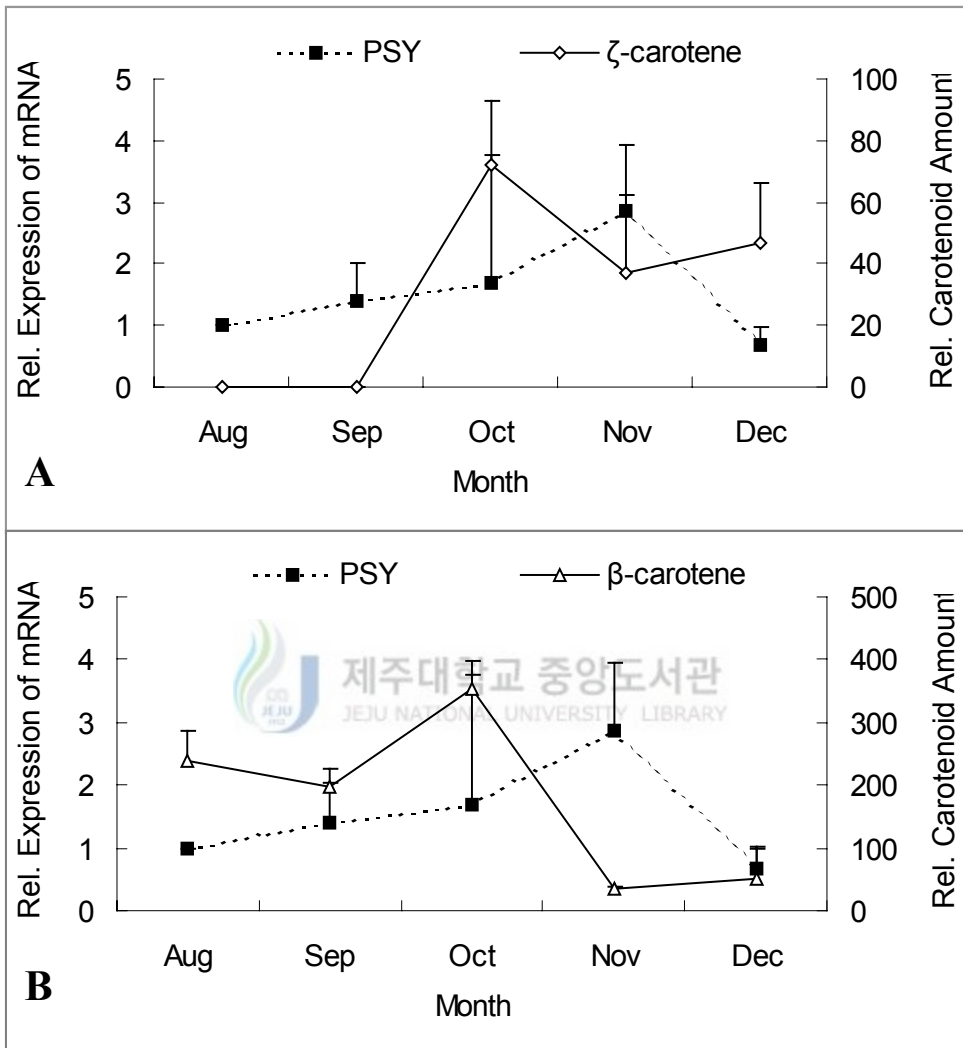
Figure 28. Relationship between β-carotene accumulation and *PSY* expression.

Gene expression assayed by real time RT-PCR and carotenoid content derived

HPLC chromatogram profile.
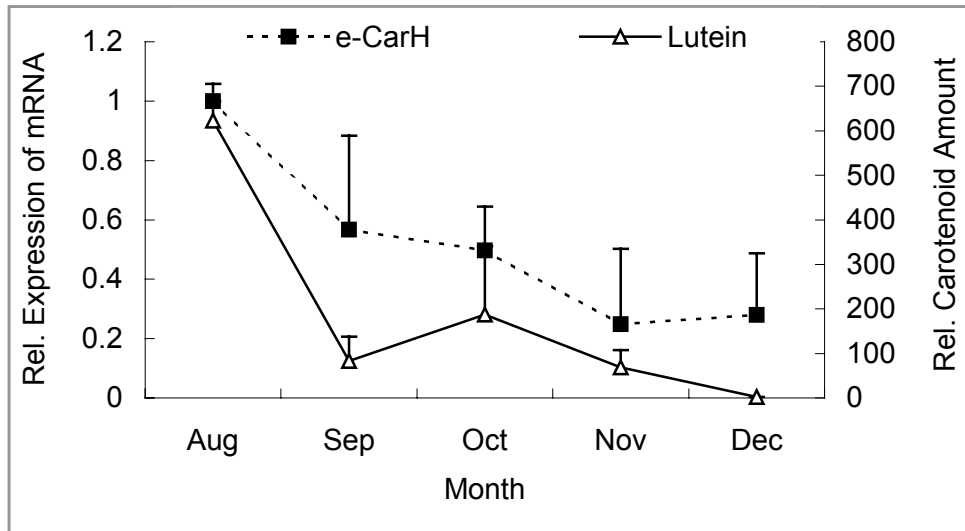
Figure 29. Relationship between utein accumulation and *ε-CarH* expression.

Gene expression assayed by real time RT-PCR and carotenoid content derived HPLC chromatogram profile.

