석 사 학 위 논 문

감귤의 Phytoene Synthase 및 β-Carotene Hydroxylase cDNAs 특성

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2000 년 12 월

Molecular Characterization of cDNAs Encoding Phytoene Synthase and β-Carotene Hydroxylase in *Citrus*

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ABBREVIATIONS

BHT : Buthylated hydroxytoluene

bp : Base pairs

BR : Breaker

Chx : β-Carotene hydroxylase

Chx : Gene coding for Chx

Psy : Phytoene synthase

CHX1 and CHX2 : β-Carotene hydroxylase of *Citrus*

CHX1 and *CHX2* : Gene coding for CHX1 and CHX2 of *Citrus*

CrtL-b : Lycopene β-cyclase

CrtR-b : β-Ring hydroxylase

CrtR-e : ε-Ring hydroxylase

CV : Coefficient of variation

DAF : Days after anthesis

DDW : Distilled deionized water

DMAPP : Dimethylallyl pyrophosphate

EDTA : Ethylenediamine tetraacetic acid

FG : Full green

FPP : Farnesyl pyrophosphate

FY : Full yellow

GGPP : Geranylgeranyl pyrophosphate

Ggps : Geranylgeranyl diphosphate synthase

GPI : Glucosephosphate isomerase

GPP : Geranyl pyrophosphate

HPLC : High pressure liquid chromatography

hr : Hour(s)

Ipi : IPP isomerase

IPP : Isopentenyl pyrophosphate

kb : Kilo base pair(s)

LcyE (CrtL-e) : Lycopene ε-cyclase

LiCl : Lithium chloride

MeOH : Methanol

MG : Mini-green

min : Minute(s)

MTBE : Methyl tert-buthyl ether

NaOAc : Sodium acetate

ORF : Open reading frame

PCR : Polymerase chain reaction

Pds (crtP) : Phytoene desaturase

PEG : Polyethylene glycol

PPPP : Prephytoene pyrophosphate

Psy (crtB) : Phytoene synthase

Psy : Gene coding for Psy

Psy1 : Gene coding for Psy1 of *Citrus*

Psy1 : Phytoene synthase of *Citrus*

PTFE : Polytetrafluoroethylene

PVDF : Polyvinylidene difluoride

R_f : Retardation factor

rpm : Revolution per minute

R_t : Retention time

SD : Standard deviation

SDS : Sodium dodecyl sulfate

SG : Small green

TLC : Thin-layer chromatography

Tris : Tris(hydroxymethyl)aminomethane

UTR : Untranslated Region

UV : Ultraviolet

Vde : Violaxanthin deepoxidase

YG : Young green stage of *Citrus* fruit ripening

Zds (crtQ) : ζ-Carotene desaturase

Zep (aba2) : Zeaxanthin epoxidase

SUMMARY

Citrus fruit accumulate β-carotene, ζ-carotene and β-cryptoxanthin in flesh, and cryptoxnathin, antheraxanthin, and violaxanthin in peel. It is expected that gene expression of Psy and Chx in *Citrus* fruits contribute to the accumulation of β-cryptoxanthin, and *Psy1* and *CHX1* involve in the ripening of flesh and peel of *Citrus*. So, for studies on characterization of expression patterns of cDNA encoding Psy1 and CHX1 were isolated from *Citrus* fruit library.

To determine β-cryptoxanthin content of *Citrus* fruits produced in Jeju island, the peel and flesh of matured *Citrus* fruits were used for the extraction of carotenoid. The crude extract was prepared, and the target component was confirmed by TLC analysis. Content of β-cryptoxanthin, determined by high performance liquid chromatography, in peel and flesh of *Citrus unshiu* Marc. cv. Miyagawa was dominantly higher than those of the native *Citrus* fruits, *Citrus platymamma* Hort. ex. Tanaka and *Citrus eythrosa* Hort. ex. Tanaka. Contents of β-cryptoxanthin in the fresh peel of *Citrus unshiu* Marc. cv. Miyagawa, *Citrus platymamma* Hort. ex. Tanaka and *Citrus eythrosa* Hort. ex. Tanaka were found to be 5.26, 1.66, and 0.88 mg%, respectively. On the other hand, β-cryptoxanthin contents in the flesh of *Citrus unshiu* Marc. cv. Miyagawa, *Citrus platymamma* Hort. ex. Tanaka, and *Citrus eythrosa* Hort. ex. Tanaka were 0.78, 0.32, and 0.45 mg%, respectively.

The accumulation of β-cryptoxanthin is controlled by gene expression involved in carotenoid biosynthesis. In this study, we have focused on phytoene synthase (Psy) and β-carotene hydroxylase (Chx) out of carotenoid biosynthesis genes.

First, we isolated a cDNA clone encoding phytoene synthase (Psy1) from the fruit cDNA library of *Citrus* (*Citrus unshiu* Marc.). Sequence analyses and phylogenetic dendrogram revealed that the cDNA contains an open reading frame of 437 amino acids (47 kDa), which showed significant similarity to those of fruit-producing plants. RNA blot analysis showed that the mRNA is expressed

in the flesh and peel of fruits, leaves, and flowers, as single transcripts. Also, during ripening of flesh of fruits, the *Psy1* transcripts were detected in all stages and its expression markedly increased to the maximum level in the latest stage. A similar pattern was also detected in peel, but gradually. However, the level of *Psy1* transcripts is consistent in the process of leaf development. Results revealed that the *Psy1* gene is regulated during fruit ripening, but not in leaf development, at the transcriptional level.

For studies on Chx, cDNA clones (*CHX1* and *CHX2*) encoding β-carotene hydroxylase were isolated from *Citrus* fruit and leaf cDNA libraries. Sequence analyses indicated that the clones showed polymorphism and significant similarity to those of fruit-producing plant. RNA blot analysis showed that its expression is ubiquitous in examined three tissues, fleshes and peels of fruits, leaves, and flowers, and detected as a single band. Also, during the development of fruits and leaves, the expression of *CHX1* transcripts was consistent in all stages. The expression pattern indicated that *CHX1* gene is not regulated during fruit ripening at the transcriptional level, which is not consistent with the fruits of bell pepper.

In summary, our studies show that *CHX1* expression vary with plant species, even having same type of fruit, while *Psy1* expression is similar to other plants Psy.

Ⅰ. INTRODUCTION

1. Biosynthesis pathway of carotenoids

The carotenoid pigments are essential components of the photosynthetic membranes in all plants, algae, and cyanobacteria and serve an extraordinary variety of functions in plants (Jones *et al.*, 1998). These pigments also accumulate in chromoplasts, providing the yellow, orange, and red colors of many flowers, fruits, and storage roots. Most carotenoids contain a linear C_{40} hydrocarbon backbone that includes between 3 and 15 conjugated double bonds. The number of double bonds largely determines the spectral properties of a given carotenoid, which typically absorbs light between 400 and 500nm. All carotenoid are derived from the isoprenoid or terpenoid pathway (Armstrong *et al.*, 1996).

Condensation of one molecule of dimethylallyl pyrophosphate (DMAPP) and three molecules of isopentenyl pyrophosphate (IPP) produces the diterpene geranylgeranyl pyrophosphate (GGPP) that forms one half of all C_{40} carotenoids. A critical step in the formation of the first C_{40} acyclic hydrocarbon carotenoid, phytoene, is the tail-to-tail condensation of two molecules of the C_{20} intermediate geranylgeranyl pyrophosphate (GGPP). The biosynthesis of phytoene from GGPP is a two-step reaction catalyzed by the enzyme phytoene synthase (Psy).

Phytoene undergoes a series of four desaturation reactions that result in the formation of first phytofluene and then, in turn, ζ-carotene, neurosporene, and lycopene. These desaturation reactions serve to lengthen the conjugated series of

carbon-carbon double bonds that constitutes the chromophore in carotenoid pigment, and thereby transform the colorless phytoene into the pink-colored lycopene. The four sequential desaturations undergone by phytoene are catalyzed by two enzymes in plants: phytoene desaturase (Pds) and ζ-carotene desaturase (Zds).

The cyclization of lycopene in photosynthetic organisms represents an important branching point in the biosynthesis pathway of carotenoids. β-Carotene, with two β-rings, is an essential end product and serves as the precursor for several other carotenoids that are commonly found in the photosynthetic apparatus of plants. α-Carotene, with one β and one ε-ring, is the immediate precursor of lutein, the predominant carotenoid pigment in the photosynthetic membranes of many green plants. Carotenoids with two ε-ring are not commonly found in plants.

Xanthophylls or oxygenated carotenoids comprise most of the carotenoid pigment in the thylakoid membranes of plants. Hydroxlation at the number three carbon of each ring of the hydrocarbons β-carotene and α-carotene will produce the xanthophylls pigments including β-cryptoxanthin and zeaxanthin, and lutein, respectively. Because the chirality of the hydroxyl on the ε-ring is opposite that of the hydroxyl on the β-ring of lutein, it is thought that different enzymes catalyze these reactions (Jones *et al.*, 1998) (Figure 1).

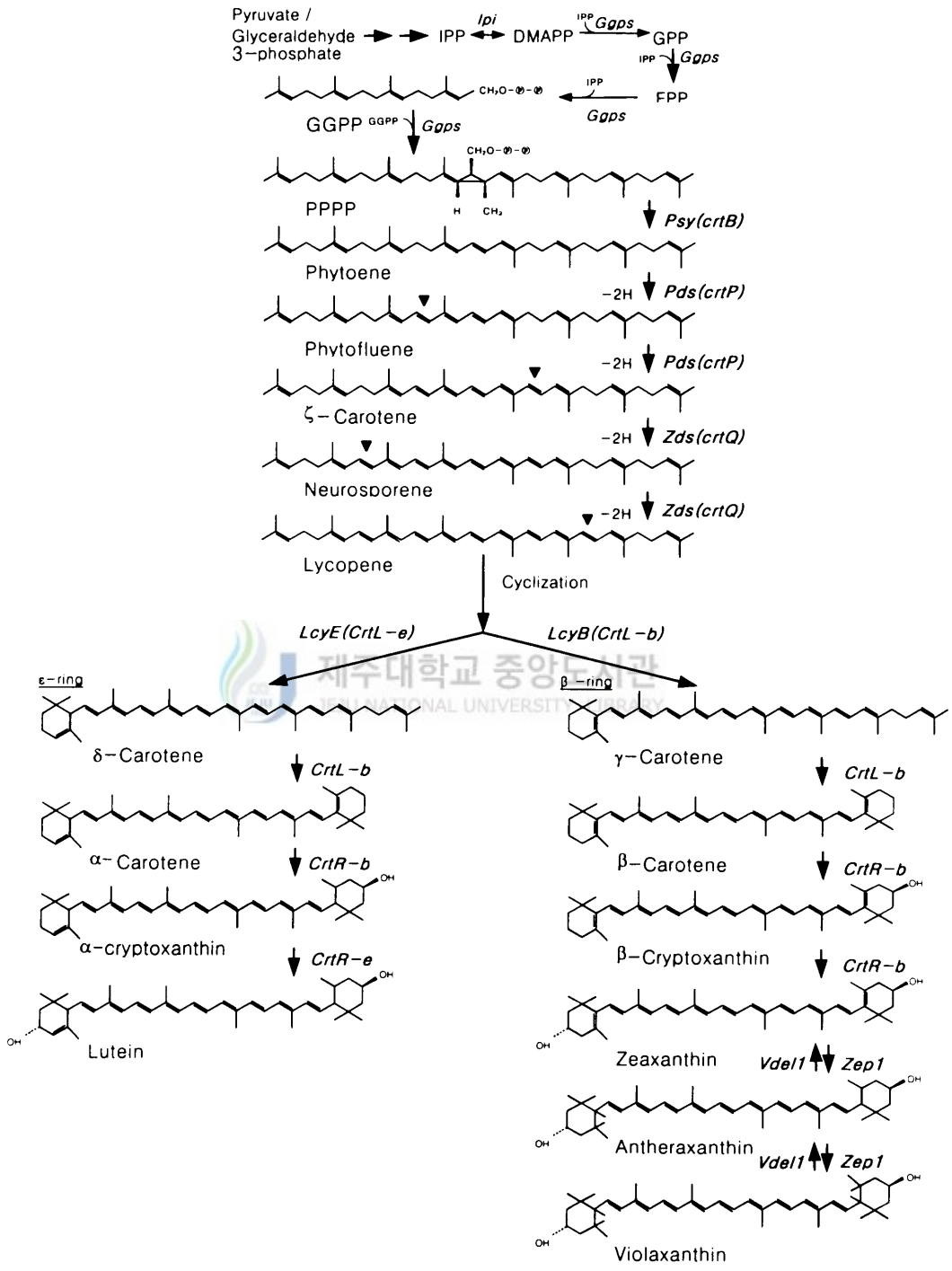

Figure 1. Biosynthetic pathway of carotenoids in plants.

2. β-Cryptoxanthin

Citrus fruits have been recognized as sources of pigment as well as biologically active compounds (Whang *et al.*, 1995; Cooper *et al.*, 1999). Carotenoids present in *Citrus* fruits and vegetables are important biological precursors of vitamin A and are widely believed to help human beings healthy (Nomura *et al.*, 1997; Riso *et al.*, 1997; Thumham *et al.*, 1998; Tee *et al.*, 1991). In addition, flavonoid, coumarins, phenyl propanoid, terpene and limonoid were found in *Citrus* fruits (Nogata *et al.*, 1996). The hydrocarbon carotenoids, lutein, and β-cryptoxanthin are the main carotenoids present in human plasma and tissue (Krinsky *et al.*, 1990). Recent studies have disclosed an inverse relationship between carotenoid consumption and the development of some cancers, showing reduction in cancer rates (Berg *et al.*, 1988; Khachik *et al.*, 1988). It was also reported that antimutagenicity was mainly associated with the fractions of the hydrocarbon carotenoids (α-, β-carotene, lycopene) and the xanthophylls (lutein, β-cryptoxanthin) (Berg *et al.*, 1988; Rauscher Berg *et al.*, 1988; 1998). In particular, β-cryptoxanthin, a major source of vitamin A, is a biologically active compound not only with having anticancer effect but also enhances the immune system (Cooper *et al.*, 1999; Thomas *et al.*, 1995).

In *Citrus* fruits, anticancer activity of carotenoids has been ascribed to its antioxidant activity (Krinsky *et al.*, 1990). Recently, Dorgan *et al.* (Dorgan *et al.*, 1998) reported that β-cryptoxanthin showed a strong anticancer activity against breast cancers. The content of carotenoids differed according to the kinds of *Citrus* fruits such as oranges, tangerines, and native *Citrus* fruits (Stewart, 1977b). Nishino *et al.* (1998) reported that large amount of β-cryptoxanthin was found in *Citrus unshiu* Marcovitch compared to the grapefruits, lemons, and oranges grown in North America. It also reported that β-cryptoxanthin showed a higher anticancer activity in mice against both skin and large intestine cancers compared

to β-carotene.

The importance of generating accurate qualitative and quantitative data on carotenoids in *Citrus* fruit has resulted in the development of analytical techniques that can separate and quantitate these compounds (Berg *et al.*, 1988). In particular, HPLC (high pressure liquid chromatograpy) has been an important tool in the separation of various classes of carotenoids isolated from food extracts (Riso *et al.*, 1997; Stewart, 1977a; Sumida *et al.*, 1999; Philip *et al.*, 1998). Although there are number of reports on the separation and characterization of carotenoids from *Citrus* fruits, little is known on the separation of β-cryptoxanthin in the native *Citrus* fruits cultivated in Jeju island. In order to utilize a biologically active pigment of *Citrus* fruits efficiently, it is necessary to determine the content of β-cryptoxanthin from various *Citrus* fruits grown in Jeju island. Therefore, β-Cryptoxanthin contents were determined from peel and flesh in three species of *Citrus* fruits cultivated in Jeju island.

