석 사 학 위 논 문

감귤의 Geranylgeranyl diphosphate synthase, Phytoene desaturase, ζ-Carotene desaturase 및 Lycopene cyclase cDNAs 특성

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

제주대학교 대학원

농화학과

남 태 식

2002년 12 월

감귤의 Geranylgeranyl diphosphate synthase, Phytoene desaturase, ζ-Carotene desaturase 및 Lycopene cyclase cDNAs 특성

지도교수 김 찬 식

남 태 식

이 논문을 농학 석사학위 논문으로 제출함

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

2002 년 12 월 10 일

남태식의 농학 석사학위 논문을 인준함

심사위원장 _____ (인)

위 원 _____ (인)

위 원 _____ (인)

제주대학교 대학원

2002 년 12 월 10 일

Molecular Characterization of cDNAs Encoding Geranylgeranyl diphosphate synthase, Phytoene desaturase, ζ-Carotene desaturase and Lycopene cyclase in *Citrus*

Tae-Sik Nam

(Supervised by professor Chan-Shick Kim)

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF AGRICULTURAL CHEMISTRY
GRADUATE SCHOOL
CHEJU NATIONAL UNIVERSITY

December 10, 2002

CONTENTS

LIST OF TABLES ---- i

LIST OF FIGURES ---- ii

ABBREVIATIONS ---- iv

SUMMARY ---- vii

Ⅰ. INTRODUCTION ---- 1

1. Biosynthesis pathway of carotenoids ---- 1

2. β-Cryptoxanthin ---- 4

3. Geranylgeranyl diphosphate synthase (Ggps), Phytoene desaturase (Pds), ζ-Carotene desaturase (Zds) and Lycopene cyclase (Lcy) ---- 5

Ⅱ. MATERIALS AND METHODS ---- 9

1. MATERIALS ---- 9

1) Plant materials ---- 9

(1) For analysis of β-cryptoxanthin contents ---- 9

(2) For isolation of cDNAs encoding Ggps, Pds, Zds and Lcy ---- 9

2) Bacterial strains and plasmids ---- 10

3) Enzymes and chemicals ---- 10

4) Sterilization of solutions and media ---- 14

5) Media ---- 14

6) Buffers and solutions ---- 15

2. METHODS ---- 17

1) Analysis of β-cryptoxanthin in peel and flesh of *Citrus* fruits ---- 17

(1) Extraction of crude carotenoids from *Citrus* fruits ---- 17

(2) Chromatography ---- 19

2) Cloning of from *Citrus* ---- 21

(1) Extraction of plant RNA ---- 21

(2) Construction of a cDNA library and screening ---- 21

(3) PCR amplification and probe preparation ---- 22

제주대학교 중앙도서관 JEJU NATIONAL UNIVERSITY LIBRARY

(4) Isolation of plasmid DNAs ---------- 23
(5) DNA manipulations ---------- 23
(6) Transformation of *E. coli* ---------- 24
(7) DNA sequencing and analyses ---------- 25
(8) Genomic DNA blot analysis ---------- 25
(9) Northern blot analysis ---------- 26
Ⅲ. RESULTS AND DISCUSSION ---------- 27
1. Contents of β-cryptoxanthin in peel and flesh of *Citrus* fruits ---------- 27
1) TLC analysis of carotenoids in *Citrus* cultivars ---------- 27
2) Analysis of β-cryptoxanthin by HPLC ---------- 30
2. Cloning and Expression Analysis of Ggps, Pds, Zds and Lcy Gene -------- 36
1) Probe preparation for screening ---------- 36
2) Isolation of cDNA clone ---------- 36
(1) Ggps ---------- 36
(2) Pds ---------- 37
(3) Zds ---------- 37
(4) Lcy ---------- 38
3) Comparison of the deduced amino acid sequence of Ggps and Lcy with polypeptides known Ggps and Lcy ---------- 49
4) Genomic Southern analysis ---------- 54
5) Expression pattern in Citurs ---------- 54
SUMMARY (국문요약) ---------- 58
REFERENCES ---------- 60
ACKNOWLEDGEMENT ---------- 66

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

LIST OF TABLES

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

Table 2. Plasmids.

Table 3. Primers.

Table 4. Operating conditions of HPLC for carotenoids.

Table 5. Taxonomic names of *Citrus* and Fortunella.

Table 6. The land of origin of *Citrus* and Fortunella.

Table 7. The nucleotide sequences of the regions highly conserved among plant *Ggps*, *Pds*, *Zds* and *Lcy* genes.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

LIST OF FIGURES

Figure 1. Biosynthetic pathway of carotenoids in plants.

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

Figure 3. TLC chromatograms of standards of β-cryptoxanthin, and carotenoids peel of *Citrus* varieties.

Figure 4. TLC chromatograms of standards of β-cryptoxanthin, and carotenoids flesh of *Citrus* varieties.