Phytoene synthase converts GGPP to phytoene, the carotenoid precursor. Expression of *PSY* was increased during maturation in fruit peel and ζ- carotene was simultaneously accumulated. However, β-carotene content was decreased in this period (Figure 28 A, B). Earlier reported the phytoene synthase (*CitPSY1*) transcript in the peel was a low level in the young fruit, and it increased toward maturation in satsuma mandarin (*Citrus unshiu*) (Kita *et al.*, 2001). As fruit maturation progressed

in satsuma mandarin (*C. unshiu*) and Valencia orange (*C. sinensis*), a increase in the expression of *CitPSY* led to massive , - accumulation in the flavedo (Kato *et al.*, 2004). In sweet orange (*C. sinensis* L. Osbeck, cv. Navelate) fruit during development and maturation accumulation of carotenoids during fruit ripening is coincident with up-regulation of the *PSY* gene (Rodrigo *et al.*, 2004). Other fruit such as tomato(Giuliano *et al.*, 1993) and pepper (Romer *et al.*, 1993) accumulation of carotenoids correlated with PSY of increase expression during fruit ripening.

ε-carotene hydroxylase catalyzes the conversion of α-carotene to lutein. Expression of ε-carotene hydroxylase was higher at early stage (August) than later stages. Relative content of lutein was decreased during fruit development and maturation in fruit peel (Figure 29). *ε-CarH* was cloned in *Arabidopsis* (Tian *et al.*, 2004). This is the first report on the relationship between *ε-CarH* expression and carotenoid biosynthesis.

APPENDIX

The nucleotide and deduced amino acid sequences of carotenoid biosynthetic

genes cloned in this experiment.

Ci - DXPS

CCACCAACACCATTGTTGGACACGATTAATTACCCAATTCATATGAAGAATCTCTCTAAAGAGGATCTTG
AACAACTGGCAGCAGAGCTTAGAGCAGATATTGTTAACAGTGTATCGAAGACAGGTGGGCATCTTAGTGC
AAACTTAGGAGTGGTGGAGCTAACACTTGCTTTGCATCGTGTTTTCAACACACCTGACGATAAAATTATA
TGGGATGTTGGCCATCAGGCTTATGTACACAAAATTCTGACTGGAAGAAGATCCAGAATGAACACCATGA
GGAAGACTTCGGGGCTTGCAGGATTCCCCAAAAGAGAGGAAAGCGTTCATGATGCATTTGGTGCAgGACA
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AAACTTCGTGAAGCCGCAAAAAGCATCACTAAGCAAATTGGTGGGCAAACACATGAAGTTGCTGCAAAGG
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GCGCCAGGGCCAGTTCTGATCCATGTTGTAACAGAGAAGGGAAAGGGCTATCCCCCAGCGGAGGCAGCAG
CTGATAGAATGCATGGAGTTGTCAAGTTCGATCCAAAAACTGGCAAGCAATTTAAGACCAAATCCCCTAC
GCTCTCGTATACACAGTACTTTGCTGAATCTTTGATAACGGAAGCTGAGACAGACGATAAGATTGTGGCC
ATCCACGCTGCAATGGGTGGTGGTACTGGCCTCAATTATTTCCAGAAAAGGTTTCCAGATCGCTGCTTCG
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TTGTGCCATCTACTCATCATTTCTGCAAAGAGGATATGATCAGGTTGTGCATGATGTAGACCTTCAAAAA
TTACCAGTCCGCTTTGCAATGGATCGGGCTGGTTTGGTTGGTGCGGATGGACCTACTCATTGTGGAGCAT
TTGACATCACATTCATGTCTTGCTTGCCCAACATGGTGGTCATGGCTCCATCGTGATGAAGCTGAGCTAAT
GCACATGGTCGCTACAGCAGCAGTTATAGATGACAGGCCCAGCTGTTTCAGATTTCCAAGGGGCAACGGA
ATTGGAGCAGTTCTCCCACCTAATAACAAAGGAACTCCACTTGAGATTGG

Ci - GGDR

TGAAGCCACACGAGTACATCGGTATGGTGAGGCGGGAGGTTCTGGACGCGTATTTGAGGGAGAGGGCGGA
GAAGAACGGGGCGAGCGTGATTAACGGGCTGTTCATGAAGATGGATTTGCCGAGGAACTTTGAGCAGCCG
TACGTTTTGTATTATACGGAGTACGATGGGACAAAGGGCGGGCGTCGGTGAGAAGAGGACGCTGGAGGTTG
ATGCTGTTATCGGAGCTGATGGTGCTAACTCCAGAGTTGCCAAGTCCATCAATGCTGGTGACTATGATTA
CGCCATTGCATTTCAGGAAAGAGTCAAAATCCCTGACGAGAAAATGGTGTACTATGAGAACTTGGCGGAG
ATGTACGTTGGTGATGATGTGTCGCCGGACTTTTATGGGTGGGTGTTCCCCAAGTGTGATCATGTGGCAG
TTGGTACAGGCACAGTGACACACAAAGGTGATATCAAGAAGTTCCAACTTGCAACTAGAAACCGGGCCAA
GGACAAAATCCTTGGTGGCAAGATCATTCGAGTGGAGGCACACCCGATTCCTGAACACCCCCCGCCCGCGG
AGGCTATCGGGGAGAGTGGCATTAGTTGGAGATGCAGCCGGGTACGTCACGAAATGCTCTGGCGAAGGCA
TTTACTTTGCGGCAAAGAGTGGAAGAATGTGTGCCGGAGGCCATCATTGAAGGGTCAGAGAATGGAACCAG
GATGATCGATGAAGGGGACTTGAGGAAGTATTTGGAGAAGTGGGATAAGACTTACTGGCCAACTTATAAG
GTACTGGATGTATTGCAGAAGGTGTTTTTACCGATCGAATCCAGCGAGGGAAGCATTCGTAGAAATGTGCG