3. Phytoene synthase (Psy) and β-carotene hydroxylase (Chx)

Carotenoids are a widespread class of pigments found in all photosynthetic and many non-photosynthetic organisms (Goodwin, 1980). In plants, carotenoids are essential in photosynthetic apparatus, including reaction centers and antenna complexes, and provide photoprotective activities in chloroplasts (Anderson and Robertson, 1960). Furthermore, these pigments are also produced in the chromoplasts of fruits and flowers, where they serve as coloring agents to attract pollinators and agents of seed dispersal (Goodwin, 1980).

The accumulation of carotenoids is a characteristic feature of fruit ripening, which occurred in the differentiation chloroplast into chromoplast (Biale, 1964). Previous studies in tomato (Fraser *et al.*, 1994), muskmelon (Gross, 1987), and bell pepper (Bouvier *et al.*, 1994) have shown that lycopene, β-carotene, and

capsanthin-capsorubin accumulate during fruit ripening, respectively, which indicated that the expression of gene involved in carotenoid biosynthesis must be controlled in the process of fruit development. It has been shown that the mRNA level of phytoene synthase (Psy) increases significantly during fruit ripening in tomato (Giuliano *et al.*, 1993; Lois *et al.*, 2000), muskmelon (Karvouni *et al.*, 1995), maize (Buckner *et al.*, 1996) and bell pepper (Römer *et al.*, 1993), which implies that the developmental regulation is controlled at the transcriptional level in fruits. Phytoene synthase is involved in the first specific and committed step of carotenoid biosynthesis, and catalyzes the condensation of two molecules of geranylgeranyl pyrophosphate (GGPP). Also, it has been reported that the transcripts of other genes, including phytoene desaturase (Römer *et al.*, 1993), geranylgeranyl pyrophosphate synthase (Römer *et al.*, 1993; Kuntz *et al.*, 1992) and β-carotene hydroxylase (Bouvier *et al.*, 1998), accumulate during fruit ripening.

β-Cryptoxanthin was synthesized by conversion of β-carotene. This step is the intermediate in the biosynthetic process of zeaxanthin from β-carotene, which is catalyzed by β-carotene hydroxylase (Chx). Epidemiological and experimental animal studies have suggested that β-cryptoxanthin may inhibit certain types of cancer (IARC 1998, Burri 2000, Narisawa *et al.*, 1999) and the onset of other diseases (Giuliano 2000). Although β-cryptoxanthin is a nutrient and has several important biological activities in humans, much less is known concerning Chx in plants, including *Citrus*. It has been known that as the fruit ripens, β-cryptoxanthin accumulates in the flesh and peel (Baldwin 1993). Also, several genes encoding Chx have been isolated from *Arabidopsis*, daffodil, tomato, and bell pepper, but the expression has been analyzed only in bell pepper (Bouvier 1998).

Carotenoid accumulation in fruits is controlled by the gene expression involved in carotenoid biosynthesis. To date, the mRNA level of many genes,

including 1-deoxy-D-xylulose 5-phosphate synthase (Lois et al. 2000), phytoene synthase (Giuliano 1993, Karvouni et al. 1995, Römer et al. 1993), phytoene desaturase (Römer et al. 1993), geranylgeranyl pyrophosphate synthase (Kuntz 1992, Römer et al. 1993), β-cyclohexynyl carotenoid epoxidase (Bouvier et al. 1994), *Chx*, and capsanthin-capsorubin synthase, increases significantly during fruit ripening, which implies that the developmental regulation is controlled at the transcriptional level. Therefore since β-cryptoxanthin accumulates during fruit ripening of *Citrus*, we anticipate that the expression of *Chx* may increase in the process of fruit development.

Although *Citrus* fruit is a nonclimacteric fruit like the bell pepper, there are differences in specific carotenoids accumulating during fruit ripening. *Citrus* fruit accumulate β-carotene, ζ-carotene and β-cryptoxanthin in flesh, and cryptoxnathin, antheraxanthin, and violaxanthin in peel (Baldwin, 1993), whereas bell pepper accumulates capsanthin and capsorubin (Bouvier *et al.*, 1994). It is presumed that these differences in carotenoid contents and kinds may result from different regulation in gene expression in fruits. Moreover, *Citrus* is a woody plant, whereas bell pepper is a herbaceous plant. Therefore, to more clearly elucidate the relationship between changes in carotenoid contents during fruit ripening and gene expression involved in carotenogenesis, in *Citrus* fruits may be useful.

In this study, we investigated the involvement of *Psy1* and *CHX1* in the ripening of flesh and peel of *Citrus* fruits and in leaf development. First, we isolated a cDNA encoding Psy1 and CHX1 and characterized the expression patterns. Results indicated that the expression of *Psy* and *Chx* is related to and distinct from, respectively, the accumulation of β-cryptoxanthin and other carotenoids.

Ⅱ. MATERIALS AND METHODS

1. Materials

1) Plant materials

(1) Analysis of β-cryptoxanthin contents

Three different *Citrus* fruits produced in Jeju island were used for the analysis: Byungkyool (*Citrus platymamma* Hort. ex. Tanaka), Dongjeongkyool (*Citrus eythrosa* Hort. ex. Tanaka), and Miyagawa wase (*Citrus unshiu* Marc. cv. Miyagawa). These cultivars were cultivated in field.

β-Carotene and β-cryptoxanthin, crystalline carotenoids used as standards, were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and Extrasynthese (Genay, France), respectively. Stock solutions of carotenoids were prepared by dissolving 1 mg of the compounds in tert-Butyl methyl ether (MTBE)/methanol (MeOH) (1/1, v/v) containing 0.1% butylated hydroxytoluene (BHT). The solutions were stored under nitrogen in dark. They were then diluted to give individual working solutions in the range of 0.1-5.0 μg/mL in the mobile. In order to assess the characteristics of the method, the linearity of the calibration graphs obtained for each carotenoid the accuracy of the chromatographic response and of the retention times were evaluated. Also, the limit of detection was defined.

(2) Psy and Chx

Citrus (*Citrus unshiu* Mac. cv. Miyagawa) was cultivated at the *Citrus* Experiment Station, Jeju (Korea) and used throughout this work. Plants were

grown under greenhouse conditions and tissues including fruits, leaves, and flowers were sampled. Their fruits were harvested at five stages of development determined by maturity and external fruit color. Developmental stages are as follows: mini-green (MG, size: 0.8-1.5 cm in diameter), 25 days after anthesis (DAF); small green (SG, size: 2-3 cm in diameter), 55 DAF; full green (FG, size: 4-5 cm in diameter), 90 DAF; breaker (BR, full size: 6-7 cm in diameter), 130 DAF; and full yellow (FY), 160 DAF. Leaves were also harvested at five stages determined by size. The stages are as follows: L1 (size: 4-7 cm in length), L2 (size: 7-9 cm in length), L3 (9-12 cm in length), L4 (12-15 cm in length), and L5 (longer than 15 cm in length).

2) Bacterial strains and plasmids

Bacterial strains, plasmids and vector used in this study are list in Table 1 and Table 2 *E. coli* JM109 was used as a host for subcloned plasmid and amplification of the plasmid DNA. *E. coli* XL1-Blue (MRF') and *E. coli* SOLR were used as the hosts for transformation. *E. coli* XL1-Blue (MRF') as a host for λ phage, and *E. coli* SOLR as a host for in vivo excision of λ-Zap clones into plasmid clones were used. Plasmid pBluescript SK+ was used as the subcloning vector of various plasmids.

3) Enzymes and chemicals

Restriction endonuclease and other DNA modifying enzymes (T4 DNA ligase, Klenow fragment, T4 DNA polymerase, calf intestinal alkaline phosphatase, and T4 polynucleotide kinase) used in DNA manipulation were purchased from Boehringer Mannheim (Germany) or Poscochem (Korea). All chemicals, unless other specific remarks, were purchased from Sigma Chemical Co. (USA). Bacto-tryptone, Bacto-yeast extract, bacto-agar, and Bacto-peptone were from Difco Laboratories. Select peptone 140 was from GibcoBRL. Hybond-N, radioactive

Table 1. Bacterial strains and f1 helper phage used in this study.

E. coli strain	Relevant genotype	Reference
E. coli JM109	*RecA*1 *supE*44 *endA*1 *hsdR*17 *gyrA*96 *RelA*1 *thi* Δ(*lac-proAB*) F′ [*traD*36 *proAB*$^{+}$ *lacI*^{q} *lacZ* ΔM15]	Yanisch-Perron *et al.*, 1985.
E. coli XL1-Blue MRF′	*Δ(mcrA)*183 *Δ(mcrCB-hsdSMR-mrr)*173 *endA*1 *supE*44 *thi*-1 *recA*1 *gyrA*96 *relA*1 *lac* [F′ *proAB lacI*^{q}*Z* ΔM15 Tn*10* (Tet^{r})]	Instruction mannual of Strategene
E. coli SOLR™	e14^{-}(mcrA) Δ(*mcrCB-hsdSMR-mrr*)171 *sbcC recB recJ umuC*::Tn5(Kan^{r}) *uvrC lac gyrA*96 *relA*1 *thi*-1 *endA*1 λ^{R} [F′ *proAB lacI*^{q}*Z* ΔM15] Su^{-} (nonsuppressing)	Instruction mannual of Strategene
Phage	**Description**	**Reference**
ExAssist™ interference-resistant helper phage	Specialized helper phage for excision of the pBluescript phagemid from the Uni-Zap XR vector	Instruction mannual of Strategene

Table 2. Plasmids and Primers.

Plasmid	Marker	Host	Relevant feature	Reference
pBluescript SK(+)	Amp	JM109, or SOLR	*E. coli* multipurpose vector derived from pUC19 (2958 bp)	Instruction mannual of Strategene
pGEM®-T	Amp	JM109	E. coli subcloning vector, having improved efficiency of PCR products, derived from pGEM-5Zf (+) vector containing T7 and SP6 promoter	U.S. Patent No. 4,766,072, Promega
pGEM T-fPsy	Amp	JM109	Subcloning vector containing 230 bp PCR products of *Psy* derived from pGEM-T	In this study
pGEM T-fChx	Amp	JM109	Subcloning vector containing 573 bp PCR products of *Chx* derived from pGEM-T	In this study

Primers	Sequence	Usage
T7 Primer	5′-GTAATACGACTCACTATAGGGC-3′	Sequencing
T3 Primer	5′-AATTAACCCTCACTAAAGGG-3′	Sequencing
CitPsy5	5′-TGGGCAATATATGTTTGGTG-3′	Sequencing
CitPsy3	5′-AACATCCTTGCCCTCTTAAT-3′	Sequencing
CPsy-S1	5′-GATATGGCTGAGAACGGTGT-3′	Sequencing
CPsy-AS1	5′-CGGCGCAAACTTCTTCCACA-3′	Sequencing
CHX3T3-2	5′-TCGAGAAGATCAGGGGAGGA-3′	Sequencing
CHX14T3-2	5′-CATGGCGGTCGGACTATTGG-3′	Sequencing
CHX3T7-2	5′-ATATGCCATAAAGAAGCATG-3′	Sequencing

* Antibiotic resistance marker of the plasmid is noted as follows : Amp, ampicillin

isotopes [α-^{32}P]dCTP and [α-^{35}S]dATP were from Amersham International. Synthetic oligonucleotides were commercially synthesized by Genotech (Korea). Water used for all procedures was distilled quality, purified by reverse osmosis to a resistance of 18 mega-ohms.

4) Sterilization of solutions and media

All bacterial growth media and solutions unless otherwise stated were sterilized by autoclaving at 15 psi for 15 min. Antibiotics and IPTG solutions were sterilized by filtration through a 0.2 μm sterile Millipore filter. To manage RNA for isolation and blotting, all solutions except for amine-containing solutions such as Tris were treated with diethyl pyrocarbonate (DEPC, 0.1%) for at least 12 hr at 37℃ and then autoclaved at 15 psi for 15 min on liquid cycle. Glassware was sterilized by heating to 180℃ for 8 hr or more

5) Media

LB medium : Bacto-tryptone 10 g, Bacto-yeast extract 5 g, NaCl 5 g per 1 liter, Ph. 7.0

TYP broth : Bacto-tryptone 16 g, Bacto-yeast extract 16 g, NaCl 5 g, K_2HPO_4 2.5g per 1 liter

SOC medium : 2% Bacto-tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 10 mM glucose, pH 7.0

NZY medium and agar plates : 0.5% Bacto-teast extract, 0.5% NaCl, 1% Casein hydrolysate, 0.2% $MgSO_4 \cdot 7H_2O$, pH 7.5, 1.5% Agar (for agar plates only)

NZY top agar : Same as NZY media with 0.7% agarose

6) Buffers and solutions

(1) Agarose gel electrophoresis of nucleic acid

TAE buffer : 40 mM Tris-HCl (pH8.0), 1 mM EDTA

TBE buffer : 0.089 M Tris, 0.089 M boric acid, 0.002 M EDTA

6x Gel loading buffer : 0.25% bromophenol blue, 0.25% xylene cyanol FF, 15% Ficoll (Type 400, Pharmacia)

(2) Plasmid preparation

Solution I : 50 mM glucose, 25 mM Tris-HCl (pH 8.0), 10 mM EDTA

Solution II : 0.2 M NaOH, 1% SDS

Solution III : 3 M potassium acetate, pH 4.8

(3) RNA isolation from plant tissue

6x Extraction buffer : 100 mM LiCl, 100 mM Tris-HCl (pH8.0), 10 mM EDTA, 1% SDS

6x Gel loading buffer : 50% (v/v) glycerol, 0.1 mg/mL bromophenol blue

(4) Others

TE buffer : 10 mM Tris-HCl (pH 8.0), 1 mM EDTA

5x TBE : 54 g Tris, 27.5 g boric acid, 20 mL 0.5 M EDTA (pH 8.0) per liter

SM buffer : 5.8g NaCl, 2.0 g $MgSO_4 \cdot 7H_2O$, 50.0 mL 1 M Tris-HCl (pH 7.5), 5 mL 2% gelatin per liter

2. Methods

1) Analysis of β-cryptoxanthin in peel and flesh of *Citrus* fruits

(1) Extraction of crude carotenoids from *Citrus* fruits

Approximately 10 g of peel (or 100 g of flesh) was mixed with 70 mL of 40% MeOH and 1 g of $MgCO_3$ by using a juice mixer (LG). The *Citrus* extract was filtered under suction. After the residue was washed with 40% MeOH, extraction was carried out using an equal volume of acetone/MeOH (7/3, v/v) containing 0.1% BHT. The mixture was stirred 2 hr and filtered through a fritted glass funnel. Filtrate was transferred to a 1 L separatory funnel, and 500 mL diethyl ether and 200mL distilled water were added. The water layer (bottom phase) was discarded and the solvent containing pigments (top phase) was placed in a vacuum rotary evaporator (Büchi) at 30℃ and evaporated to dryness. Carotenoids were dissolved in 10 mL diethyl ether. Pigments were then saponified by adding 20% methanolic KOH (10 mL) and allowed to stand, in the dark, for 2 hrs under nitrogen atmosphere with occasional stirring at room temperature. The sample was subsequently fractionated with a saturated NH_4Cl solution, and the organic layer was put aside. The aqueous layer was washed with diethyl ether until neutralized, then combined with the organic layer. The ether layer was reduced to dryness on a rotary evaporator at 30℃. The saponified samples were dissolved in an appropriate volume of MTBE/MeOH (1/1, v/v) for the HPLC analysis and filtered through a 0.45 μm PTFE syringe filter (Micro Filtration System). Figure 2 showed overall scheme for the extraction of carotenoids in peel and flesh of *Citrus* fruits.