Figure 5. HPLC elution profiles of carotenoid pigment extracted from peel of *Citrus* fruits of varieties.

Figure 6. HPLC elution profiles of carotenoid pigment extracted from flesh of *Citrus* fruits of varieties.

Figure 7. β-Cryptoxanthin content from peel of *Citrus* cultivars.

Figure 8. β-Cryptoxanthin content from flesh of *Citrus* cultivars.

Figure 9. Flow chart for experimental scheme.

Figure 10. Total RNA extracted from *Citrus* fruits

Figure 11. *Ggps* (A) and *Pds* (B) cDNA fragment subcloning.

Figure 12. *Zds* (A) and *Lcy* (B) cDNA fragment subcloning.

Figure 13. Agarose gel (1%) electrophoresis of *Eco*RI and *Xho*I digestion Products of *in vivo* excised phagemid from containing *Ggps* (A), *Pds* (B), *Zds* (C) and *Lcy* (D) cDNAs.

Figure 14. Nucleotide sequence and deduced amino acid sequence for the *Ggps* cDNA clones isolated from *Citrus* fruits.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Figure 15. Nucleotide sequence and deduced amino acid sequence for the *Pds* cDNA clones isolated from *Citrus* fruits.

Figure 16. Nucleotide sequence and deduced amino acid sequence for the *Zds* cDNA clones isolated from *Citrus* fruits.

Figure 17. Nucleotide sequence and deduced amino acid sequence for the *Lcy* cDNA clones isolated from *Citrus* fruits.

Figure 18. Alignment of the deduced amino acid sequences of *Ggps* isolated from various species.

Figure 19. Alignment of the deduced amino acid sequences of *Lcy* isolated from various species.

Figure 20. Phylogenetic relationship of the *Citrus Ggps* to *Ggps* of other various species.

Figure 21. Phylogenetic relationship of the *Citrus Lcy* to *Lcy* of other various species.

Figure 22. Genomic blot analysis of *Ggps* (A) and *Pds* (B).

Figure 23. Genomic blot analysis of *Zds* (C) and *Lcy* (D).

Figure 24. Northern blot analysis of Ggps (A, D), Zds (B, E) and Lcy (C) gene expression in five developmental stages of flesh and peel of fruits, and leaves of *Citrus*.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

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

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

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

RT-PCR : Reverse Transcriptase – Polymerase Chain Reaction

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

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

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

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

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 *Ggps, Pds, Zds* and *Lcy* in *Citrus* fruits contribute to the accumulation of β-cryptoxanthin. So, for studies on characterization of expression patterns of cDNA encoding *Ggps, Pds, Zds* and *Lcy* were isolated from *Citrus* fruit library.

To determine β-cryptoxanthin content of *Citrus* fruits produced in Korea and America, 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.

Satsuma (*Citrus unshiu* Marcovitch) showed the highest β-cryptoxanthin content in both peel and flesh. Contents of β-cryptoxanthin in the peel of Kiyomi (*Citrus* Tangor), Siranuhi (*Citrus sp.*), Seminoru (*Citrus* Tangelo), Satsuma (*Citrus unshiu* Marcovitch), Navel orange (*Citrus sinensis* Osbeck var. basiliensis Tanaka), Lemon (*Citrus limon*), Grapefruit (*Citrus* paradisi) and Valencia orange (*Citrus* sinensis Osbeck) were 0.315±0.066, 0.749±0.011, 0.811±0.002, 2.178±0.008, 0.164±0.026, 0.066±0.001, 0.048±0.001 and 0.164±0.001 mg%, respectively. And, contents of β-cryptoxanthin in the flesh of Kiyomi (*Citrus* Tangor), Siranuhi (*Citrus sp.*), Seminoru (*Citrus* Tangelo), Satsuma (*Citrus unshiu* Marcovitch), Navel orange (*Citrus sinensis* Osbeck var. basiliensis Tanaka), Lemon (*Citrus limon*), Grapefruit (*Citrus* paradisi) and Valencia orange (*Citrus* sinensis Osbeck) were 0.405±0.003, 0.427±0.004, 0.307±0.002, 0.663±0.002, 0.068±0.002, 0.008, 0.007±0.001 and 0.114±0.004 mg%, respectively.

The accumulation of β-cryptoxanthin is controlled by gene expression involved in carotenoid biosynthesis. In this study, we have focused on geranylgeranyl diphosphate synthase (Ggps), phytoene desaturase (Pds), ζ -carotene desaturase

(Zds) and lycopene cyclase (Lcy) out of carotenoid biosynthesis genes.

First, we isolated a cDNA clone encoding geranylgeranyl diphosphate synthase (Ggps) 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 426 amino acids, which showed significant similarity to those of fruit-producing plants.