CCGACGA

Ci-e-CarH
GCTTCATTGATGAAAGCACAAGAAGAGATTGATAGAGTATTGCAGGGAAGATCTGCATCCTTTGAAGACA
TAAAAGATCTGAAATTCCTAACACGTTGCATAAATGAGTCAATGCGTCTCTACCCACATCCTCCTGTCTT
GATAAGAAGAGCTCAAGTAGATGATGTGCTCCCTGGAAATTACAAGGTTAATGCTGGTCAAGATATAATG
ATTTCAGTTTACAATATCCACCATTCCTCACAGGTCTGGGAGAGAGCAGAAGAGTTTTTACCAGAAAGGT
TTGACTTGGAAGGCCCCATGCCCAATGAAACAAATACAGATTTTAGATTCATTCCTTTCAGCGGAGGGCC
TCGGAAGTGTGTTGGAGATCAGTTAGATCTGG

Ci-ZEP-T7
TTCATCTATGTTCTACAATTCAGTTAATCTTTCAACAACTGTTTTCTCAAGAACCCACTTCCCAGTTCCT
GTATACAAACATTCTTGCATTGATTTTTCACGTTATGATCACTGTATTAACTACAAATTCAGGACCGGAA
CAAGTGGGCAAAGCAAGAACCCGACGCAAATGAAAGCTGCAGTGGCAGAGTCCCCTACTAATAACAGTGA
CAGTGAAAACAAGAAATTGAGGATTTTGGTGGCTGGTGGTGGGATTGGAGGACTTGTATTTGCTTTGGCT
GCTAAAAGGAAAGGATTTGAAGTGCTGGTGTTTGAGAAAGATATGAGTGCTATAAGAGGTGAGGGACAAT
ACAGAGGTCCAATTCAGATACAAAGCAATGCATTGGCTGCTTTGGAAGCTATTGATTTGGATGTTGCTGA
GGAAGTTATGAGAGCTGGATGTGTTACCGGTGATAGAATTAATGGTTTGGTTGATGGGATTTCTGGTTCT
TGGTACATCAAGTTTGATACTTTCACTCCTGCAGCAGAAAAGGGACTTCCAGTCACAAGAGTAATTAGCC
GAATGACTTTGCAACAAATACTTGCTAAAGCGGTTGGGGATGAAATCATTTTGAATGAAAGTAATGTTAT
TGATTTTAAGGATCATGGAGATAAGGTCTCCGTAGTGCTTGAGAATGGACAGTGTTATGCTGGTGATCTT
CTC

Ci-ZEP-SP6
ACACTGCCTGAAGAATTTCACCCGCTTCCTTTCTCTCTGAATTATTATTTGGAGGAGTTCCCATCACCTT
CACCCGAAAAATTGCCTTTTTATCAGAGCCAAACTCAATAGTGTCTGACGGACGAAACCGAGCAGGAAAG
TTTGAAGATACCCTATACCTTCGCCCTTCATTGTCTGTGACATAGGTTCCATGTTCGCTCTGCAAATCAA
TCAGGTAGAAGGCACCATCTTTATAGCTGATACGAGCATGCATTTTGGACACCTGCGCTGAAGGGATCAC
AATCGATGTTCGAGAAAAATCCTCGTGACTTTCACTTCCAATTAAATAGGGTTCATTCTCATGTGATACA
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CACGCTCCAATGCGTCATCGTCACGAAACCATGTTCGTAAGTTGTCACTTGCCTTGTCCGAGAGCTTGCA
ACACGGTGACCTACCTTCAAGTTTTGAGCTGTTGCCACCTAAGACCCAACTAAGCATTAAGGGCATTGCT
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TCCAAGTCCACACCTAAATAAGCCTTGTAAGTTGAAGCCATCACTGCGGCCGATCTTGCCAGTCCATGGA
TA

Ci-VDE
TGCGGTCTCAAGAAAGAAGTGTGTACCACAGAAATCTGATTTAGGGGAATTTCCTGTCCCTGATCCTGCC
ATTCTAGTTAAAAGTTTTAACATCAAAGACTTCAATGGGAAGTGGTACATTTCTAGTGGTTTAAATCCTT
CCTTCGATACTTTTGATTGCCAATTGCATGAATTCCATACAGAATCCAGCAAACTCATGGGAAATTTATC
GTGGAGAATAAGAACTCCAGATGGTGGCTTTTTCACCCGATCAGCTATGCAGAGATTTGTTCAAGATCCA
ATTCATCCTGGGATACTCTATAATCACGACAATGAATACCTTCACTATAAAGATGACTGGTATATATTGT
CATCCAAGATACAGAATGAACCAGATGACTATGTCTTTGTGTACTATCGGGGCAGCAAT

Ci-lyc-b-T7
GCCAAGGATACTGTACTCAAAACTCATAACAAGCTTGAATTCTTGCCCCAAGTTCACGGGGCTTTGGAAA
AATCCAGTAGTTTAAGCTCATTGAAGATTCAGAACCAGGAGCTTAGGTTTGGTCTCAAGAAGTCTCGTCA
AAAGAGGAATATGAGTTGTTTCATTAAGGCTAGTAGTAGTGCTCTTTTGGAGCTAGTTCCTGAAACCAAG
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TCGGTGGTGGCCCAGCTGGGCTTGCTGTTGCTCAGCAAGTTTCAGAGGCGGGGCTTTCGGTTTGCTCGAT

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TGATCCATCTCCCAAATTGATTTGGCCAAATAATTATGGTGTTTGGGTGGATGAATTTGAGGCCATGGAT
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TGGTGTTAAGTTCCACCAAGCTAAAGTTATTAAGGTTATTCATGAAGAGTCCAAATCTTTGTTGATTTGC
AATGATGGTGTGACAATTCAGGCTGCCGTGGTTCTTGATGCTACGGGATTCTCTAG