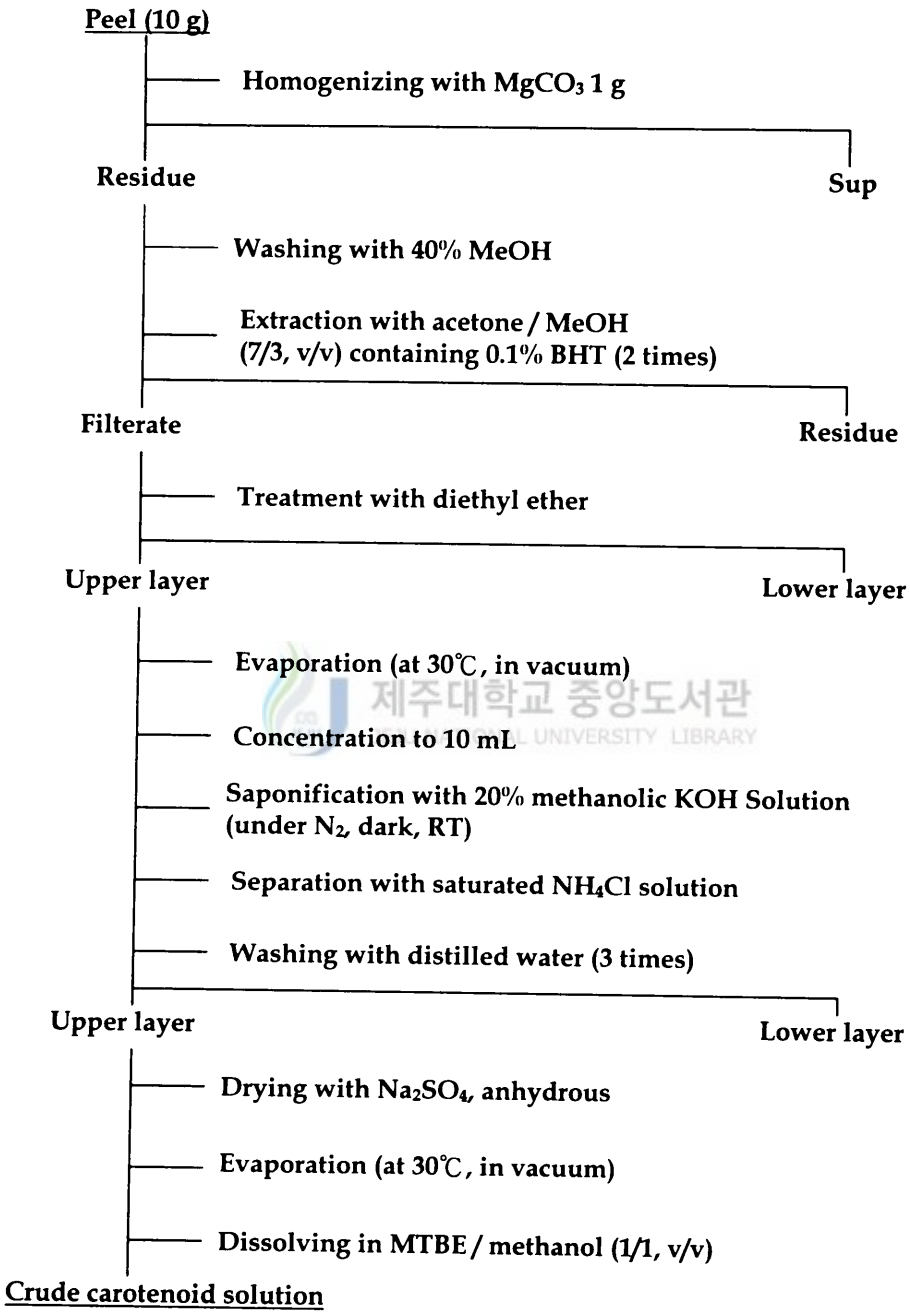

Figure 2. Extraction scheme of carotenoids in peel and flesh of *Citrus*.

(2) Chromatography

Carotenoids were analyzed by thin-layer chromatography (TLC) using silica gel plates (Silica gel 60 F254, Merck). Hexane/acetone (75/25, v/v) was used as the mobile solvent for separation. The HPLC equipment consisted of a Spectra-Physics (SpectraSYSTEM) P4000 pump (Spectra-Physics Analytical, Inc.), an UV1000 UV/vis detector (Spectra-Physics Analytical, Inc.), and an AS3500 autosampler (TSP Inc.). The separation was carried out by using a μBondapak™ C18 reverse phase column (3.9×30mm, particle size 10 μm) (Waters Chromatography, Milford) and a guard column containing C18 material similar to the analytical column. The mobile phase was prepared using an HPLC grade MeOH, water and high grade MTBE. Prior to analysis, the solvent was filtered through a 0.5 um PTFE membrane filter (ADVANTEC MFS) and degassed using an ultrasonic bath. The identification of the major components of solvent extracts obtained from *Citrus* fruits were achieved by comparing their HPLC retention times with those of known reference compounds and UV-visible absorption spectra. Carotenoids were separated at room temperature under gradient conditions changing MTBE/MeOH/water from (95:1:4) to (25:71:4) for 13 min. Samples were injected onto the column via an automatic sampler equipped with a sample loop (20 μL). The column temperature was maintained at 35℃, and peak responses were measured at 445 nm. With reference carotenoids, the linearity of the calibration graphs was confirmed and the retention times were determined. Table 3 indicated the condition for HPLC analysis.

Table 3. Operating conditions of HPLC for carotenoids.

Apparatus	Spectra-Physics (SpectraSYSTEM)
Detector	UV1000 UV/Vis detector
Wave length	445 nm
Column	μBondapak™ C18 (10 μm)
Flow rate	1 mL/min
Injection volume	20 μL
Column temperature	35℃
Mobile phase	
A solvent	MTBE / MeOH / H_2O (95/1/4, v/v/v)
B solvent	MTBE / MeOH / H_2O (25/71/4, v/v/v)

Gradient table

Time (min)	A (%)	B (%)
0	100	0
12	100	0
25	0	100
30	100	0
35	100	0

2) Cloning of *Psy* and *Chx* from *Citrus*

(1) Extraction of plant RNA

Extraction of plant total RNA was performed using the hot phenol RNA isolation procedure as described by Verwoerd *et al.* (1989). Plant tissue was ground to a fine powder with liquid nitrogen, resuspended with preheated mixture (80℃) of 5 mL Extraction buffer and an equal amount of phenol, and was homogenized by vortexing for 30 seconds. The mixture was mixed with 1/2 volume of chloroform:isoamyl alcohol and vortexed again 30 seconds. The suspension was centrifuged at 4℃ for 15 min at 9250xg and the upper aqueous phase was removed. After addition of one volume 4 M LiCl, the solution was mixed and stored at -70℃ for 1 hr. The pellet was collected by centrifuging at 4℃ for 15 min at 9250xg, washed with 70% ethanol, and dried under vacuum. The pellet was dissolved in DEPC-treated water. If the RNA is not to be used immediately, the RNA solution was mixed with 1/10 volume 3 M NaOAc and 2.5 volumes 95% ethanol, and placed at -70℃.

(2) Construction of a cDNA library and screening

Total RNA was extracted from full yellow *Citrus* fruits using the hot phenol RNA isolation procedure (Verwoerd *et al.* 1989). Poly(A)$^+$ RNA was isolated by PolyATtract mRNA Isolation System III (Promega). A *Citrus* fruit cDNA library was constructed by using the Zap-cDNA synthesis and Gigapack II gold cloning kits (Stratagene) according to the manufacturer's instructions. *In vivo* excision of pBluescript SK+ plasmids was done in the *E. coli* SOLR strain. The library was screened with the radiolabeled PCR products, as described below, by standard plaque lift methods (Sambrook *et al.*, 1989). After prehybridization for 1-2 hr at 42℃ in 30% formamide, 5x Denhardt's solution, 5x SSPE, and 100 μg/mL

denatured salmon sperm DNA, filters were washed twice in 2x SSC and 0.05% SDS for 15 min at 42℃ and twice in 0.2x SSC and 0.1% SDS for 15 min at 68℃.

(3) PCR amplification and probe preparation

For the amplification of *Psy* cDNA, the sense primer (DCitPsy5: 5'-ATG[A/G]T[A/T/C]GAAGGAATG[A/C]G[G/A/T]ATGGA-3') and the antisense primer (DCitPsy3 : 5'-GCATC[T/C]TC[G/T]CC[A/T/C]AC[A/G]TCT CT-3') were used. Also, for the amplification of *CHX1* cDNA, the sense primer (DCitChx5: 5'-TCTTCCA[A/G]TTCCTT[A/G]GGTCC-3') and the antisense primer (DCitChx3: 5'-[G/T]AGGAAGAA[A/G]TC[A/G]GAGAGGT-3') were used. These PCR primers were synthesized on the basis of the conserved regions of the previously reported sequences of *Psy* and *Chx* in plants, respectively. Template for PCR was generated by reverse transcription as described by Sambrook *et al.* (1989), using 1 μg of mRNA, 1 μg of random hexamers as primers, and 200 units of M-MLV reverse transcriptase (Promega) in a total volume of 20 μL. PCR amplification was performed in the DNA thermal cycler (Perkin-Elmer Cetus). The reaction mixture (50 μL) contained 20 μL of the cDNA reaction mixture as described above, 100 pmol each of sense and antisense strand primers, 0.4 mM deoxyribonucleotide triphosphates, 2.0 units of *Taq* DNA polymerase (Perkin-Elmer Cetus), and *Taq* DNA polymerase reaction buffer (50 mM KCl, 10 mM Tris-HCl, pH 9.0, at 25℃, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.1% Triton X-100). The denaturation of DNA was carried out at 94℃ for 5 min for the first cycle and then for 1 min. Primer annealing and extension reactions were performed at 50℃ for 1 min and at 72℃ for 1 min, respectively. Total 30 cycles of amplification were performed. PCR products were visualized on 1% agarose gels stained with ethidium bromide and purified by GenCleanII kit (BIO 101, Inc). The 560 bp for *Psy* and 573 bp for *Chx* fragments were cloned into pGEM-T vector (Promega), which is called pGEM T-fPsy and pGEM T-fChx respectively, by using *E.coli*

JM109 as host. Then it was radioactively labeled by use of a random primer labeling kit (Promega) with [α-^{32}P] dCTP for the probe preparation.

(4) Isolation of plasmid DNAs

Small quantities of plasmid DNA were prepared from *E. coli* cells by the alkaline lysis method of Birnboim and Doly (1979). In order to sequence plasmids, the plasmid DNA is prepared using Wizard Miniprep (Promega, Cat. No. A7500) from a 3 mL overnight of *E. coli*.

(5) DNA manipulations

DNA samples were digested with restriction endonucleases according to the supplier's recommendations. Plasmid DNAs and/or their digests were analyzed by horizontal agarose (Promega) gel (0.8%-1.5%, w/v) electrophoresis for 0.5-100 kb and MetaPhor agarose (FMC Bioproducts) gel (2-3%, w/v) for 50-500 bp, with TAE buffer system or vertical polyacrylamide gel (6%, w/v) electrophoresis with TBE buffer system (Voytas, 1987).

Agarose gels were routinely used to analyze the double-stranded DNA fragments. After running, the gel bands were visualized by soaking into 1 μg/mL of ethidium bromide in the electrophoresis buffer for about 20 min and then transilluminating the long wavelength UV light. If necessary, specific DNA bands were eluted and purified from the agarose gel using GENECLEAN II kit (Bio 101 Inc.) or JetSorb (GENOMED Inc.) according to the manufacturer's guidance. Ligation reactions of DNA fragments were carried out with T4 DNA ligase in a volume of 10 μL for 12 hr at 16℃ and 22℃ for cohesive and blunt ends, respectively. Other DNA modifying enzymes were used to manipulate the DNA fragments according to the supplier's recommendations.

(6) Transformation of *E. coli*

(6-1) Preparation of Competent cells

A single colony of *E. coli* cells was inoculated into 3 mL LB media (1% NaCl, 1% Bacto-tryptone and 0.5% yeast extract, pH 7.0) and grow overnight. The 1.5 mL of overnight culture was inoculated into 100 mL LB media in a 250mL flask. The culture was incubated at 37℃ with shaking, to an ABS_{600} of 0.3-0.4 (about during 2hr 45min). This procedure requires that cells be growing rapidly (early- or mid-log phase). The culture was aliquoted into 250 mL sterile centrifuge tubes and leaved the tubes on ice to 10 min. The cells were centrifuged for 5 min at 5000 rpm, resuspended gently each pellet in 10 mL ice-cold solution of 50 mM $MgCl_2$ and 80 mM $CaCl_2$ solution, and then stored on ice for 10 min. The latter two steps were repeated twice. Each pellet was resuspended in 14 mL of ice-cold 100 mM $CaCl_2$ and the suspension was mixed with an equal volume of 50% glycerol to yield competent cells. The tube was standed for 4 hr or more at 4℃. Competent cells were distributed with 500 μL into the chilled Eppendorf tube, flash frozen in liquid nitrogen, and then placed at -70℃ deep freezer.

(6-2) Transformation procedure

DNA samples (about 200 ng) was diluted into 100 μL with DDW, and then 10 μL of solution (0.5 M $MgCl_2$ and 0.1 M $CaCl_2$) and 8 μL of 30% PEG6000 solution were added. The mixture was added to the 100 μL of rapidly thawed competent cells and mixed gently. After standing on ice for 30 min, cell suspension was heat-shocked at 42℃ for 90 seconds and rapidly cooled on ice for 5 min. The mixture was diluted with appropriate volume of SOC medium and incubated at 37℃ for 60 min with gently shaking. Then appropriated volume of cells were spread on the LB agar plate supplemented with ampicillin, and incubated for 18 hr at 37℃.

(7) DNA sequencing and analyses

Nucleotide sequencing using the dideoxy chain termination method (Sanger *et al.*, 1977) was done using Sequenase Version 2.0 kit (United State Biochemical), and T3 promoter, T7 promotor, and custom-made (DNA International), for a double strand to avoid errors. Sequencing reaction was carried out using [α-^{35}S]dATP (1 m*ci*/mmole) according to the supplier's instruction. Each reaction mixture was then incubated at 80℃ for 3 min and an alquot of 2.5 μL was loaded onto a 6% polyacrylamide gel containing 8 M urea in TBE. The gel was then dried to on a sheet of Whatman No. 1 paper and exposed to an X-ray film (Fuji) for 24 hr. Computer analyses for the nucleotide and amino acid sequences were done by PCGENE software (IntelliGenetics Inc., Release 6.60).