For studies on Pds and Zds, cDNA clones encoding phytoene desaturase and ζ -carotene desaturase were isolated from *Citrus* fruit and leaf cDNA libraries. Sequence analyses and phylogenetic dendrogram of Pds and Zds revealed that the cDNA contains an open reading frame of 553 and 570 amino acids.

And, we isolated a cDNA clone encoding lycopene cyclase (Lcy) from the fruit cDNA library of *Citrus*. Sequence analyses and phylogenetic dendrogram revealed that the cDNA contains an open reading frame of 504 amino acids, which showed significant similarity to those of fruit-producing plants.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Ⅰ. INTRODUCTION

1. Biosynthesis pathway of carotenoids

Plant carotenoid are 40-carbon isoprenoids with polyene chains that may contain up to 15 conjugated double bonds. Because of their chemical properties carotenoids are essential components of all photosynthetic organisms (Joseph, 2001). Carotenoids form one of the largest groups of pigments that are widely distributed in plants, and they are responsible for the yellow, orange and red coloration of tissues (Zhu *et al.*, 2002). These yellow, orange, and red pigments protect against photooxidation, harvest light for photosynthesis, and serve a number of other important functions. Most of the carotenoids important in photosynthetic organisms are xanthophylls or oxygenated carotenoids (Sun *et al.*, 1996). Certain cyclic carotenoids, such as β-carotene, are precursors of vitamin A in animals and are of current interest as nutritional factors important for cancer prevention (Matsumura *et al.*, 1997).

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

The biosynthesis of carotenoids occurs within the chloroplasts of plants and algae (Cunningham *et al.*, 1996). Condensation of three molecules of isopentenyl pyrophosphate (IPP) and one molecule of dimethylally pyrophosphate (DMAPP) 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 geranylgeranyl pyrophosphate (GGPP) is a two-step reaction catalyzed by the enzyme phytoene synthase (PSY). This reaction yields the first C_{40} carotenoid, phytene, the backbone of all plant carotenoids (Zhu *et al.*, 2002).

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 there by transform the coloress 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.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Xanthophylls or oxygenated carotenoids comprise most of the carotenoid pigment in the thylakoid membranes of plants (Cunningham and Gantt, 1998). Several of the enzymes involved in xanthophyll biosynthesis, carotenoids modified with oxygen containing groups, have also been characterized, e.g., the β-cryptoxanthin and zeaxanthin from bacteria and higher plants (Linden, 1999)(Figure 1).