Ci-lyc-b-SP6
TGGTCACCTTAATCTGTATCTTGTACCAAGTTGTTGATCATGTTAACCAAAGGAAGAGTGCCCTTTGCCA
TGATCTCTAGCCTAGAAGTATTAGAGGCATGTGAGAATAGAGAAAGCCCAAAAACTAAAAGCTCGGGGAG
AAACAATCTCGATGATAAGAAACCATGCCAATAACGGGGCTCCAGATCAAAAAAAGCATCGAAAAACCTT
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TTTCTATGGGCCACAAATCTTTCCAAACTTCAGCAGACAATTTGTGTCCTGAAATGCTTCTGTCAGAACT
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GAAGGGTGCACCATCCCAGCGGTACCACCTATTCCAACAACTCTTTGAGGAAGCACTGGAAGGGGCCCAC
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ATCCTGTTTGACGAA

Ci-lyc-e
TTTATACTACCGCCAATATCAATTGGTAATGGTATTTTGGATTTGGTGGTGATTGGTTGTGGCCCAGCTG
GTCTTGCTTTGGCTGCAAAATCAGCGAAGTTGGGATTAAATGTTGGACTTATTGGCCCGGATCTCCCTTT
CACAAACAATTATGGTGTGTGGGAAGATGAATTTAGAGATCTTGGACTTGAAGGGTGTATCGAACATGTC
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AGTGGAAAGCATTACCGAATCTACCAGTGGTCATCGTCTTGTAGCTTGTGAACATGATATGATTGTCCCC
TGCAGGCTTGCTACTGTTGCTTCTGGAGCAGCATCAGGGAAGCTATTGGAATATGAGGTGGGGGGTCCCA
AAGTTTCTGTCCAAACAGCTTATGGTGTGGAGGTTGAGGTGGAAAATAATCCATATGATCCAAGCCTTAT
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GATCCAACTGGAGC

Ci-PDS-T7
GGACACATGCTTGCCTCAGCCAATCTCCCTTTCCCCCCGTGCAGCAAGCAGCACATAGTCCTGTACAATTG
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TGCCATCTCTTTCCAGTTTTCAGGTAACTGAAGCTTGAGGATATCAACAGGTGTGGCAAATACATAAGCA
TCTCCGTCAATCACATTGCCATTAGTTAGTAAAAAAATTCTTCACAGTTCCATCATCATTGAGCTCAATTT

Ci - PDS - SP6

TTATTGCTTTCAAACGCGAAATTAATTCAACTTAATTTTGTTGGTTTCACTGTTGTCATTGTTTGGTCTT
CAGTTTGATAAATTAAAAAGGTTAAAAAAAAAAGATGAGCCTTTGCTTCAGCGTTTCTGAAAGTGCTTTC
AACTTGCGATATGGTTTCCGAGATAGTGAACCGATGGGTCAGAGCCTGAAAATTCGAGTTAAAACGGGGA
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GTTGTAATTGCTGGTGCAGGTTTGGCTGGTTTATCAACTGCAAAATATTTGGCAGATGCAGGCCACAAGC
CTTTGTTACTGGAAGCAAGAGATGTTCTAGGTGGAAAGGTAGCTGCCTGGAAAGATGGGGACGGGAACTG
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ATTAATGATCGGTTGCAGTGGAAGGAGCACTCTATGATTTTTGCAATGCCAAACAAGCCCGGAGAATTCA
GCCGATTTGATTTTCCTGAAGTTCTTCCGGCTCCGCTAATGGGATATTGGCCATTTTAAGGAATAATGAA

Ci - FDS

ATGAGTGATCTGAAGTCAAGATTCATTGAGGTGTATGGCGTTTTGAAACAAGAGCTGCTGAATGATCCTG
CATTTGAGTTCGATCACGATTCTCGCCAATGGGTTGATCGTATGCTGGATTACAATGTACCTGGAGGAAA
GCTGAACCGAGGGCTATCTGTTGTTGACAGCTACAGGTTACTGAAAGAAGGAAAAGAACTAACAGATGAT
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TGCTGCAAATGATGGTGTATTACTACGCAACCATATCTCCAGAATTCTCAAGAATCATTTTAGAGACAAG
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TGGAGTATGAGAAAGAGAGTTATGAAAAGCTGACAAAATCCATTGAAGCTCATCCTACTAAGGAAGTTCA
AGCCGTGCTAAAATCATTCTTGGCAAAGATATACAAGAGACAGAAGTGA

Ci - HMGR

AAGCTCGGTGATTGCAGACGAGCGGCTGCCATTCGCCGCGAGGCATTGCAGAAAATGACCGGGAGGTCGC
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TTACGTGCAGATTCCGGTGGGAATTGCCCGGGCCGTTGTTGCTTGATGGGTTTGAATACTCGGTTCCCATG
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AGTAGATTTGCGAGGCTGCAACATATTCAGTGCTCTATTGCAGGTAAGAATCTTTACATCAGATTTTGCT
GTACCACTGGTGATGCGATGGGCATGAACATGGT

Ci - HMGS

ATGGACATTTATTTCCCTCCTACTTGCGTCCAGCAGGAAGCTTTGGAGGCTCATGATGGTGCAAGCAAAG
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CCTTGCTAGTGAATATCCGGTTGTTGATGGGAAACTTTCTCAGACATGCTACCTCATGGCTCTTGATACT

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TGTTACAAATGTTTATGCAGCAAGTACGAGAAACTGGAGGGAAAACAATTTTCTCTTTCTAATGCTGATT
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AGGTCCAACCATCCACTTTAATACCGAAGCAAGTTGGTAACATGTACACAGCATCTCTGTATGCAGCATT
GGCTTCCCTTATTCACAACAAACACAGTGAATTGGATGGCAAGCGAGTGGTTTTGTTCTCGTATGGAAGT
GGTTTGACTGCAACGATGTTCTCATTGAAACTCAGTGAAGGTCATCATCCCTTCAGCTTGTCAAACATCC
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Ci - CCD