(8) Northern blot analysis

Total RNA was isolated from fruits in five developmental stages (MG, SG, FG, BR, and FR) and leaves in five stages (L1, L2, L3, L4, and L5) as well as flowers. The fruits were divided into two parts, flesh (juice sacs/pulp segments) and peel, except for the mini-green fruit of 25 DAF. The harvest stages of each material were described above. The RNA was fractionated on a denaturing agarose (1.0%) gel, and then transferred to the nylon membrane (Hybond-N from Amersham). Filters were prehybridized at 42℃ for 1-2 hr in 50% formamide, 5x SSPE, 5x Denhardt's solution, 0.1% SDS, and 100 μg/mL denatured salmon sperm DNA. The hybridization to the labeled probe with [α-^{32}P] dCTP and random primer was done overnight in a hybridization buffer. The filters were washed twice at room temperature for 10 min in 2x SSC and 0.1% SDS, once at 65 ℃ for 15 min in 1x SSC and 0.1% SDS, and twice at 65℃ for 15 min in 0.1x SSC and 0.1% SDS. After the filters (Hybond-N from Amersham) were stripped of probe according

to the manufacturer's instruction, the same blot was hybridized with partial cDNA of 18S rRNA labeled with [α-^{32}P] dCTP. The signal intensity of blot was analyzed by the scanning densitometer (Pharmacia) to adjust a little difference in loading amount among RNA samples. The probe was PCR products amplified by use of degenerate primers, as described above, and *Psy1* and *CHX1* cDNA as template, labeled with [α-^{32}P] dCTP.

(9) Genomic DNA blot analysis

Genomic DNA was isolated from young leaves of *Citrus* plants, by the method of Dellaporta *et al.* Genomic DNA was digested with *Sac*I, *Xba*I, and *Hin*dIII separated on 0.7% agarose gels, and then blotted onto a Hybond-N (Amersham). Hybridization and washing of filters were done as described in northern blot analysis.

Ⅲ. RESULTS AND DISCUSSION

1. Contents of β-cryptoxanthin in peel and flesh of *Citrus* fruits

Crude carotenoids were successfully extracted from peel and flesh of *Citrus* fruits, and their composition analysis was performed with a standard β-cryptoxanthin using the TLC method. Most crude carotenoids contained more than four different compounds (Figure 3). From crude carotenoids prepared from peel and flesh in *Citrus* fruits, β-cryptoxanthin was confirmed. The separation obtained was not affected by the coexisting substances in the *Citrus* fruits, and the spots indicated the R_f value of 0.39 as did the standard β-cryptoxanthin. Dongjeongkyool contained very low content of β-cryptoxanthin. On the other hand, Miyagawa showed the highest content of β-cryptoxanthin compared with Byungkyool and Dongjeongkyool. It indicated that β-cryptoxanthin content was greatly variable according to the kinds of *Citrus* fruits.

HPLC analysis was carried out to determine β-cryptoxanthin content from crude carotenoids. Because saponification with methanolic potassium hydroxide led to an increase in free β-cryptoxanthin of *Citrus* fruits (Khachik *et al.*, 1988), saponified carotenoids were used for analysis. Fortunately, carotenoids are coloured and adsorb maximally at about 450 nm, these compound can be easily determined and quantitated in a spectrophotometer in the visible range (Tee *et al.*, 1991). As shown in Figure 4, carotenoids were fractionated as various major peaks. Retention time of β-cryptoxanthin and β-carotene as standards were 18 and 24 min, respectively. Flesh of Byungkyool contained several isomeric pigments with a retention time of 6 min and relatively small amount of β-cryptoxanthin. On the other hand, both the peel and flesh of Miyagawa wase showed a large amount of β-cryptoxanthin, and relatively very small amount of other compounds. In particular, carotenoids prepared from the flesh of

Miyagawa wase contained highly purified β-cryptoxanthin.

β-Cryptoxanthin content in *Citrus* fruits are shown in Table 4. Contents of β-cryptoxanthin in fresh peel of Miyagawa wase, Byungkyool, and Dongjeongkyool were found to be 5.26, 1.66, and 0.88 mg%, respectively. On the other hand, β-cryptoxanthin contents in the flesh of Miyagawa wase, Byungkyool, and Dongjeongkyool were 0.78, 0.32, and 0.45 mg%, respectively. It was reported that β-cryptoxanthin content extracted from fruit mix (pear, peach strawberry, lemon, orange, and fried fruit) was 0.12 mg% (lyophilised samples) and increased to 0.15 mg% after saponification (Riso *et al.*, 1997). The β-cryptoxanthin content of *Citrus* juice cultivars varied between 8-160 ug% according to the type of cultivars (Stewart, 1977b). A carotenoid extract from Orlando tangelo juice showed 15.3 ug% of β-cryptoxanthin by HPLC (Stewart, 1977a). Conclusively, the β-cryptoxanthin content was higher in the peel than in the flesh of the native *Citrus* fruits cultivated in Jeju island. In particular, the peels of Miyagawa wase showed relatively high content of β-cryptoxanthin and thus is an important source for β-cryptoxanthin. These results indicate that the β-cryptoxanthin contents is significantly higher in the cultivars cultivated in Jeju island than in other cultivars culrivated in other regions.

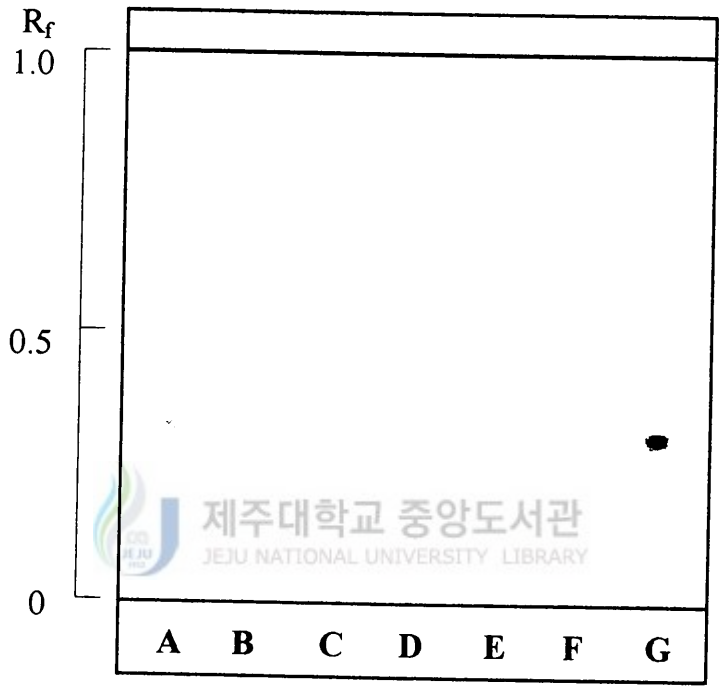

Figure 3. TLC chromatograms of standards of β-cryptoxanthin, and carotenoids *Citrus* varieties. A : Standard of β-cryptoxanthin, B : Peel of Byungkyool, C : Flesh of Byungkyool, D : Peel of Dongjeongkyool, E : Flesh of Dongjeongkyool, F : Peel of Miyagawa wase, G : Flesh of Miyagawa wase.

TLC conditions : 1) Plate; Silica gel 60 F254 TLC (Merck), 2) Solvent system; Hexane/Acetone (75/25, v/v)

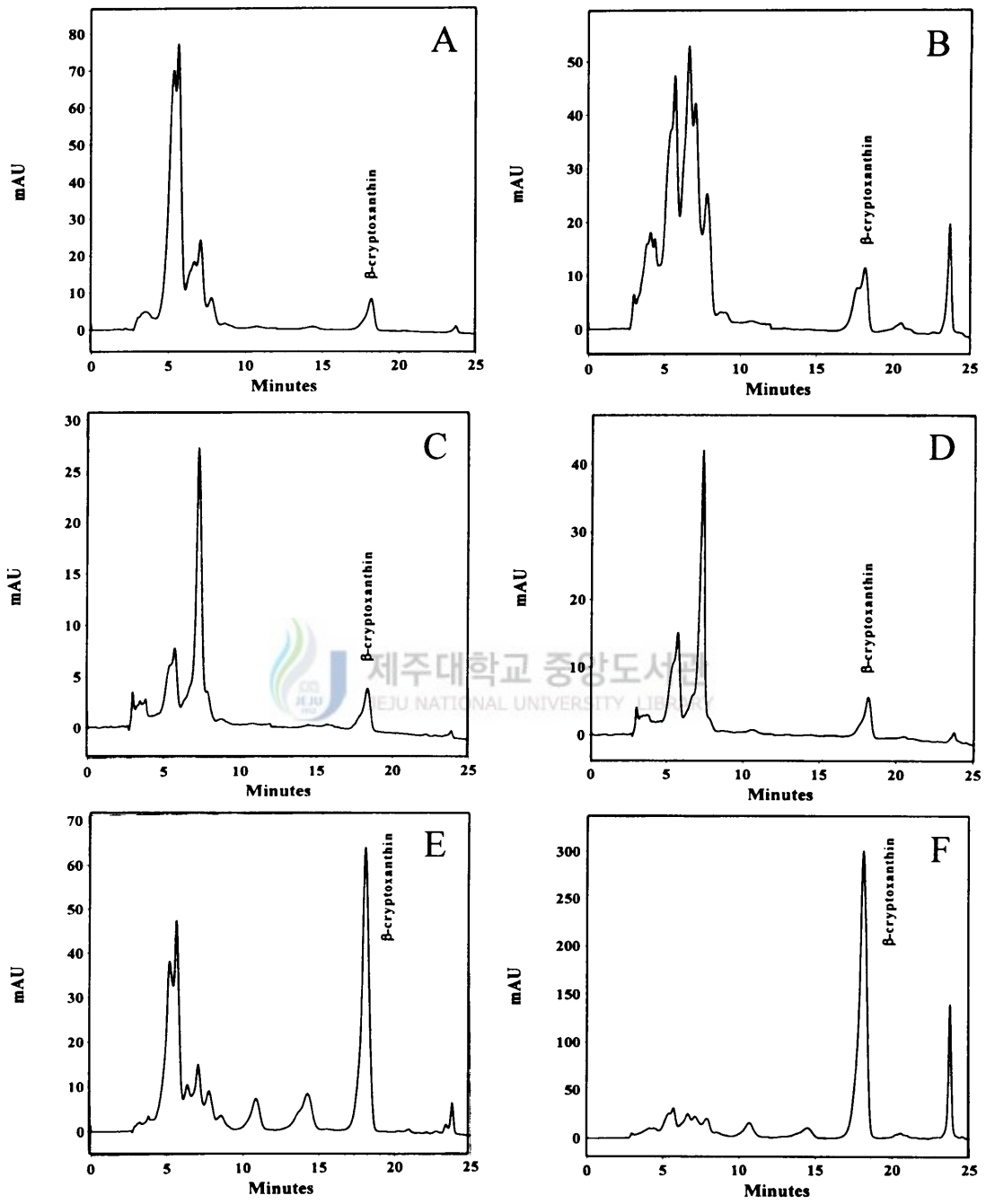

Figure 4. HPLC elution profiles of carotenoid, extracted from *Citrus* fruit of varieties. A : Peel of Byungkyool, B : Flesh of Byungkyool, C : Peel of Dongjeongkyool, D : Flesh of Dongjeongkyool, E : Peel of Miyagawa wase, F : Flesh of Miyagawa wase

Table 4. β-Cryptoxanthin content from three *Citrus* cultivars.

Cultivar	Content (mg%*)	
	Peel	Flesh
Miyagawa wase	5.26	0.78
Byungkyool	1.66	0.32
Dongjeongkyool	0.88	0.45

*mg% : content of β-cryptoxanthin mg/100g of samples.

2. Cloning and Expression Analysis of Psy and Chx Gene

To study of the Psy and Chx showing fruit specificity of expression, the experimental scheme (Figure 5) was followed throughout this work.

1) Probe preparation for screening

Total RNA was extracted from *Citrus* and was analyzed by 1% agarose gel electrophoresis (Figure 6A). Four kinds of degenerate oligonucleotides (Table 5) were designed from the sequence of the highly conserved regions of the known Psy and Chx genes (Figure 7). The oligonucleotides were used as primers for PCR reaction to amplify a partial *Psy* and *Chx* cDNA fragment from mRNA of *Citrus* fruit. The 230 and 560 bp for *Psy* and 573 bp for *Chx* PCR products were amplified, as shown in Figure 6B and 6C, respectively. The DNA fragments were cloned into pGEM-T® vector (Figure 6D and 6E) and sequenced using T7 and T3 primers.

2) Isolation of cDNA clone

(1) Psy1

By using degenerate primers (DCitPsy5 and DCitPsy3), 230 bp cDNA fragment encoding Psy1 was amplified by PCR reaction (Figure 6B). Fiffteen positive clone were isolated out of 450,000 plaques (Figure 8). We screened full cDNAs from the *Citrus* fruit cDNA library and isolated a cDNA clone, which is called Psy1 (Figure 9A). It has been reported that plant *Psy*, including that of the tomato (Bartley and Scolnik *et al.*, 1993; Fraser *et al.*, 1999), bell pepper (Römer *et al.*, 1993), and muskmelon (Karvouni *et al.*, 1995), exists in two more isoforms encoded by different genes, which suggests that *Citrus* might have additional isoforms of *Psy*, besides *Psy1*. Two isoforms of *Psy* in tomatos showed differential

expression patterns according to fruits and leaves. However, we could not isolate any clones having different sequences from *Psy1*, from the *Citrus* leaf cDNA library as well as the fruit library.

The length of the *Psy1* cDNA clone (GenBank accession number AF220218) is 1506 bp. The *Psy1* cDNA contains 1311 bp coding region, 111 bp 5′ UTR (untranslated region), and 84 bp 3′ UTR, which has an uninterrupted open reading frame deriving 47 kDa polypeptide (Figure 9). Putative polyadenylation signal was found at the positions 1488-1493 (AATGAA).

(2) CHX1

A 573 bp cDNA fragment encoding CHX1 was amplified by PCR reaction with degenerate primers (DCitChx5 and DCitChx3)(Figure 6C). Fourteen positive clone were isolated out of 450,000 plaques (Figure 8). We screened full cDNAs from the *Citrus* fruit cDNA library and isolated a cDNA clone, which is called *CHX1* (GenBank accession number AF296158) (Figure 9B). It has been reported that plant *Chx*, including that of the tomato (GenBank accession number Y14809 and Y14810) and the bell pepper (Bouvier *et al.*, 1998) exists in two more isoforms encoded by different genes, which suggests that *Citrus* might have additional isoforms of *Chx*, besides *CHX1*. However, we didn't any isoform from the *Citrus* leaf and fruit cDNA libraries. We did isolate an other clone, though, which is called *CHX2* (GenBank accession number AF315289), and is polymorphic to CHX1, from *Citrus* leaf and fruit cDNA library (Figure 10). There are differences in 6 nucleotides and 3 amino acids between CHX1 and CHX2.

The *CHX1* cDNA contains 936 bp coding region, 130 bp 5' UTR, and 173 bp 3' UTR, which has an uninterrupted open reading frame deriving 34 kDa polypeptide (Figure 9). The polypeptide contains conserved histidine residues important for the iron coordination of enzymatic function (Bouvier *et al.*, 1998). Putative polyadenylation signals were found at positions 1131-1136 (AATGAA), 1140-115 (AATAAA), 1193-1198 (AATAAT), and 1207-1212 (AATGAA).