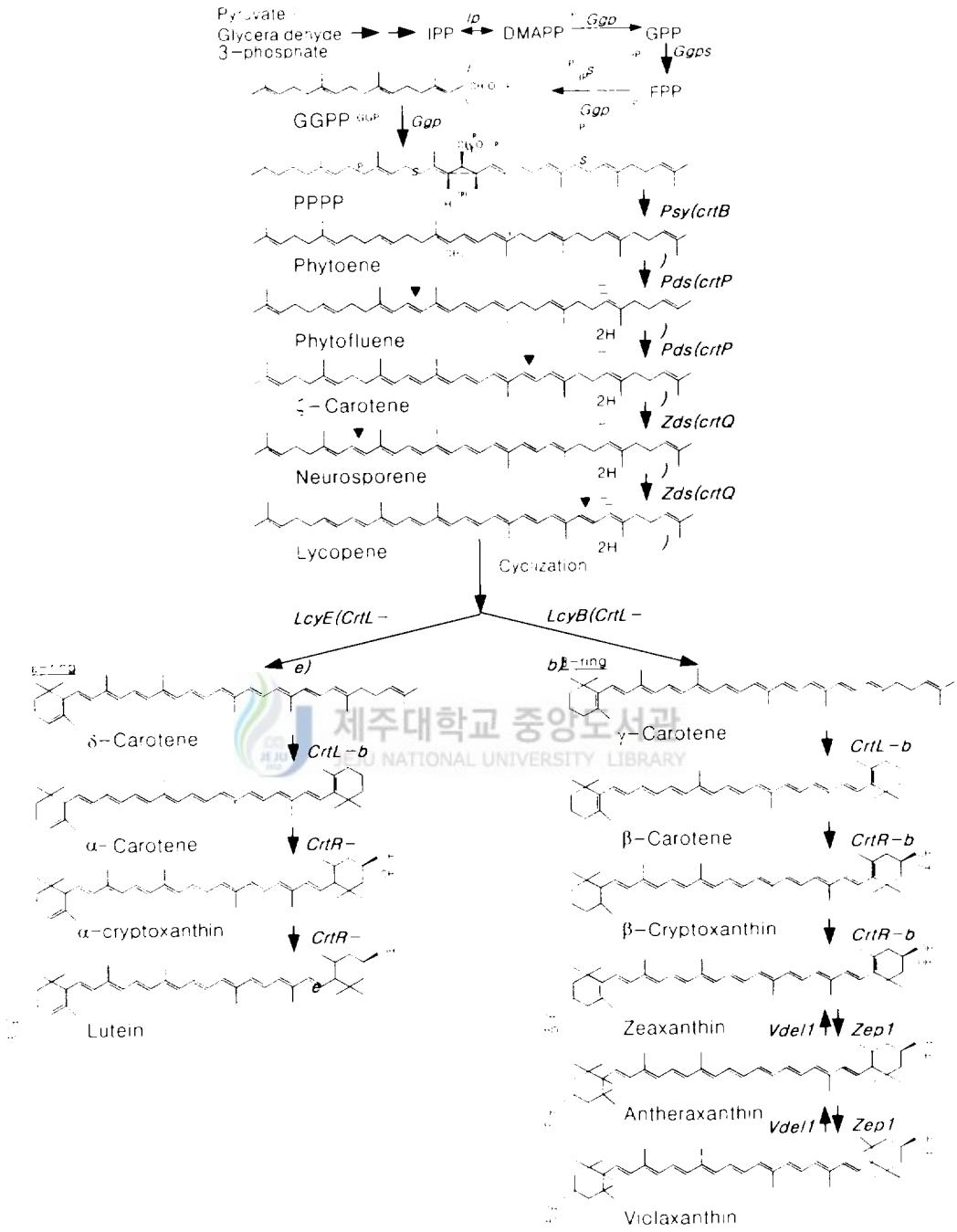


Figure 1. Biosynthetic pathway of carotenoids in plants.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

2. β-Cryptoxanthin

Carotenoides in citrus fruits is widely believed to protect human health (Nishino *et al.*, 1996). Various natural carotenoids were proven to have anticarcinogenic activity (Nishino *et al.*, 2000). Some epidemiological studies have confirmed an inverse relation between carotenoid consumption and the development of some cancers (Slattery *et al.*, 1997; Cooper *et al.*, 1999). Anticancer activity of carotenoids from citrus fruits was due to mainly their antioxidants function (Tee and Lim, 1991).

Recently, carotenoids have attracted attention due to reported beneficial health effects (Rauscher *et al.*, 1998; Nishino *et al.*, 1998). In particular, β-cryptoxanthin functions as protective role against human disease (Hart and Scott, 1995; Sumida *et al.*, 1999). β-cryptoxanthin was suggested to stimulate the expression of an anti-oncogene (Nishino *et al.*, 2000). Dorgan et al. reported that β-cryptoxanthin shows the effect of anticancer activity on breast cancer (Dorgan *et al.*, 1998). *In vivo* experiments for preventing skin cancer or colon cancer with mouse, β-cryptoxanthin shows higher anticancer activity than that of β-carotene (Nishino *et al.*, 1998)

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

In recent years, there has been particular emphasis on obtaining more accurate data on the types and concentrations of various carotenoids in foods for various health and nutrition activities (Tee and Lim, 1991). The data on the individual carotenoid content of citrus fruits has become increasingly important. A wide variety of separation and detection and quantitation procedures have been used in studies of carotenoids (Tee and Lim, 1991). Nishio et al. reported that *Citrus unshiu* Marcovitch contained great amount of β-cryptoxanthin than those of grapefruits, lemons and oranges cultivated in America (Nishino *et al.*, 1998). Although there are some reports concerning the separation of carotenoids from citrus fruits, the systematic analysis of β-cryptoxanthin from domestic citrus

fruits and foreign citrus fruits were not reported.

3. Geranylgeranyl diphosphate synthase (Ggps), Phytoene desaturase (Pds), ζ-Carotene desaturase (Zds) and Lycopene cyclase (Lcy)

Carotenoids are isoprenoid polyene pigments synthesized by photosynthetic and some non-photosynthetic organisms (Lagarde and Vermaas, 1999). Carotenoids serve structural functions in the photosynthetic pigment protein complexes of the reaction centers and the light-harvesting antenna, where they are bound to specific chlorophyll/carotenoid-binding proteins (Ronen *et al.*, 1999).

In recent years, plant genes coding for carotenoid biosynthetic enzymes have been cloned using various approaches. All known plant genes encoding carotenoid biosynthetic enzymes are encoded in the nucleus, formed on free cytoplasmatic ribosomes and subsequently imported into the plastids, where they are further targeted to their final destinations (Lintig *et al.*, 1997).