CCGACGTCGCATGCTCCCGGCCGCCATGGCGGCCGCGGGAATTCGATTGCCTGAGTGCCTGAATGGGGAA
TTCGTCAGGGTTGGTCCTAATCCCAAGTTTGCCCCTGTGGCCGGATACCACTGGTTTGATGGAGATGGCA
TGATTCATGGTCTGCGCATCAAAGACGGAAAGGCTACATATGTCTCCCGTTTTTGTGAGGACTTCACGTCT
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CTCATGGTTAACATGCAAATGCTTAGAGCAAAGTTGAAAGTACTGGATGTTTCATATGGAAATGGGACAG
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CAAAGTTCTGGAAGATGGAGATCTGCAAACACTTGGTATGCTTGACTATGACAAGAGATTACAACATTCC
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CATAATGCCAATGCTTGGGAGGAGGATGAAGTGGTTCTGATCACTTGCCGGCCTAGACAAGCCGGATCTAG
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CTTGAAAACTGGCCTAGCTTCACAAAAGAGATTATCGGCATCCGCTGTTGATTTTCCTAGGGTGAATGAG
TGCTACACTGGCAGGAAGCAAAGATATGTGTATGGAACAATACTAGATAGCATTGCAAAAGTCACAGGGA
TCATCAAATTTGATCTGCATGCTGAACCAGACGAAGAGAAAACAAAGCTTGAAGTTGGAGGAAATGTGAG
AGGCATCTTTGATCTGGGCCCTGGAAGATTTGGTTCAGAGGCTGTTTTTGTTCCTAGAGAGCCTGGAACC
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GTCGACCATATGGGAGAGCTCCCAACGCGT

Ci - I PI

ATACGACTCACTATAGGGCGAGCTCGGTACCCGGGGCGAATTCCAAGCTT
   1 ATGGGTGACGCTACTACTGATGCCGGGATGGACGCTGTCCAGCGCCGCCTCATGTTTGAA
     M G D A T T D A G M D A V Q R R L M F E
  61 GACGAATGCATTTTGGTGGATGAGAATGATCGCGTTGTTGGTCATGAAAACAAATACAAC
     D E C I L V D E N D R V V G H E N K Y N
 121 TGTCACTTGATGGAAAAGATTGAGTCTTTGAATTTGCTACACAGAGCCTTCAGCGTATTT
     C H L M E K I E S L N L L H R A F S V F
 181 TTGTTTAACTCAAAATATGAGCTACTCCTTCAGCAACGCTCTGGAACCAAAGTTACCTTC
     L F N S K Y E L L L Q Q R S G T K V T F
 241 CCTCTTGTGTGGACTAACACCTGCTGCAGCCATCCTCTGTACCGTGAATCTGAGCTTATT
     P L V W T N T C C S H P L Y R E S E L I
 301 GAGGAGAACGCTCTTGGGGTGAGGAATGCTGCACAAAGAAAGCTTTTGGATGAGCTGGGC
     E E N A L G V R N A A Q R K L L D E L G
 361 ATTTGTGCTGAAGATGTGCCAGTTGATGAGTTCACTCCACTGGGTCGCATTCTGTACAAG
     I C A E D V P V D E F T P L G R I L Y K
 421 GCCCCTTCTGATGGCAAGTGGGGGGGAGCATGAACTTGACTACTTACTTTTCATTGTCCGA
     A P S D G K W G E H E L D Y L L F I V R
 481 GATGTTAGTGTTAACCCAAATCCTGATGAAGTAGCCGAGTATAAATATGTCAACCGGGAA

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        D  V  S  V  N  P  N  P  D  E  V  A  E  Y  K  Y  V  N  R  E
541 CAGTTAAAAGAGCTTTTGAGGAAAGCAGATGCTGGAGAAGAATGTTTGAAGCTGTCTCCT
        Q  L  K  E  L  L  R  K  A  D  A  G  E  E  C  L  K  L  S  P
601 TGGATTCAGACTGGTTGTGGACAATTTCTTGTTCAAGTGGTGGGATCACCTCGAAAAAGG
        W  I  Q  T  G  C  G  Q  F  L  V  Q  V  V  G  S  P  R  K  R
661 TACCCTTAAAGATCTGGATCCCCTCTAGAGTCGACCTGCAGGCATGCAAGCT
        Y  P  *
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Ci-GGPS T7

```
GCTGCAAGAATTGTCAGAGCTATTGCGGAATTGGCTAAATATATCGGAGCTGATGGACTTGTCGCCGGCC
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CAAAACTGCAGCTTTATTGGAGGCTGCTGTTGTTCTTGGAGCCATATTGGGCGGCGGAACTGACAACGAA
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TGACCAAGTCTTCAAAAGAATTGGGGAAGACTGCTGGCAAAGATTTGGTGGCCGATAAGTTAACTTATCC
CAAGTTGCTGGGGATCGAAGAATCGAAGAAGTTAGCTGATAAGTTGAATAAAGATGCTCAACAGCAATTG
TCGGAATTTGATCAGGAAAAGGCCGTGCCTTTGATTGCTTTGGCTAACTATATTGCTTATAGGCAGAAT
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Ci-GGPS T3

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ATGAGTTGCGTCAATCTTGCGGCATGGACTCAAACATGCTCAATCTTTAACCAAGCTAGCAGTCGCAGAT
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CCCCGTTTCGATCTCCGCAGTTCAAACTCTTGAGGAAAACCCCGCCCCAAGCCCCACTTTCGATTTCAAG
TCCTACATGATCCAAAAAGCAAGTACCGTCAACCAAGCCTTAGACGCCGCCGTTTCGCTCAAGGACCCCG
TGAAGATCCACGAATCCATGCGATACTCTTTACTGGCGGGCGGGCAAGCGGGTGCGCCCCGTGCTCTGCCT
CGCCGCTTGTGACCTTGTTGGTGGCCACGAGTCCATGGCCATGCCAGCTGCATGCTCTATCGAGATGATC
CACACCATGTCCTTAATTCACGACGATTTGCCTTGCATGGATAACGACCCTCTTCGTCGAGGGAAGCCCA
CGAACCACACGATCTACGGCGAA
```