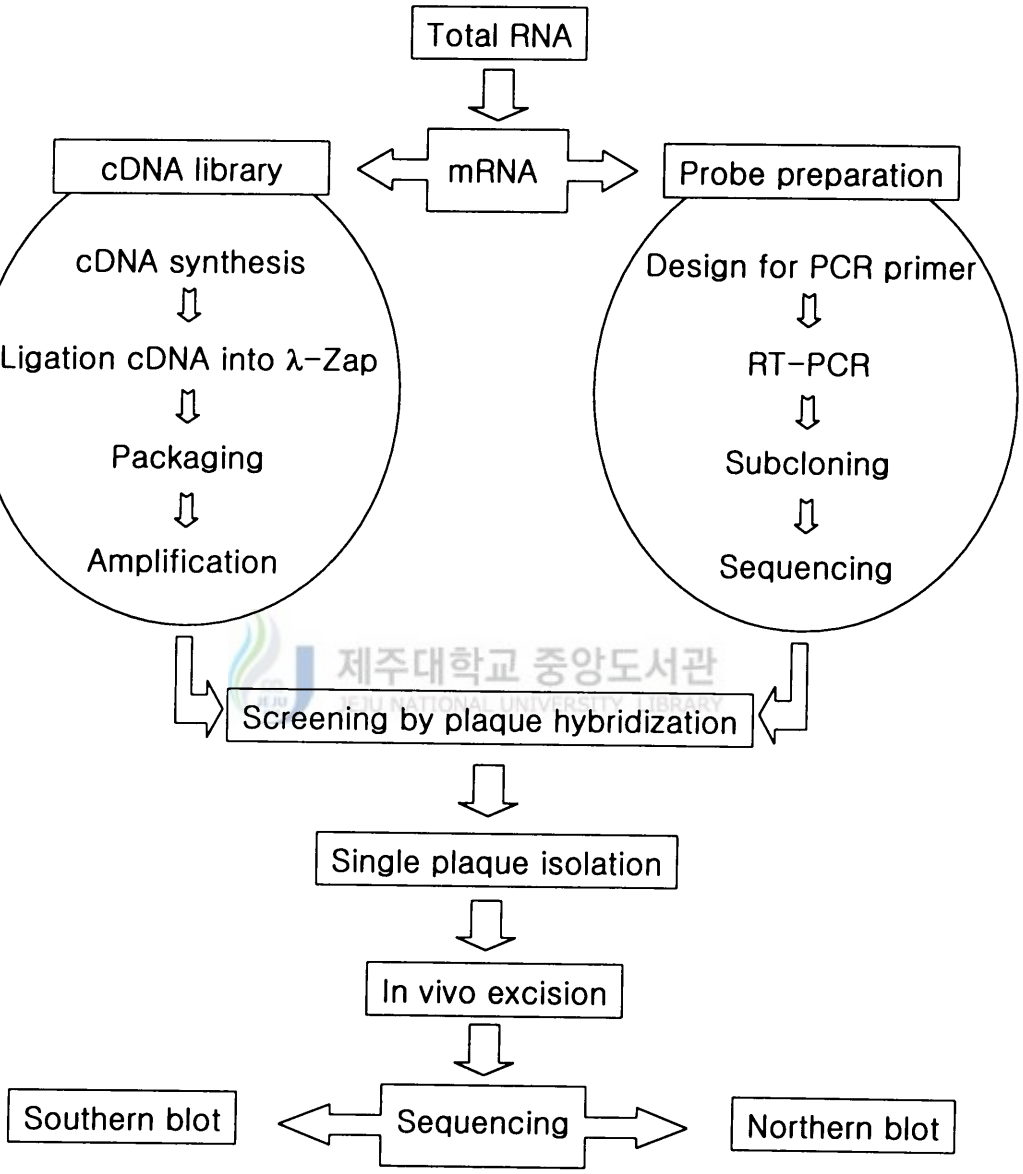

Figure 5. Flow chart for experimental scheme.

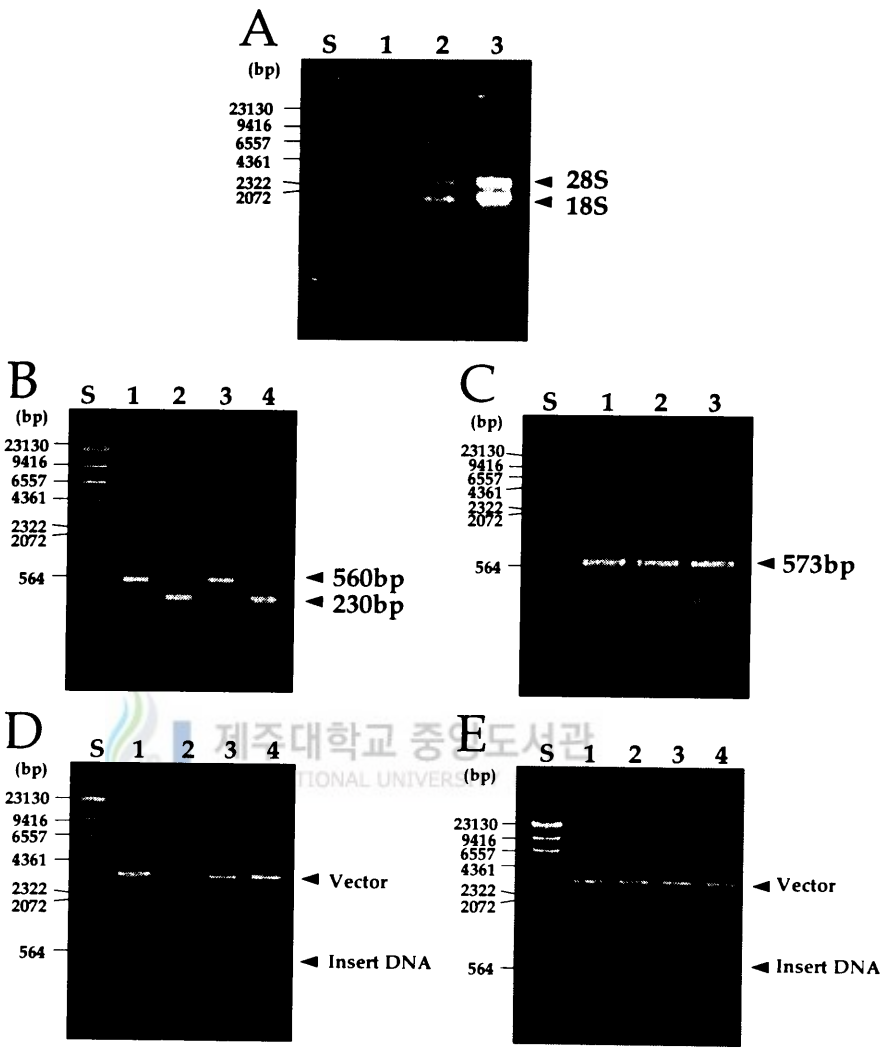

Figure 6. Amplification of *Psy1* and *CHX1* cDNA fragments by RT-PCR and their subcloning. (A) Total RNA extracted from *Citrus* fruits. (B) Poly(A)$^+$ RNA from *Citrus* fruit was used as a template. RT was done with MMLV reverse transcriptase and random hexamer. PCR was amplified with *Taq* DNA polymerase and synthetic oligomer. The four combinations of primers used in PCR of *Psy* cDNA fagment are as follows: lane 1 and 3 for CitpPsy5 and CitPsy3 (product size: 560bp), lane 2 and 4 for DCitPsy5 and DCitPsy3 (product size: 230bp). Lane 1 and 2 is amplified from flesh of fruits, and lane 3 and 4 is from peel. (C) The primers used in PCR of *Chx* cDNA fagment are as follows: lane 1, 2 and 3 (573 bp product) for CHX5f and CHX3f. Arrowhead indicates amplified PCR product. (D, E) The PCR products of *Psy* and *Chx* were subcloned into pGEM-T vector. The plasmids for Psy (D) and Chx (E) digested with *EcoR*I, and then electrophoresed onto agarose gel. S (bp) represents the molecular standards of λ/*Hin*dIII.

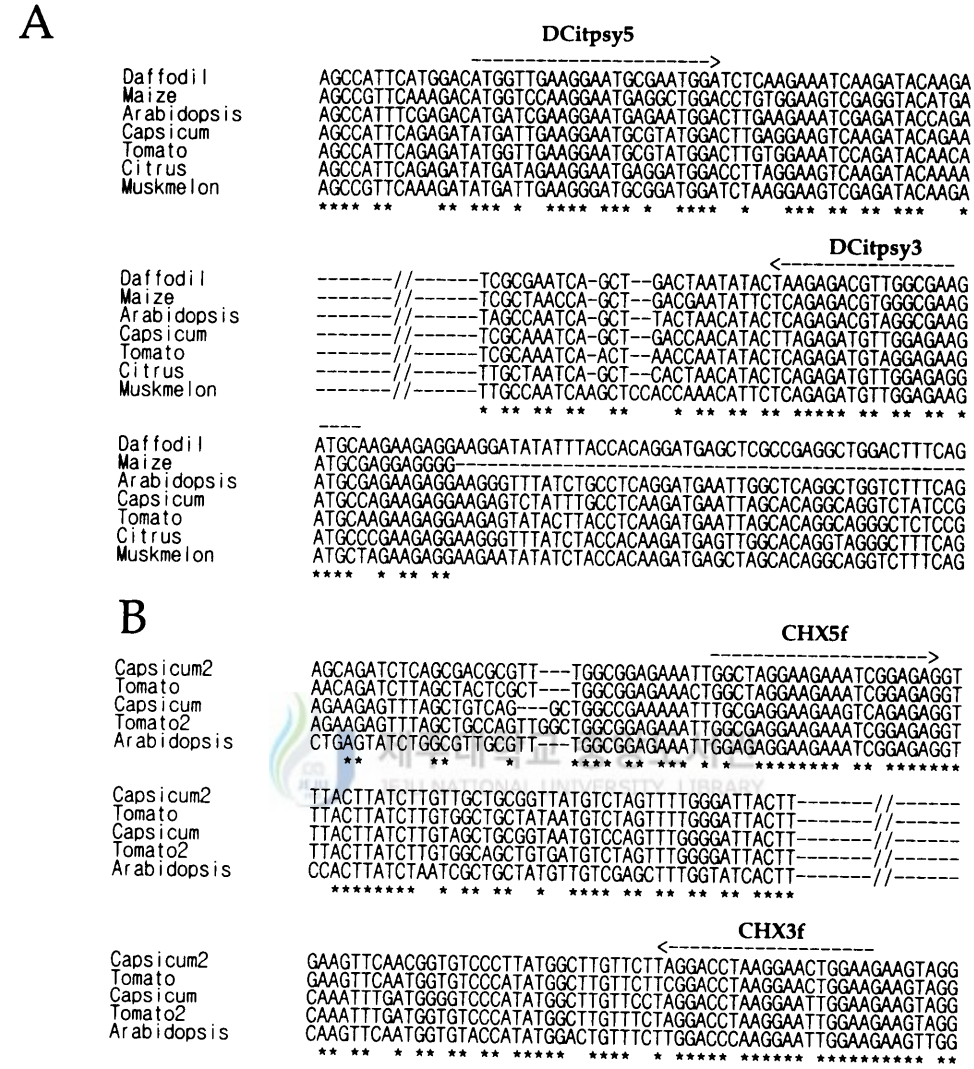

Figure 7. Design of degenerate oligomers. Multiple sequence alignment was done with cDNA encoding Psy (A), including Daffodil (GenBank accession number X78814), Maize(U32636), *Arabidopsis* (AF009954), *Capsicum* (X68017), Tomato (X60441), *Citrus* (AF152892), and Muskmelon (Z37543) and Chx (B), including Capsicum2 (Y09722), Tomato (Y14809), *Capsicum* (Y09225), Tomato2 (Y14810), and *Arabidopsis* (AF125576). This alignment was generated by Clustal X (version 1.64b) (Thompson et al., 1994). An asterisk indicates identical nucleotides. Gaps were introduced to maximize identities, represented by a dash.

Table 5. The nucleotide sequences of the regions highly conserved among plant *Psy* and *Chx* genes. On the basis of these regions, degenerated oligonucleotides were synthesized and used for PCR amplification.

	Primers	Sequence
Psy	DCitPsy5	5′-ATGRTHGAAGGAATGMGDATGGA-3′
	DCitPsy3	5′-GCATCYTCKCCHACRTCTCT-3′
Chx	CHX5f	5′-KAGGAAGAARTCRGAGAGGT-3′
	CHX3f	5′-TCTTCCARTTCCTTRGGTCC-3′

* Symbols for mixed mer;
D=(G+A+T), H=(A+T+C), K=(G+T), M=(A+C), R=(A+G), Y=(C+T)

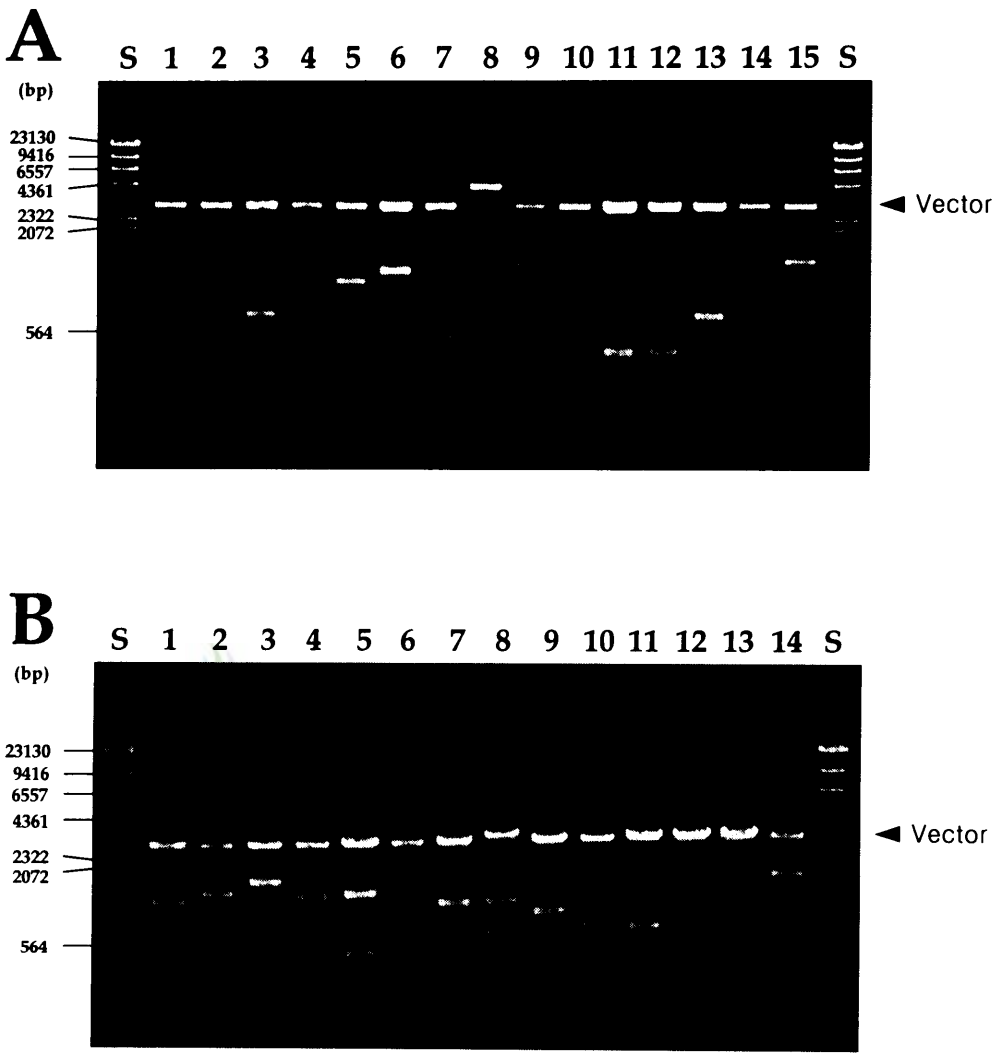

Figure 8. Agarose gel (1%) electrophoresis of *Eco*RI and *Xho*I digestion products of *in vivo* excised phagemid from containing *Psy1* (A) and *CHX1* (B) cDNAs. Clones were isolated through plaque hybridization twice and *in vivo* excised into pBluescript SK+ phagemids. S (bp) represents the molecular standards of λ/*Hin*dIII.