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Geranylgeranyl diphosphate synthase

Geranylgeranyl diphosphate synthase (GGPS), a member of the short-chain isoprenyl diphosphate synthase family, catalyzes the consecutive condensation of three molecules of isopetenyl pyrophosphate (IPP) with dimethylally pyrophosphate (DMAPP) to give a C_{20} compound which is a precursor to diterpenes, catotenoids and chlorophylls (Sitthithaworn *et al.*, 2001; Wang and Ohnuma, 1999). A geranylgeranyl diphosphate synthase has been isolated as a soluble and functional homodimer from the chromoplasts of pepper (Dogbo and Camara, 1987), and the corresponding cDNA has been identified and sequenced (Kuntz *et al.*, 1992). Immunolocalization experiments confirmed a predominant

localization in the chromoplast for geranylgeranyl diphosphate synthase in pepper fruits (Cunningham and Gantt, 1998).

Phytoene desaturase and ζ-Carotene desaturase

Phytoene undergoes a series of four desaturation reation that result in the formation of first phytofluene (7,8,11,12,7',8'-hexahydro-ψ,ψ-carotene) and then, in turn, ζ-carotene (7,8,7',8'-tetrahydro-ψ,ψ-carotene), neurosporene (7,8-dihydro-ψ,ψ-carotene), and lycopene (ψ,ψ-carotene). The four sequential desaturations undergone by phytoene are catalyzed by two related enzymes in plants: phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS) (Cunningham and Gantt, 1998).

The enzyme phytoene desaturase (PDS) catalyzes the conversions of phytoene to phytofluene to ζ-carotene. Evidence suggests that phytoene desaturase catalyzes regulated steps in carotenoid and abscisic acid (ABA) biosynthesis in many organisms (Linden *et al.*, 1991). For example (i) phytoene desaturase steady-state transcript levels increase before and during carotenoid accumulation in several fruits (Giuliano *et al.*, 1993); (ii) studies in fungi and cyanobacteria indicate that the enzyme is subject to feedback regulation by the pathway intermediate, lycopene, and (iii) phytoene synthase has also been shown to catalyze the first ratelimiting step in carotenoid biosynthesis in the cyanobacterium Synechococcus (Hable *et al.*, 1998). Phytoene desaturase has been cloned from soybean (Bartley *et al.*, 1991b), pepper (Hungueney *et al.*, 1992), and tomato (Bartley. G. E. and Scolnik. P. A., unpyblished data, GenBank accession number M88693; pecker *et al.*, 1992) (Giuliano *et al.*, 1993). The enzyme ζ-carotene desaturase (ZDS) catalyzes the conversions of ζ-carotene to neurosporene to lycopene. A cDNA encoding a ζ-carotene desaturase (ZDS) was recently identified by functional analysis in E. coli of a pepper cDNA that predicts a polypeptide distantly resembling the known plant phytoene desaturase

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

(Albrecht *et al.*, 1995). Phytoene desaturase and ζ-carotene desaturase both have amino acid sequence signatures that are conserved in pyridine nucleotide-disulphide oxidoreductases (Hugueney *et al.*, 1992).

The desaturases are demonstrably membrane-associated in plants, although the predicted amino acid sequences are not particularly hydrophobic overall (Cunningham and Gantt, 1998).

Lycopene cyclase

The cyclization of lycopene is an important branch point in the pathway of carotenoid biosynthesis. Cyclization at both ends of the symmetrical, linear lycopene leads to β-carotene, an essential carotenoid that also serves as a precursor for the production of several other carotenoids with roles in photosynthesis (Cunningham *et al.*, 1996). Lycopene β-cyclase (LCY-B/CRTL-B) catalyzes a two-step reaction that creates one β-ionone ring at each end of the lycopene molecule to produce β-carotene, whereas lycopene ε-cyclase (LCY-E/CRTL-E) creates one ε-ring to give δ-carotene (Hirschberg, 2001). Most of the carotenoids common in plants and algae have two β or modified β rings (Cunningham *et al.*, 1996). There is a high degree of structural resemblance, 30% identity in amino-acid sequence, between Lcy-B and Lcy-E in both tomato and *Arabidopsis* (Hirschberg, 2001). The apportioning of substrate into the pathways leading to β,ε-carotenoids (e.g. the abundant lutein) and to β,β-carotenoids (e.g. β-carotene, zeaxanthin, and violaxanthin) could be determined quite simply by the relative amounts and/or activities of the ε and β-cyclase enzymes (Cunningham and Gantt, 1998).

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

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

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 *Ggps, Pds, Zds* and *Lcy* in the ripening of flesh and peel of Citrus fruits and in leaf development. First, we isolated a cDNA encoding *Ggps, Pds, Zds* and *Lcy* and characterized the expression patterns. Results indicated that the expression of *Ggps, Pds, Zds* and *Lcy* is related to and distinct from, respectively, the accumulation of β-cryptoxanthin and other carotenoids.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Ⅱ. MATERIALS AND METHODS