Ci-GAPDH

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CACCGACTACATGACCTACATGTTTAAATACGACAGCGTTCACGGTCAATGGAAGCACCATGAATTGAAG
GTTAAGGACGATAAGACCCTTCTCTTTGGTGAGAAGCCTGTCACTGTTTTCGGCGTTAGGAACCCAGAGG
AGATCCCATGGGCTGAGACTGGAGCCGAGTATGTTGTGGAGTCGACTGGAGTCTTTACTGACAAGGATAA
GGCTGCTGCCCATTTGAAGGGTGGTGCTAAGAAAGTTATCATCTCAGCCCCCAGCAAGGATGCTCCTATG
TTTGTTGTGGGTGTCAGTGAAAACGAGTACAAACCAGAGCTCAACATTGTGTCCAATGCTAGCTGCACCA
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GCTGCTTCATTTAACATCATTCCTAGCAGTACTGGAGCCGCTAAGGCTGTTGGAAAGGTCTTGCCTGCTT
TGAATGGAAAACTGACTGGTATGGCTTTCCGTGTACCCACTGTTGATGTCTCAGTGGTCGACCTCACAGT
GAGGCTGGAGAAGGATGCTTCTTATGATGAAATTAAAGCAGCCATCAAGGAGGA
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Ci-b-Car H

```
  1 ATGGCGGTCGGACTATTGGCCGCCATAGTCCCGAAGCCCTTCTGTCTCCTCACAACAAAA
       M  A  V  G  L  L  A  A  I  V  P  K  P  F  C  E  E  E  E  E
 61 CTTCAACCCTCTTCGCTCCTCACAACAAAACCCGCTCCCCTTTTTGCCCCTCTCGGTACC
       L  Q  P  S  S  L  L  T  T  K  P  A  P  L  F  A  P  L  G  T
121 CGCCACGGCTTCTTTAATGGCAAAAACCGAAGAAAAATCAACTCTTTCACCGTATGTTTT
       R  H  G  F  F  N  G  K  N  R  R  K  I  N  S  F  T  V  C  F
181 GTTTTAGAGGAGAAAAAACAAAGCACCCAGATCGAGACTTTCACGGAGGAGGAGGAGGAG
       V  L  E  E  K  K  Q  S  T  Q  I  E  T  F  T  E  E  E  E  E
241 GAGTCGGGTACCCAGATCTCGACTGCTGCCCGCGTGGCCGAGAAATTGGCGAGAAAGAGA
       E  S  G  T  Q  I  S  T  A  A  R  V  A  E  K  L  A  R  K  R
```

301 TCCGAGAGGTTCACTTATCTCGTTGCTGCCGTCATGTCTAGTTTTGGTATCACTTCCATG
     S   E   R   F   T   Y   L   V   A   A   V   M   S   S   F   G   I   T   S   M
361 GCTGTCATGGCTGTTTATTACAGGTTCTGGTGGCAAATGGAGGGTGGAGAGGTGCCTTTA
     A   V   M   A   V   Y   Y   R   F   W   W   Q   M   E   G   G   E   V   P   L
421 GCTGAAATGTTTGGCACATTTGCTCTCTCTGTTGGTGCTGCTGTGGGCATGGAGTTTTGG
     A   E   M   F   G   T   F   A   L   S   V   G   A   A   V   G   M   E   F   W
481 GCACGATGGGCTCATAAAGCTCTGTGGCATGCTTCTTTATGGCATATGCACGAGTCTCAC
     A   R   W   A   H   K   A   L   W   H   A   S   L   W   H   M   H   E   S   H
541 CATCGACCAAGAGAGGGTCCTTTTGAGCTAAACGATGTGTTTGCCATAATCAACGCAGTT
     H   R   P   R   E   G   P   F   E   L   N   D   V   F   A   I   I   N   A   V
601 CCAGCCATAGCCCTTCTCTCTTTTGGCTTCTTCCACAAAGGCCTTGTACCTGGTCTCTGT
     P   A   I   A   L   L   S   F   G   F   F   H   K   G   L   V   P   G   L   C
661 TTTGGTGCTGGACTTGCCATTACGGTGTTTGGGATGGCCTACATGTTCGTCCACGATGGT
     F   G   A   G   L   A   I   T   V   F   G   M   A   Y   M   F   V   H   D   G
721 CTCGTTCACAAAAGGTTCCCTGTGGGTCCCATTGCCGACGTGCCTTATTTCCGGAGAGTC
     L   V   H   K   R   F   P   V   G   P   I   A   D   V   P   Y   F   R   R   V
781 GCTGCCGCTCACCAGCTTCACCACTCGGATAAATTCCACGGTGTTCCATATGGGCTCTTT
     A   A   A   H   Q   L   H   H   S   D   K   F   H   G   V   P   Y   G   L   F
841 CTCGGACCTAAGGAGCTTGAAGAAGTGGGGGGACTAGAAGAATTGGAGAAGGAGATCAGT
     L   G   P   K   E   L   E   E   V   G   G   L   E   E   L   E   K   E   I   S
901 AAGAGAATCAAATCATACAACAGGGTTCCAAAATAA
     K   R   I   K   S   Y   N   R   V   P   K   *