A

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1     GTTCTCTGACAAGCTTTCTCTGATTTCTGGTATCAGGATTCAGCTACAGAGAGTTGAAGTTACAGGGGCCTCAAAATTTTCTTTTTATCC

91    AAAAATAATAATTTTTACAGCATGTCTGTTACATTGCTGTGGGTTGTATCACCTAACTCACAATTGTCCAATTGCTTCGGGTTCGTCGAT
                                M  S  V  T  L  L  W  V  V  S  P  N  S  Q  L  S  N  C  F  G  F  V  D

181   TCAGTTCGAGAGGAAAACAGGCTGTTTTATTCATCAAGATTTCTTTACCAACATCAAACCCGGACTGCTGTGTTTAATTCTAGACCTAAG
      S  V  R  E  E  N  R  L  F  Y  S  S  R  F  L  Y  Q  H  Q  T  R  T  A  V  F  N  S  R  P  K

271   CAGTTTAATAATAGTAATAAGCAGAGACGGAATTCTTATCCTTTAGATACAGATTTGAGGCATCCTTGCTCATCTGGAATCGACTTGCCT
      Q  F  N  N  S  N  K  Q  R  R  N  S  Y  P  L  D  T  D  L  R  H  P  C  S  S  G  I  D  L  P

361   GAAATATCATGTATGGTTGCTAGCACTGCTGGAGAAGTGGCCATGTCTTCAGAAGAAATGGTTTACAATGTTGTGCTCAAGCAGGCAGCC
      E  I  S  C  M  V  A  S  T  A  G  E  V  A  M  S  S  E  E  M  V  Y  N  V  V  L  K  Q  A  A

451   TTGGTTAATAAGCAACCAAGTGGGGTTACTCGTGATCTTGATGTGAACCCAGATATTGCTTTACCCGGAACTTTAAGTCTGCTCAGTGAA
      L  V  N  K  Q  P  S  G  V  T  R  D  L  D  V  N  P  D  I  A  L  P  G  T  L  S  L  L  S  E

541   GCTTATGATCGTTGTGGAGAAGTTTGCGCCGAGTATGCTAAGACATTTTACTTGGGAACTTTGCTGATGACCTCTGAAAGGCGAAGGGCT
      A  Y  D  R  C  G  E  V  C  A  E  Y  A  K  T  F  Y  L  G  T  L  L  M  T  S  E  R  R  R  A

631   ATATGGGCTATATATGTGTGGTGTAGGAGGACAGATGAGCTCGTTGATGGGCCTAATGCTTCACACATAACTCCAACAGCTTTAGACAGG
      I  W  A  I  Y  V  W  C  R  R  T  D  E  L  V  D  G  P  N  A  S  H  I  T  P  T  A  L  D  R

721   TGGGAGTCCAGGTTGGAAGACCTTTTCCGGGGTCGTCCATTTGATATGCTTGATGCTGCATTATCAGATACAGTAACCAAATTTCCTGTC
      W  E  S  R  L  E  D  L  F  R  G  R  P  F  D  M  L  D  A  A  L  S  D  T  V  T  K  F  P  V

811   GACATTCAGCCATTCAGAGATATGATAGAAGGAATGAGGATGGACCTTAGGAAGTCAAGATACAAAAACTTTGATGAATTATACTTGTAT
      D  I  Q  P  F  R  D  M  I  E  G  M  R  M  D  L  R  K  S  R  Y  K  N  F  D  E  L  Y  L  Y

901   TGTTATTATGTTGCTGGGACCGTAGGGCTAATGAGTGTTCCAGTTATGGGCATAGCACCTGACTCACAGGCAACAACAGAGAGCGTCTAC
      C  Y  Y  V  A  G  T  V  G  L  M  S  V  P  V  M  G  I  A  P  D  S  Q  A  T  T  E  S  V  Y

991   AATGCAGCATTGGCACTAGGGATTGCTAATCAGCTCACTAACATACTCAGAGATGTTGGAGAGGATGCCCGAAGAGGAAGGGTTTATCTA
      N  A  A  L  A  L  G  I  A  N  Q  L  T  N  I  L  R  D  V  G  E  D  A  R  R  G  R  V  Y  L

1081  CCACAAGATGAGTTGGCACAGGCAGGGCTTTCAGATGATGACATATTTGCTGGAGAGGTGACCATTAAATGGAGAAACTTCATGAAGAAC
      P  Q  D  E  L  A  Q  A  G  L  S  D  D  D  I  F  A  G  E  V  T  I  K  W  R  N  F  M  K  N

1171  CAAATTAAGAGGGCAAGGATGTTCTTTGATATGGCTGAGAACGGTGTGACCGAGCTGAGTGAAGCTAGTCGATGGCCGGTATGGGCTTCA
      Q  I  K  R  A  R  M  F  F  D  M  A  E  N  G  V  T  E  L  S  E  A  S  R  W  P  V  W  A  S

1261  TTGCTGTTGTACCGGCAAATACTGGATGAGATTGAGGCCAATGATTACAACAACTTCACAAAGAGAGCTTATGTGAGTAAAGCCAAGAAG
      L  L  L  Y  R  Q  I  L  D  E  I  E  A  N  D  Y  N  N  F  T  K  R  A  Y  V  S  K  A  K  K

1351  ATAGCTGCACTACCAATTGCATATGCAAAATCCCTCTTACGCCCGTCAAGAATATATACCAGTAAGGCTTAAACTGAACATTTAACATCA
      I  A  A  L  P  I  A  Y  A  K  S  L  L  R  P  S  R  I  Y  T  S  K  A

1441  AAGTTTAGAAGCATATATAGTTGAAACGGATTCAAAGTGGAAAAAAAAAATGAAAATGATTGCTTGT
```


Figure 9. (A) Nucleotide sequence and deduced amino acid sequence for the *Psy1* cDNA clones isolated from *Citrus* fruits.

B

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1     CAAGAAGTCCAGAATTTCACGCTCACGCCATTAAACACCACAATCCACAATCCACTTCACATCAACTCTTCCTCTTTTCAAGTGCTTTTA

91    CTCTAAAACCCAAAACCTCGTAAACAAACAAAACCCCACCATGGCGGTCGGACTATTGGCCGCCATAGTCCCGAAGCCCTTCTGTCTCCT
                                                M  A  V  G  L  L  A  A  I  V  P  K  P  F  C  L  L

181   CACAACAAAACTTCAACCCTCTTCGCTCCTCACAACAAAACCTGCTCCCCTTTTTGCCCCTCTCGGTACCCGCCACGGCTTCTTTAATGG
        T  T  K  L  Q  P  S  S  L  L  T  T  K  P  A  P  L  F  A  P  L  G  T  R  H  G  F  F  N  G

271   CAAAAACCGAAGAAAAATCAACTCTTTCACCGTATGTTTTGTTTTAGAGGAGAAAAAACAAAGCACCCAGATCGAGACTTTCACGGAGGA
        K  N  R  R  K  I  N  S  F  T  V  C  F  V  L  E  E  K  K  Q  S  T  Q  I  E  T  F  T  E  E

361   GGAGGAGGAGGAGTCGGGTACCCAGATCTCGACTGCTGCCCGCGTGGCCGAGAAATTGGCGAGAAAGAGATCCGAGAGGTTCACTTATCT
        E  E  E  E  S  G  T  Q  I  S  T  A  A  R  V  A  E  K  L  A  R  K  R  S  E  R  F  T  Y  L

451   CGTTGCTGCCGTCATGTCTAGTTTTGGTATCACTTCCATGGCTGTCATGGCTGTTTATTACAGGTTCTGGTGGCAAATGGAGGGTGGAGA
        V  A  A  V  M  S  S  F  G  I  T  S  M  A  V  M  A  V  Y  Y  R  F  W  W  Q  M  E  G  G  E

541   GGTGCCTTTAGCTGAAATGTTTGGCACATTTGCTCTCTCTGTTGGTGCTGCTGTGGGCATGGAGTTTTGGGCACGATGGGCTCATAAAGC
        V  P  L  A  E  M  F  G  T  F  A  L  S  V  G  A  A  V  G  M  E  F  W  A  R  W  A  H  K  A

631   TCTGTGGCATGCTTCTTTATGGCATATGCACGAGTCTCACCATCGACCAAGAGAGGGTCCTTTTGAGCTAAACGATGTGTTTGCCATAAT
        L  W  H  A  S  L  W  H  M  H  E  S  H  H  R  P  R  E  G  P  F  E  L  N  D  V  F  A  I  I

721   CAACGCAGTTCCAGCCATAGCCCTTCTCTCTTTTGGCTTCTTCCACAAAGGCCTTGTACCTGGTCTCTGCTTTGGTGCTGGACTTGGCAT
        N  A  V  P  A  I  A  L  L  S  F  G  F  F  H  K  G  L  V  P  G  L  C  F  G  A  G  L  G  I

811   TACGGTGTTTGGGATGGCCTACATGTTCGTCCACGATGGTCTCGTTCACAAAAGGTTCCCTGTGGGTCCCATTGCCGACGTGCCTTATTT
        T  V  F  G  M  A  Y  M  F  V  H  D  G  L  V  H  K  R  F  P  V  G  P  I  A  D  V  P  Y  F

901   CCGGAGAGTCGCTGCGGCTCACCAGCTTCACCACTCGGATAAATTCCACGGTGTTCCATATGGGCTCTTTCTCGGACCTAAGGAGCTTGA
        R  R  V  A  A  A  H  Q  L  H  H  S  D  K  F  H  G  V  P  Y  G  L  F  L  G  P  K  E  L  E

991   AGAAGTGGGGGGACTAGAAGAATTGGAGAAGGAGATCAGTAAGAGAATCAAATCATACAACAGGGTTCCAAAATAATCAATTTAATGGGA
        E  V  G  G  L  E  E  L  E  K  E  I  S  K  R  I  K  S  Y  N  R  V  P  K

1081  GGACCAATTTTTGGATCAATTTGTCAGTGTACAGAAACAATAGTGTTATTAATGAAAAAAATAAATTATGAATGCTTATGGGTGGATTAC

1171  TGTTGTAAAGTTTATGATGTTAAATAATATATAGTCAATGAAAGATAACAAAAGGGATATATTAATTAT
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Figure 9. (B) Nucleotide sequence and deduced amino acid sequence for the *CHX1* cDNA clones isolated from *Citrus* fruits. The putative polyadenylation signals are represented by underlined.

```
CHX1  MAVGLLAAIVPKPFCLLTTKLQPSSLLTTKPAPLFAPLGTRHGFFNGKNRRKINSFTVCF
CHX2  MAVGLLAAIVPKPFCLLTTKLQPSSLLTTKPAPLFAPLGTHHGFFNGKNRRKLNSFTVCF
      ************************************:***********:*******

CHX1  VLEEKKQSTQIETFTEEEEEESGTQISTAARVAEKLARKRSERFTYLVAAVMSSFGITSM
CHX2  VLEEKKQSTQIETFTDEEEEESGTQISTAARVAEKLARKRSERFTYLVAAVMSSFGITSM
      ***************:********************************************

CHX1  AVMAVYYRFWWQMEGGEVPLAEMFGTFALSVGAAVGMEFWARWAHKALWHASLWHMHESH
CHX2  AVMAVYYRFWWQMEGGEVPLAEMFGTFALSVGAAVGMEFWARWAHKALWHASLWHMHESH
      ************************************************************

CHX1  HRPREGPFELNDVFAIINAVPAIALLSFGFFHKGLVPGLCFGAGLGITVFGMAYMFVHDG
CHX2  HRPREGPFELNDVFAIINAVPAIALLSFGFFHKGLVPGLCFGAGLGITVFGMAYMFVHDG
      ************************************************************

CHX1  LVHKRFPVGPIADVPYFRRVAAAHQLHHSDKFHGVPYGLFLGPKELEEVGGLEELEKEIS
CHX2  LVHKRFPVGPIADVPYFRRVAAAHQLHHSDKFHGVPYGLFLGPKELEEVGGLEELEKEIS
      ************************************************************