1. MATERIALS

1) Plant materials

(1) For analysis of β-cryptoxanthin contents

Citrus fruits cultivated in Korea and America were used for determination of β-cryptoxanthin. Table 5 showed the land of origin and taxonomic names of eight *Citrus* cultivars. The pulp and peel separated from *Citrus* fruits were stored at -70℃. β-Cryptoxanthin and β-carotene as standards and butylated hydroxytoluene (BHT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Crystalline carotenoids were obtained from Extrasynthese (Genay, France). A 5 mg of β-cryptoxanthin was dissolved in 25 ml of methyl tert-butyl ether containing 1% BHT and methanol (1:1, v/v). The concentrations for the carotenoid standards ranged from 0.1-0.5 μg/ml. Other chemicals used were HPLC or analytical grade.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

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) For isolation of cDNAs encoding *Ggps*, *Pds*, *Zds* and *Lcy*

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

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), 87 DAF; breaker (BR, full size: 6-7 cm in diameter), 145 DAF; and full yellow (FY), 170 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.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

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

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Table 2. Plasmids.

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-fGgps	Amp	JM109	Subcloning vector containing about 500bp PCR products of *Ggps* derived from pGEM-T	In this study
pGEM T-fPds	Amp	JM109	Subcloning vector containing about 500bp PCR products of *Pds* derived from pGEM-T	In this study
pGEM T-fZds	Amp	JM109	Subcloning vector containing about 500bp PCR products of *Zds* derived from pGEM-T	In this study
pGEM T-fLcy	Amp	JM109	Subcloning vector containing about 500bp PCR products of *Lcy* derived from pGEM-T	In this study

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

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Table 3. Primers.

Primers	Sequence	Usage
T7 Primer	5′-GTAATACGACTCACTATAGGGC-3′	Sequencing
T3 Primer	5′-AATTAACCCTCACTAAAGGG-3′	Sequencing
GGPS5	5′-GAYGAYYTNCCYTGYATGGA-3′	PCR
GGPS3	5′-AYRTCATCNAYHACYTGAWA-3′	PCR
PDS5	5′-GTTTGGTCTTCAGTTTGATA-3′	PCR
PDSF3	5′-TAGAGTGCTCCTTCCACTGC-3′	PCR
ZDS5	5′-GAYGTTTAYTTRAGYGGTCC-3′	PCR
ZDS3	5′-GTCTTYTGATCRGGYCTGAA-3′	PCR
LCY5	5′-GGTGGCGGCCCGGCTGGGCT-3′	PCR
LCY3	5′-TCCAATCCATGAAAACCATC-3′	PCR

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Mixed Base	Code Name	Mixed Base	Code Name	Mixed Base	Code Name	Mixed Base	Code Name
A + G	R	C+T	Y	A+G+C+T	N	A+T+C	H
A+T	W	G+T	K	G+A+C	V	G+C	S
G+A+T	D	A+C	M	G+T+C	B		

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 121℃ 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 121℃ for 15 min on liquid cycle. Glassware was sterilized by heating to 160℃ for 10 hr or More

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

5) Media

LB medium : Bacto-tryptone 10 g, Bacto-yeast extract 5 g, NaCl 10 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

Suspension solution : 50 mM Tris-HCl (pH 7.5), 10 mM EDTA, 100μg/ml Dnase-free Rnase A

Lysis solution : 0.2 M NaOH, 1% SDS

Neurralising solution : 3 M potassium acetate, pH 4.8

Binding buffer : 6M guanidine hydrochloride

TE buffer : 10mM Tris-HCl, pH 8.5, 1mM EDTA

Washing solution : 80% isopropanol

(3) RNA isolation from plant tissue

6x Extraction buffer : 100 mM LiCl, 100 mM Tris-HCl (pH8.0), 10 mM EDTA, 1% SDS, 0.1% DEPC