Ci-ZDS

CATGTGATGTCCCTGGAATTAAAAGATTGCTTCCATCATCGTGGAGGGAAATGAAATTTTTCAACAATAT
TTATGAGCTAGTTGGAGTTCCTGTTGTCACAGTGCAGCTTAGATACAATGGTTGGGTTACTGAGTTGCAA
GACCTAGAACGGTCAAGACAATTGAGGCAAGCTGTGGGGTTAGATAACCTTTAATATACTCCAGATGCAG
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GCTCCAATTCGTTTTGACGCCTGGAGATCCTTATATGCCCTTACCAAATGATGAAATCATAAGGAGAGTG
GCAAAGCAGGTTTTAGCTCTATTTCCATCATCCCAAGGTTTAGAAGTTATTTGGTCATCTTTCGTCAAAA
TCGGGCAATCTTTGTGCGGCGAGGGACCTGGTAAAGACCCCTTCAGACGCGATCAAAAGACACCGGTGAA
GAACTTCTTCCTCGCCGGCTCATATACAAAACAGATAGTATGGAAGGAGCAACTTTGTCTCGGTAGACAAG
CCTCAGGCTACATATGCAATGCCGGGGAAGAATTAGTAGCACTGAGGAAGCAGCTTGCTGCCTTTAAATC
TCTAGAACAAATGGAAGCTCCAACTACTACTGATGATGAACTAAGTCTTGTCTGATCACAATCTGTTTGA

Ci-PSY

AGCTCATCTGTCCTCCTACACCACACATATATAGCCCATATAGCCCTTCGCCTTTCAGGGGTCATCAGCA
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CAGACTTAAAGTTCCGGGTAAAGCAATATCTGGGTTCACATCAAGATCACGAGTAACCCCACTTGGCTGC
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CAGCAGTGCTAGCAACCATACATGATATTTCAGGCAAGTCGATTCCAGATGAGCAAGGATGCCTCAAATC
TGTATCTAAAGGATAAGAATTCCGTTTCTGCTTTTTTACTATTATTAAACTGCTTAGGTCTAGAATTAAAC
ACAGCAGTCCGGGTTTGATGTTGGTAAAGAAA

Ci-capsanthin/capsorubin synthase (CCS) gene-T3

TTTCTATTATCCATCATTTCAAGTGCACGAATGATACCGACATCCACTTTTTAAATTCGTTGTATTCATA
ACATGTTACTGTGTACTGCAAGAGACGCTAATTATTCAAGAACATTTAATCTTCAAATGGTTTCAAGGGC
AAGATTCCCCATCATTTTAACCAGAGGAAGAGGGCACTTGGTAACAATATCAAACCTGGAAGAATTCGAG
GCGTGTCCAAAGAGAGACAAGCTTAGGCCAGCAAGCTCTGCAAGAGACAACCTTGAGGACAGAAACCCAT
GCCAGTAGTGAGGATTCAAATCAAAGAAAGCATCAAAGAATCTCCTAGTCCCCTTCAAATCCAGCTTCAA

CAAAGTCTCCATACCAAATGAATAAAACTCCCTATTGCATCTTCTGTCAATTGGCCACAACCCATTCCAC
ACTTTCTGATGAAGTGGCCTGCCTCTGATCATCCTGGTTGAGCCAAGGCACTCAGCTATTGCATCAGCCA
ACGCAGGGGCCAGAGCCATGGTCCGAGCCACCATATACCCAGTTGAAGGATGGACTAAACCAGACGTGCT
GCCAATAGCCATCACACTTTGCGGGATCACAGGCAGAGGACCTCCCATTGGAATCAAACATTTTTCATCT
TCAATCACTCTTTTAACTCTAATTCCCATATGCCTTA

Ci-capsanthin/capsorubin synthase (CCS) gene-T7

TCATTTCTCTGTGCTCTGGCATGGCAACTCTTCTTAGCCCGTTTTCTCCTTCTCCTTTAGCTAAAGTTTC
GCAAATAATTGATTCAACATCATCACATTCATTTTCGCTATTTCCATTAGGCCGCCAAAATGCATGTTCA
AGAAAGGCCGGATCATCATCATCATCACAGGATCCGGACAAGCAAGTTTGGTAACTTCCTAGAGTTGACAC
CGGAGTCGGAACCTGAATTCTTAGTCTTTGATCTCCCCTGGTTTCATCCGTCCGATCGTATTCGATATGA
CGTGATCATCATTGGCACTGGACCTGCCGGCCTCCGTCTAGCTGAGCAAGTCTCATCGCGTCATGGTATC
AAGGTATGTTGTGTTGATCCTTCACCTCTTTCTACGTGGCCTAACAACTATGGAGTTTGGGTTGATGAGT
TTGAAGACATAGGACTTATAGACTGTTTGGACAAAACTTGGCCGATGACTTGTGTTTTTTATTAATGATCA
CAAGACCAAGTATCTAGACAGGCCCTACGGTCGTGTTAGTAGAAATATTTTGAAGACAAAGTTATTAGAG
AATTGTGTTTTAAATGGCGTTAGGTTTCATAAGGCTAAAGTTTGGCATGTGAATCATCAGGAGTTCGAGT
CTTCGATTGTTTGTGATGATGGAAATGAGATTAAGGCTAGCTTGATTGTTGATGCTAGTGGCTTTGCTAG
TAGTTT