CHX1  KRIKSYNRVPK
CHX2  KRIKSYNRVPK
      ***********
```

Figure 10. Comparison of the deduced amino acid sequences of *CHX1* and *CHX2*. This alignment was generated by Clustal X (version 1.64b). An asterisk indicates the identical amino acids. The shaded letters are important histidine residues for the iron coordination.

3) Genomic Southern analysis

The genomic DNA isolated from *Citrus* leaves was digested with *Sac*I, *Xba*I, and *Hin*dIII, and then separated on 0.7 % agarose gels. As shown in Figure 11, the digested DNA fragments were hybridized with the *Psy1* and *CHX1* cDNA probe and washed at high stringency (0.1xSSPE and 0.1% SDS, 68℃). The genomic blot pattern showed 1 to 3 bands in each DNA sample, which indicated that *Psy1* and *CHX1* is present as low copy in *Citrus* genome.

4) Comparison of the deduced amino acid sequence of Psy1 and CHX1 with polypeptides known Psy and Chx

(1) Psy1

The deduced amino acid sequences were compared between *Citrus* and other organisms. The sequence of *Psy1* has the highest homology with muskmelon (79%). The Psy1 sequence has a high degree of similarity with various species of plants (50-79%), algae (47%), and cyanobacteria (53%), but not with bacteria and fungi (5-20%) (Figure 12A). The phylogenetic dendrogram showed similar patterns, which indicated that Psy isolated from fruits can be categorized into one group (Figure 13A). Therefore, these results suggested an evolutionary link among the fruit-producing plants.

(2) CHX1

The deduced amino acid sequences were compared between *Citrus* and other organisms. The sequence of *CHX1* has the highest homology with tomato (70%) (GenBank accession number Y14809) and bell pepper (70%), which is similar to the case of *Citrus Psy1* (GenBank accession No. AF220218). Its sequence has a high degree of similarity with various species of plants (64-70%), but not with bacteria and fungi (25-28%) (Figure 12B). The phylogenetic dendrogram showed

similar patterns, which indicated that *Chx* isolated from fruits can be categorized into one group (Figure 13B). Therefore, these results suggested an evolutionary link among the fruit-producing plants.

5) Expression pattern in *Citrus*

(1) *Psy1*

We investigated the expression patterns of *Citrus Psy1* gene in the process of fruit and leaf development (Figure 14). *Citus* fruits and leaves were harvested at five stages determined by maturity and internal fruit color, and size of fruit. Fruit tissues were partitioned into flesh and peel, except for the mini-green fruit of 25 DAF. As shown in Figure 14, the transcripts of *Citrus Psy1* were detected in all investigated tissues including fruits, leaves, and flowers. The *Psy1* transcript was consistent during leaf development, which suggested that the expression of *Psy1* gene is not regulated. During ripening of the flesh, the expression level of *Psy1* was lowest at the mini-green stage and markedly increased to the maximum at the full yellow stage. The accumulation of the expression of *Psy1* gene in fruit peel showed a similar expression pattern to flesh, except for a lesser extent in increase, which may be associated with the difference in carotenoids accumulated in each part of fruits. These patterns of *Psy1* in *Citrus* were more similar to the bell pepper (Römer *et al.*, 1993), a nonclimacteric fruit, while the *Psy* transcripts in tomato (Giuliano *et al.*, 1993) and muskmelon (Karvouni *et al.*, 1995), climacteric fruits, declined a little in their level after the breaker stage of fruit development, and are tissue-specific in their expressions.

However, there are some differences between the *Citrus* and the bell pepper. Two *Psy* transcripts were detected in pepper fruits and leaves, but a single one was detected in *Citrus* fruits and leaves (Römer *et al.*, 1993). Our results, including cDNA screening, genomic blot, and northern blot analysis, suggested that *Psy1* may be present as single copy in the *Citrus* genome.

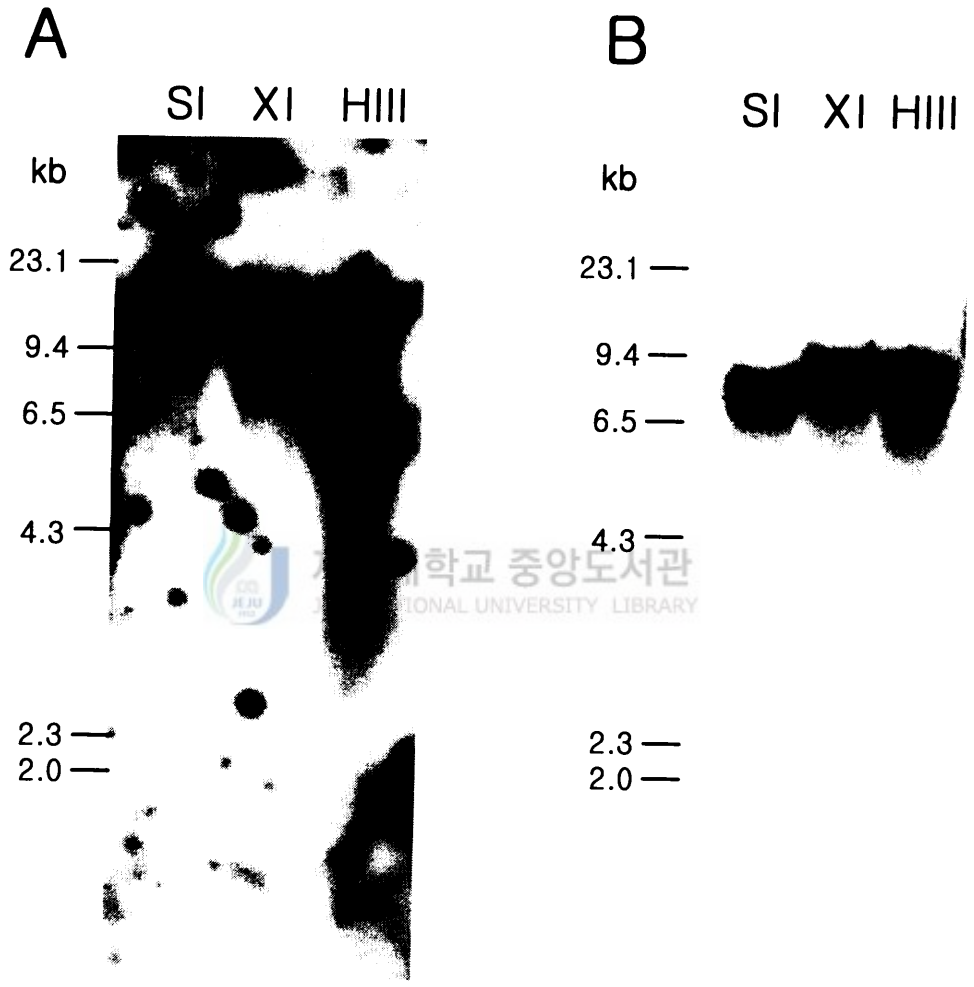

Figure 11. Genomic blot analysis of *Psy1* (A) and *CHX1* (B). Genomic DNA (10μg) was digested with *Sac*I (SI), *Xba*I (XI), and *Hin*dIII (HIII) for each DNA sample. Size markers (kb) are indicated on the left.

A

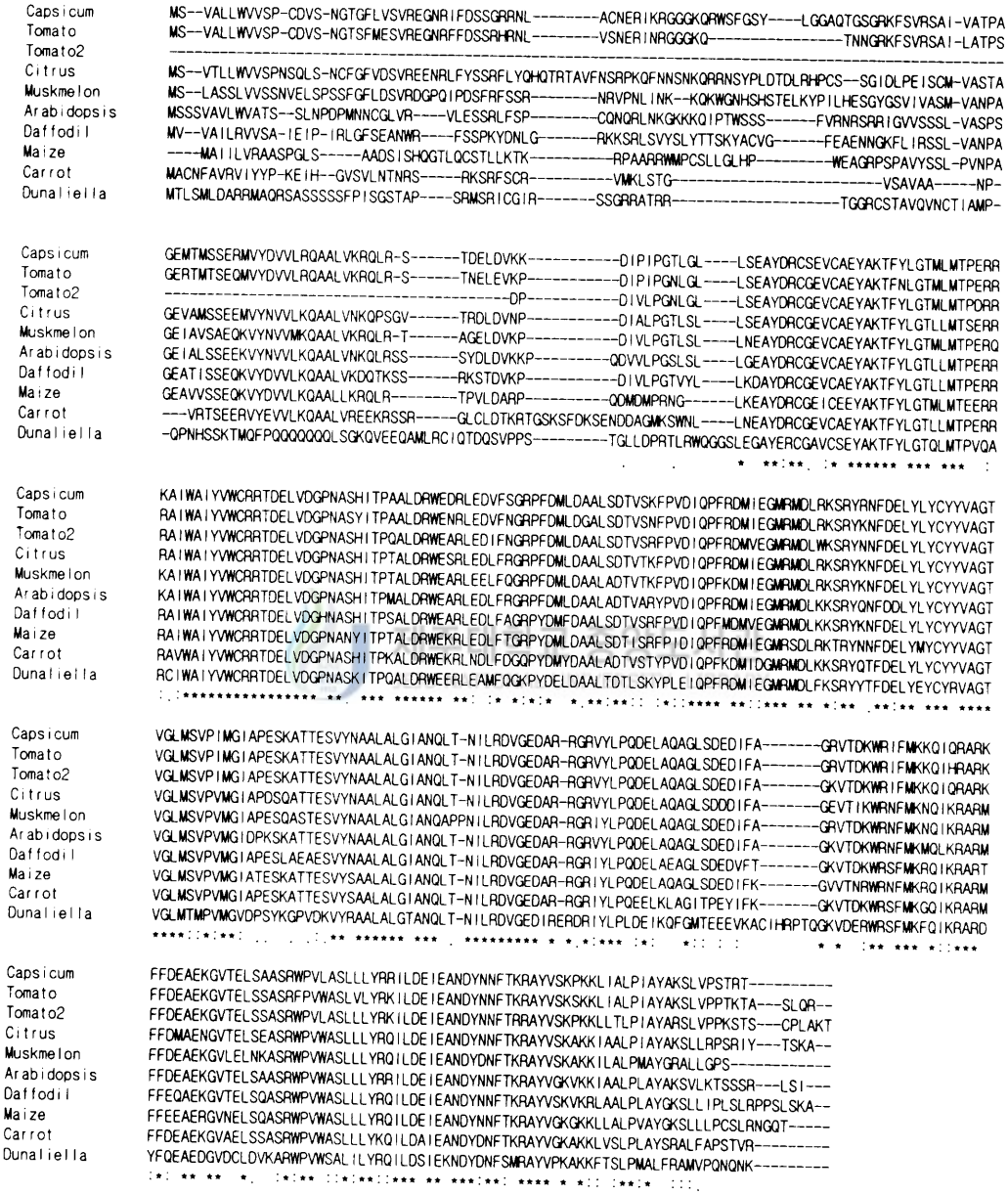

Figure 12. (A) Alignment of the deduced amino acid sequences of *Psy1* isolated from various species. Multiple sequence alignment was done with cDNA encoding PSY (A), including *Capsicum* (X68017), Tomato (M84744), Tomato2 (L23424), *Citrus* (AF220218), Muskmelon (Z37543), *Arabidopsis* (AF009954), Daffodil (X78814), Maize (U32636), Carrot (AB032797), and Dunaliella (U91900)

B

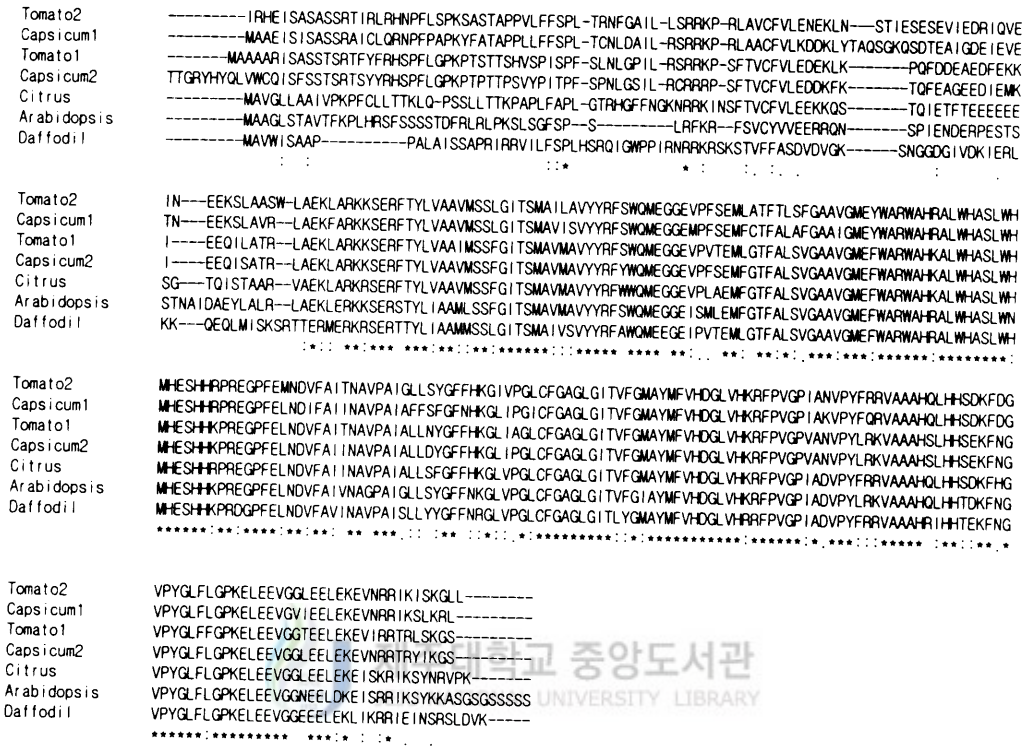

Figure 12. (B) Alignment of the deduced amino acid sequences of *CHX1* isolated from various species. Multiple sequence alignment was done with cDNA encoding CHX (B), including Tomato2 (Y14810), *Capsicum*1 (Y09225), Tomato1 (Y14809), *Capsicum*2 (Y09722), *Citrus* (AF296158), *Arabidopsis* (AF125576), and Daffodil (AJ278882). Names are GenBank accession numbers. Stars (*) represent the perfectly matched amino acids. The positions in the well-matched amino acids represented by '.'. The bold letters represent the different amino acid residues. This alignment was generated by Clustal X (version 1.64b) (Thompson et al., 1994). An asterisk indicates identical amino acid residues. The amino acid residues showing similar character are represented by a dot.

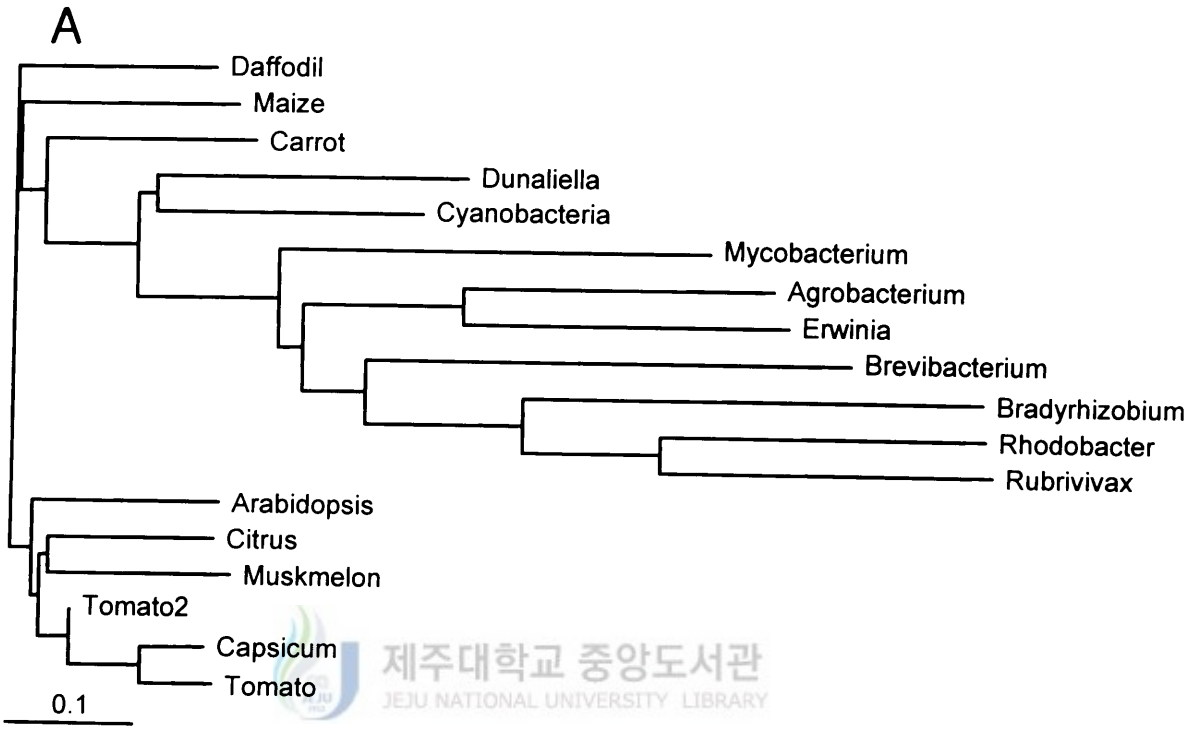

Figure 13. (A) Phylogenetic relationship of the *Citrus Psy1* to *Psy* of other various species. The *Psy* cDNA sequences used for amino acid translation and GenBank accession numbers are: Carrot (AB032797), *Citrus* (AF220218), *Arabidopsis* (AF009954), Maize (U32636), Daffodil (X78814), *Dunaliella* (U91900), Muskmelon (Z37543), bell pepper (X68017), Tomato (M84744), Tomato2 (L23424), *Mycobacterium* (AJ133724), *Agrobacterium* (D58420), *Rhodobacter* (X52291), *Erwinia* (M38423), *Bradyrhizobium* (AF218415), *Rubrivivax* (AB034704), *Brevibacterium* (AF139916), and *Cyanobacteria* (AB001284). Phylogenetic analysis is based on the deduced amino acid sequences of *Psy* from various species. The tree was generated by Clustal X (version 1.64b) and TreeView (version 1.64b).

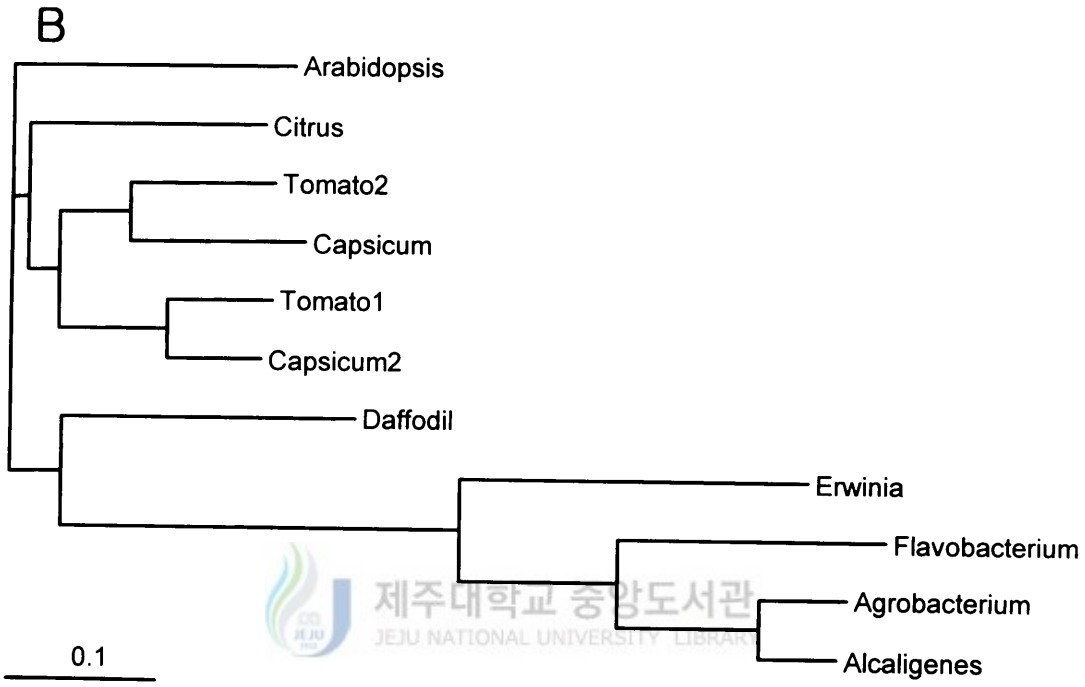