Ci-Squan

ATTCATTTGCAAAGAGGTAGAATCGATCGATGACTATGATGAATATTGTTACTATGTAGCAGGACTTGTT
GGATTAGGTCTGTCCAAGCTTTTCTATGCCTCTGGGACAGAAGATCTGGCTCCAGATAGTCTTTCCAACT
CGATGGGTTTATTTCTTCAGAAAACAAATATCATTCGAGATTATCTGGAAGATATTAATGAGATACCGAA
GTGTCGCATGTTTTTGGCCTCGTGAGATCTGGAGTAAATATGTTAATAAACTTGAGGACTTAAAATATGAG
GAAAACTCTGACAAAGCAGTACAGTGCTTGAATGATATGGTCACCAATGCTCTCTGATGCATGTGGAAGATT
GTTTGAAGTACATGTCTGCTTTAAGGGATCATGCTATATTCCGATTCTGTGCTATCCCTCAGATCATGGC
AAT

Ci-bCClS T3

CAATAGGATATACAACAAGAGAAACAGTCGCAGAAGCAGTACTTACCATGCCAAGGAAAATCAAGAAAGT
TTATTATATGCCGCATCTCTTGAATTTAGACTCCTAAGACAACATGGTTATGATATACATGCACATGGAA
CTCTTTCTAGTTTCATGGATGAGAAGGGGAAGTTTAAGTCATGCCTCGGAGACGATATCAAAGGAATTTT
AGCATTGTATGAAGCTGCATATCTTTTGGGAGAAGAAGAGAGCACCATCTTCCATGAAGCTATCAATTTC
ACAACCACACATCTCGAAGAATACGTGAAGAAGCATAATGATGATGATGATGATGATGATGATGGATATT
TTTCAGCATTAGTGAAGCATGCATTGGAACTTCCCTTACACTGGAGGATGGTAAGATTGGAGGCAAGGTG
GTTCATTGATGTATATGAAAGAGGAACGGACATGAACCCAGTTTTGGTCGAGCTTGCTAAACTGGACTTT
AATTCTGTGCAAGCAGCACACCAAGACGAGCTCAAGTATGTGTCTTGGTGGTGGAAGAAGACCGGACTTG
GAGAGTTGCATTTTGCAAGGGACAGGTACTGGAAAATTTCTTCTGGGCTTTGGGGGAGATATGGGAGCC
TCAATTTGGATACTGTAGA

Ci-bCClS T7

GACTTTATGGGGKGAATGAGTAATGGCAATACACGATTTTTACTATTGTCTTGAACGGTATGCCCATCTC
CGTTTTGGTATGTGCATTGAGCCATTCTCGCTAGATTCATTGCAAACTGGACAAAATTGTTGGGCAGTAA
TAAGTGATCAGGATTTTCATCTCCTTTTCTTTTATTGTTCATCTTCACCCATGCTGCTGTGATCAAATCT
CTTATGTGTAGACGAGCATCGCTTTCTGAAGCTCCAGTTTCATGCATGTAACATTGAATTGCCTTAGGAA
CATCTCCTCTTTTCACCTCATCCGATGATGTTGCCAGGTCATTGGCAAGTCGAAAAATCATTGATGGCCA
ACGAATTATATGG

Ci-HPT T3

AAATACAAGTCCAGAACACCCGCTTTTGACCCAAACGTACAGTAAAAGTTCGGATCCCAAATGTTTTGTC

TCCTTCAAGATCAGGTACATCCTTAAATAATGCTATAACTACTGAAAAGAAGCTCATGAATGCTGTCGCA
AAGATTAGAGGCTTTGAAAATACTGCTGGTCTTCTGTACACATGAGTCTGGAAATTGCCACTACA

CI-HPT T7
AAATACAAGTCCAGAACACCCGCTTTTGACCCAAACGTACAGTAAAAGTTCGGATCCCAAATGTTTTGTC
TCCTTCAAGATCAGGTACATCCTTAAATAATGCTATAACTACTGAAAAGAAGCTCATGAATGCTGTCGCA
AAGATTAGAGGCTTTGAAAATACTG

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ABSTRACT IN KOREAN

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abscisic acid

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[(*Citrus unshiu* Marcov x *C. sinensis* Osbeck)

x *C. reticulata* Blanco]

.

HPLC

LC/MS   .

,   ,   .

,   real time

quantitative RT–PCR   Northern blot   .

(lutein),

(β-cryptoxanthin),   -   (ζ-carotene),   phytofluene,

(capsathin),   (zeaxanthin),   -   (β-carotene)

.   ,

.   -

,   ,   , phytofluene,

,   -   .

glyceraldehydes-3-phosphate

dehydrogenase, *ent*-copaly diphosphate synthase, geranylgeranyl pyrophosphate

reductase, HMG CoA reductase, HMG CoA synthase, 1-deoxy-D-xylulose-5-

phosphate synthase, 1-deoxy-D-xylulose-5-phosphate reductoisomerase, isopentenyl

diphosphate isomerase, *FPP* synthase, squalene synthase, Sesquiterpene synthase 2,

(+)-limonene   synthase   2,   (E)-beta-ocimene   synthase,   terpene   synthase,

geranylgeranyl pyrophosphate synthase, phytoene synthase, phytoene desaturase,

lycopen beat cyclase, lycopen epsilon cyclase, beta-carotene hydroxylase, epsilon-

carotene   hydroxylase,   zeaxanthin   epoxsidase,   violaxanthin   de-epoxidase,

9,10[9',10']carotenoid   cleavage   dioxygenase,   capsanthin/capsorubin   synthase,

tocopherol polyprenyltransferase   .

  β-carotene   hydroxylase   phytoene   synthase

                         ,   (11   )

                 .   , ε-carotene   hydroxylase

. *DXPS*   (8)

      ,   , *IPI*   .

                β-carotene hydroxylase   phytoene synthase

                                                    β, β-xanthophyll

β-carotene hydroxylase   phytoene synthase

    β, β-xanthophyll, phytofluene, z-carotene   .