Figure 13. (B) Phylogenetic relationship of the *Citrus CHX1* to(h of other various species. The *Chx* cDNA sequences used for amino acid translation and GenBank accession numbers are: Daffodil (AJ278882), *Arabidopsis* (AF125576), Tomato2 (Y14810), Tomato1 (Y14809), Bell pepper2 (Y09722), Bell pepper1 (Y09225), *Citrus* (AF296158), *Flavobacterium* (U62808), *Agrobacterium* (D58420), *Alcaligenes* (D58422), and *Erwinia* (M87280). Phylogenetic analysis is based on the deduced amino acid sequences of *Chx* from various species. The tree was generated by Clustal X (version 1.64b) and TreeView (version 1.64b).

The expression profile of *Psy* in bell pepper during fruit ripening was a little different from *Citrus*. In bell pepper, the *Psy* transcript was not detected in green fruits nor even in mature green fruits (Römer *et al.*, 1993), while the *Psy1* transcript was detected in all processes of fruit development. These phenomena are consistent with the accumulation pattern of specific carotenoids in bell pepper and in *Citrus*. Therefore, these results suggest that although the expression of *Psy* could be related to fruits types, climacteric or nonclimacteric fruits, its expression varies with plant species, even those having the same type of fruit.

In summary, we isolated a cDNA clone encoding Psy1 and investigated the expression patterns during fruit and leaf development in *Citrus*, which is a nonclimacteric fruit-producing woody plant. The *Psy1* transcripts were detected in the leaf, fruit, and flower. The expression of *Citrus Psy1* showed a differential pattern during fruit development, while its expression was consistent during leaf development. The expression analysis of other genes, including phytoene desaturase and β-carotene hydroxylase, involved in carotenogenesis could lead to the an illumination of the relationship between gene expression and carotenoid biosynthesis in *Citrus*.

(2) *CHX1*

We investigated the expression patterns of *Citrus CHX1* gene in the process of fruit and leaf development (Figure 14). As shown in Figure 14, the transcripts of *Citrus CHX1* were detected in all investigated tissues including fruits and leaves, and flowers. The level of *CHX1* transcript didn't change during fruit, leaf development, which indicated that the gene expression of *CHX1* is not regulated at the transcriptional level. The expression pattern of *CHX1* gene is not consistent with the accumulation of β-cryptoxanthin in fruit (Baldwin, 1993). Therefore these results indicated that CHX1 is not a regulatory enzyme in carotenoid

biosynthesis of *Citrus*, though Chx is the enzyme directly involved in biosynthesis of β-cryptoxanthin. These patterns of *CHX1* in *Citrus* were different from *Ca1* bell pepper, though both fruits are nonclimacteric. These results suggest that other enzymes including phytoene synthase could be regulatory enzymes in β-cryptoxanthin accumulation in *Citrus* fruits, which is supported by our results (Figure 14).

In summary, we isolated two cDNA clones, showing the polymorphism, encoding CHX1 and CHX2, and investigated the expression patterns during fruit and leaf development of fruit and leaf in *Citrus*, which is a nonclimacteric fruit-producing woody plant. The transcripts were detected in leaf, fruit, and flowers. The expression showed a consistent pattern during the development of fruit and leaf, which indicated that the expression of CHX1 and CHX2 genes, unlike the bell pepper (Bouvier, 1998), is not regulatory at the transcription level in *Citrus*.

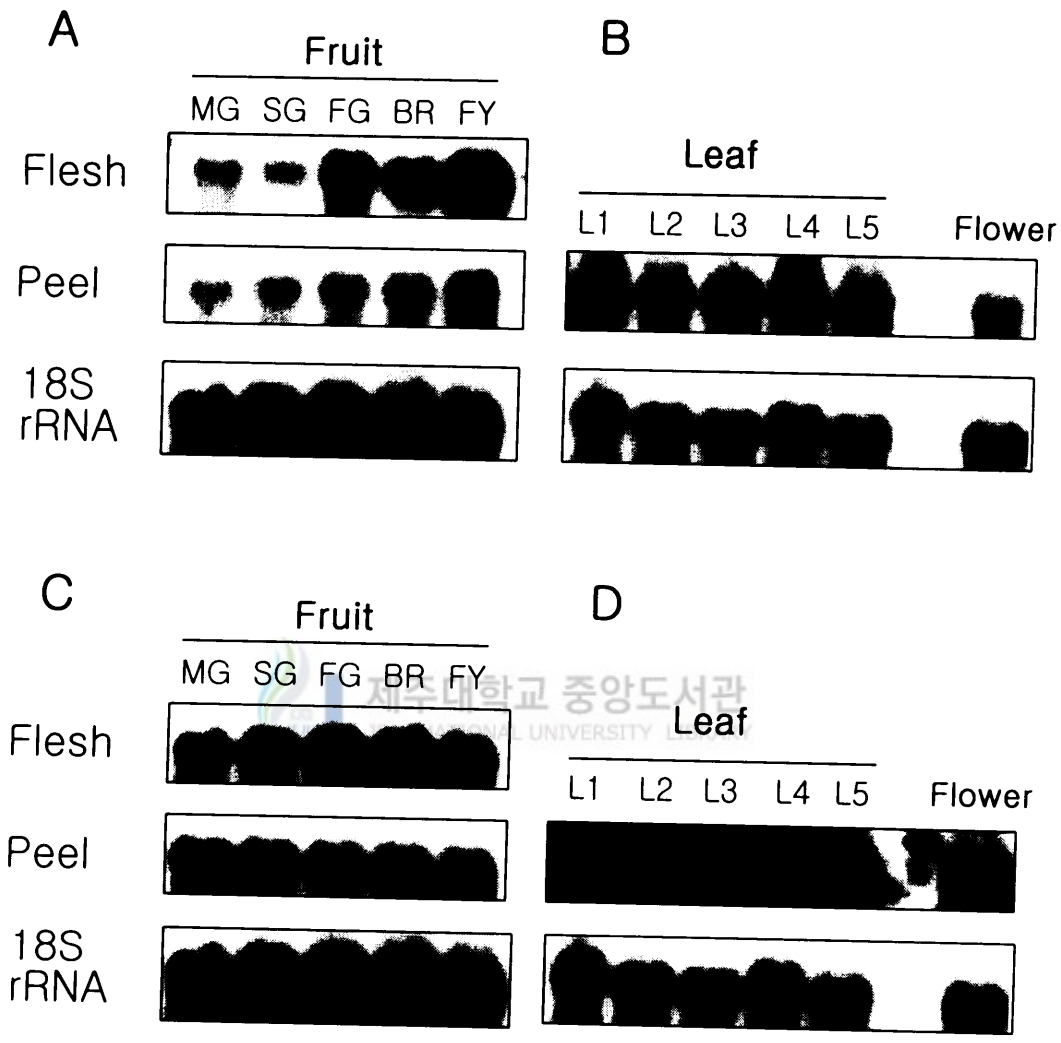

Figure 14. Northern blot analysis of *Psy1* (A, B) and *CHX1* (C, D) gene expression in five developmental stages of flesh and peel of fruits, and leaves, and in flowers of *Citrus*. Total RNA (20μg/lane) was separated on a 1.2% formaldehyde gel, transferred to Hybond N membrane (Amersham Life Science, Amersham, UK), and hybridized with *Psy1* and *CHX1* cDNA probes. The same blot was hybridized with an 18S rDNA probe.

SUMMARY (국문요약)

감귤 중 과육에는 β-carotene 와 ζ-carotene, β-cryptoxanthin 이 많고 , 과피에는 cryptoxnathin 과 antheraxanthin, violaxanthin 이 많이 축적되어 있다. 따라서 카로티노이드 생합에 관여하는 Psy 와 Chx 의 유전자 발현이 β-cryptoxanthin 의 축적과 밀접하게 관련되어 있을 것으로 생각되어진다. 따라서, Psy1 과 CHX1 를 암호화하는 cDNA 의 발현양상의 특성을 연구하기 위해 감귤과육 library 로부터 분리되여 졌다.

제주도에서 생산되는 세 종류의 감귤 품종의 과피와 과육으로부터 β-cryptoxanthin 함유량을 측정하였다. 추출한 색소농축물은 TLC (Thin-layer chromatography) 분석을 통해 확인하였고, β-cryptoxanthin 을 비롯한 여러 카로티노이드의 분석은 HPLC (High Performance Liquid Chromatography)를 통해 수행하였다. 과피와 과육 중의 β-cryptoxanthin 함량은 궁천조생에서 현저히 많았고, 제주 재래감귤인 병귤과 동정귤에서도 β-cryptoxanthin 함량은 일반 오렌지에 비해 높았으나, 그 함량은 궁천조생에 비해 훨씬 적었다. 그 함량은 궁천조생과 병귤, 동정귤의 과피에서 각각 5.26 mg%와 1.66 mg%, 0.88 mg%이고, 과육에서는 각각 0.78 mg%와 0.32 mg%, 0.45 mg%로 측정되었다.

β-cryptoxanthin 생합성은 여러 유전자의 발현에 의해 조절되지만, 본 연구에서는 이들 유전자 중, 카로티노이드 생합성의 첫단계인 phytoene 의 생합성에 관여하는 phytoene synthase 와, β-cryptoxanthin 의 생합성에 직접 관여하는 β-carotene hydroxylase (Chx)의 과육에서의 발현특성을 규명하였다. 이를 위해 먼저 phytoene synthase (Psy)와 β-carotene hydroxylase (Chx)의 유전자를 분리한 후, 발현 양상을 조사하였다.

우선, 감귤 (*Citrus unshiu* Marc.)의 과육 cDNA library 에서 phytoene synthase (Psy1)를 암호화하는 cDNA 클론을 분리하였다. 염기서열분석과

유연관계조사를 통해 이 클론은 과실수의 Psy 와 유사한 서열특성을 가지고 있음을 알 수 있었다. Psy1 클론은 437 개 아미노산 (47 kDa)을 이루는 한 개의 ORF 를 포함하고 있었다. 발현양상을 조사하기 위해 RNA blot 분석을 수행한 결과, 과실의 과육이 성숙하는 동안 *Psy1* 유전자의 발현은 모든 성숙단계에서 일어나고, 성숙됨에 따라 현저하게 증가하는 양상을 보였다. 과피에서도 이와 비슷한 양상이 나타나지만, 발현증가는 훨씬 적었다. 이러한 결과를 통해 *Psy1* 유전자의 발현은 과실이 성숙하는 동안 전사수준에서 조절되고 있음을 알 수 있었다.

감귤에서의 Chx 에 관한 연구를 위해 먼저 과육과 잎 cDNA library 로부터 두개의 클론을 분리하였다. 염기서열 분석과 유연관계 조사를 통해 이들 두 클론은 다형현상을 보여주며, 다른 과일 생산 식물의 Chx 와 높은 상동성을 가지고 있었다. RNA blot 분석을 통해 조사된 *CHX1* 의 발현은 잎과 꽃, 과육의 모든 조직에서 이루어지고 있고, 과실과 잎의 발달단계에 따른 일정한 양상을 보여주었다. 즉, 감귤에서 *CHX1* 유전자의 발현은 고추와는 달리 전사수준에서 조절되지 않음을 보여준다. 이러한 결과는 카로티노이드의 축적이 과일의 성숙과 관련된다고 알려져 있지만, 과일 type 이 같을지라도 식물 종에 따라 그 발현은 다양할 수 있음을 제시해 준다.

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이번 ISC2000 학회발표에 같이 참여하였을 뿐만 아니라 여러가지 면에서 가르침을 주시는 원예과학전공 문두길 교수님께 감사를 드립니다. 그리고, 실험에 대해 많은 부분을 지적해 주신 화학과 이남호 교수님과 실험실원들께 진심으로 감사 드립니다. 또한, 강지용 학장님, 그리고 화학과 이선주 교수님, 생물학과 김세재 교수님, 식품공학과 고영환 교수님, 허단 교무과장님께도 감사를 드립니다.

같은 실험실에 있었을 뿐만 아니라 항상 친형처럼 사랑을 베풀어주신

고세광 선배님(선생님)과 형수님, 학과의 실험을 이끌어 가고 계시는 문창룡 선생님, 전 조교 선생님이셨던 강봉천 선생님과 형수님께도 진심으로 감사드립니다. 또한, 동료이자 힘을 실어주는 친구인 원예과학전공의 (강)석범에게도 감사를 드립니다.

같은 유전공학 연구실의 4 년을 같이한 친동생이나 다름없는 남태식 후배님, 그리고 이제 새로운 멤버로 잘 적응하고 있는 안재우 후배님, 지금은 실험실을 떠났지만 다른 곳에서 열심히 하고 있을 오상훈 선배님과 양권민 후배님께 감사드립니다. 또한, 2 년동안 서로의 학문에 매진하였으며 앞으로도 계속 힘차게 달려갈 대학원생들 모두에게도 감사 드립니다.

항상 바쁘다는 이유로 거의 활동을 못했지만, 항상 저의 그늘이 되여주신 Calliope 人들과 Billows 회원님들께도 진심으로 감사 드립니다.

마지막으로 제가 이 자리에 오기까지 많은 뒷바라지와 희생, 사랑을 베풀어주신 어머님과 형님, 형수님, 혁제, 혁준, Francisca 와 하늘에서 지켜보고 계실 아버님께 이 모든 영광을 돌립니다.

2000 년 12 월 어느 깊어 가는 새벽....

"인생은 한 권의 책과 같다. 어리석은 이는 그것을 마구 넘겨 버리지만, 현명한 인간은 열심히 읽는다. 단 한 번밖에 인생을 읽지 못한다는 것을 알고 있기 때문이다." - 상 파울