4M LiCl solution : 4M LiCl, 0.1% DEPC

3M NaOAc solution : 3M NaOAc, pH 5.2, 0.1% DEPC

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

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

(4) cDNA library screening

Denaturing solution : 0.5N NaOH, 1.5M NaCl

Neutralizing solution : 1M Tris-HCl (pH8.0), 1.5M NaCl

2x SSC solution : 0.3M NaCl, 0.03M sodium citrate

20x SSPE : 3M NaCl, 0.2M NaH_2PO_4, 0.02M EDTA, pH 7.4

(5) Others

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

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

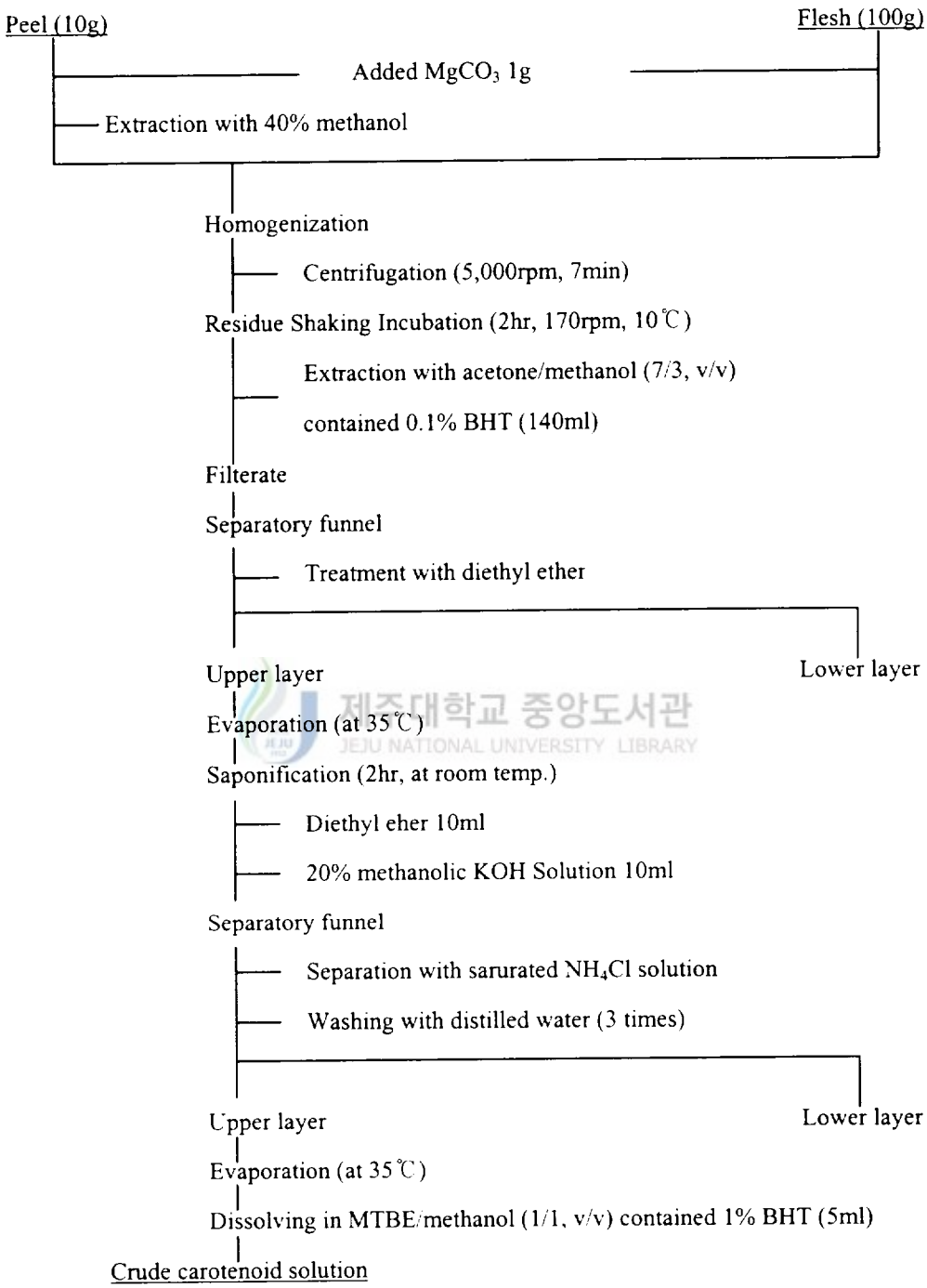
2. METHODS

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

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

The extraction of carotenoids was performed using the method reported by Ko *et al.* A 10 g peel (or 100 g flesh) was mixed with 70 mL of 40% methanol containing 1 g of $MgCO_3$ using juice mixer (LG, Korea). The supernatant was collected after centrifugation (7,000 rpm, 7 min, 10℃) and residue was mixed with 140 ml of acetone/methanol (7/3, v/v) solution containing 0.1% BHT for 1 hr and then was filtered by vacuum filtration. The extraction was repeated to recover carotenoid pigments. Filtrate was transferred to 1 L of separatory funnel and was mixed with 150 ml of distilled water, 250 ml of ethyl ether and 100 ml of 10% NaCl. After standing for 1 hr, top phase containing carotenoid pigments was collected and then was concentrated using vacuum evaporator at 35℃. Crude carotenoids were dissolved in 20 ml of ethyl ether containing 10% methanolic KOH. The saponification was conducted at room temperature in the dark for 2 hr. The sample was subsequently partitioned into a saturated solution of NH_4Cl and ethyl ether using separatory funnel and then the organic layer was collected. The aqueous layer was washed with diethyl ether and the organic layers combined were washed several times with water. Organic layer was reduced to dryness using a rotary evaporator at 30℃. The saponified samples were dissolved in 5 ml of MTBE/methanol (1/1, v/v) containing 1% BHT and was filtered through 0.45 μm PTFE filter (Micro Filtration System, CA, USA). The solutions were stored under nitrogen in dark and then were diluted to prepare working solutions in the range of 0.1-5.0 μg/mL. Figure 2 showed overall scheme for the extraction of carotenoids in peel and flesh of *Citrus* fruits.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY



제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

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

(2) Chromatography

The carotenoides extracted from citrus fruits were analyzed on silica gel TLC plates (Silica gel 60 F254, Merck, USA). For separation, hexane/acetone (75/25, v/v) was used as the mobile solvent. The separation of carotenoids was performed by HPLC. HPLC equipment consisted of a Spectra-Physics (SpectraSYSTEM) P4000 pump (Spectra-Physica Analytical, Inc., CA, USA), an UV 1000 UV/Vis detector (Spectra-Physics Analytical, Inc., CA, USA), and an AS3500 autosampler (TSP Inc., USA). A μBondapakTM C18 reverse phase column (3.9 x 30 mm, particle size 10 μm) (Waters Chromatography, Milford, MA, USA) and a guard column containing C18 material similar to the analytical column was used. The mobile phase was prepared using an HPLC grade methanol, water and high-grade methyl tert-butyl ether (MTBE) for gradient conditions from (95:1:4) to (25:71:4) for 13 min. The solvent was filtered through a 0.5 μm PTFE membrane filter (ADVANTEC MFS, Inc., CA, USA) and degassed in an ultrasonic bath. 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. The identification of β-cryptoxanthin from solvent extracts of citrus fruits was achieved by comparing the retention time of a known standard. Table 4 indicated the condition for HPLC analysis.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Table 4. Operating condition of HPLC for carotenoids.

Solvent Degassers	: Spectra-Physics Analytical, Inc. part number A0099-504
Gradient pumps	: Spectra-Physics Analytical, Inc. part number A0099-510 (P4000)
Autosamplers	: Thermo Separation Products Inc. part number A0099-587 (AS1000)
UV/Vis Detectors	: Spectra-Physics Analytical, Inc. 8/91 part number A0099-540 (UV2000)
Column	: μBondapakTM C18 125 Å 10μm 3.9×300mm HPLC Column, Waters

Mobile phase

A solvent = Methanol : Methyl tert-buthyl ether : H_2O = 95 : 1 : 4

B solvent = Methanol : Methyl tert-buthyl ether : H_2O = 25 : 71 : 4

Injection volume	: 20$\mu\ell$
Column temperature	: 35℃
Flow rate	: 1ml/min
Wave length	: 445 nm

Gradient table

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

제주대학교 중앙도서관 JEJU NATIONAL UNIVERSITY LIBRARY

2) Cloning of *Ggps*, *Pds*, *Zds* and *Lcy* 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℃.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

(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 *Ggps* cDNA, the sense primer (GGPS5: 5′-GA[C/T]GA[C/T][C/T]T[A/G/C/T]CC[C/T]TG[C/T]ATGGA-3′) and the antisense primer (GGPS3: 5′-A[C/T][A/G]TCATC[A/G/C/T]A[C/T][A/T/C]AC[C/T]TGA[A/T]A-3′) were used, and for the amplification of *Pds* cDNA, the sense primer (PDS5: 5′-GTTTGGTCTTCAGTTTGATA-3′) and the antisense primer (PDSF3: 5′-TAGAGTGCTCCTTCCACTGC-3′) were used. Also, for the amplification of *Zds* cDNA, the sense primer (ZDS5: 5′-GA[C/T]GTTTA[C/T]TT[A/G]AG[C/T]GGTCC-3′) and the antisense primer (ZDS3: 5′-GTCTT[C/T]TGATC[A/G]GG[C/T]CTGAA-3′) were used, and for the amplification of *Lcy* cDNA, the sense primer (LCY5: 5′-GGTGGCGGCCCGGCTGGGCT-3′) and the antisense primer (LCY3: 5′-TCCAATCCATGAAAACCATC-3′) were used.

These PCR primers were synthesized on the basis of the conserved regions of the previously reported sequences of *Ggps, Pds, Zds* and *Lcy* 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 53℃ for 1 min and at 72℃ for 40sec, respectively. Total 40 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 *Ggps*, *Pds*, *Zds* and *Lcy* fragments were cloned into pGEM-T vector (Promega), which is called pGEM T-fGgps, pGEM T-fPds, pGEM T-fZds and pGEM T-fLcy 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*.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

(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 5 mL LB media 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, at 4℃, 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 cells were centrifuged for 5 min at 2610rpm, at 4℃. 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.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

(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 carbenicillin, 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 aliquot 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).

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

(8) 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 *Bam*HI, *Eco*RI, *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.

(9) Northern blot analysis

Total RNA was isolated from fruits in five developmental stages (MG, SG, FG, BR, and FY) 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. The signal intensity of blot was analyzed by the scanning densitometer (Pharmacia) to adjust a little difference in loading amount among RNA samples.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

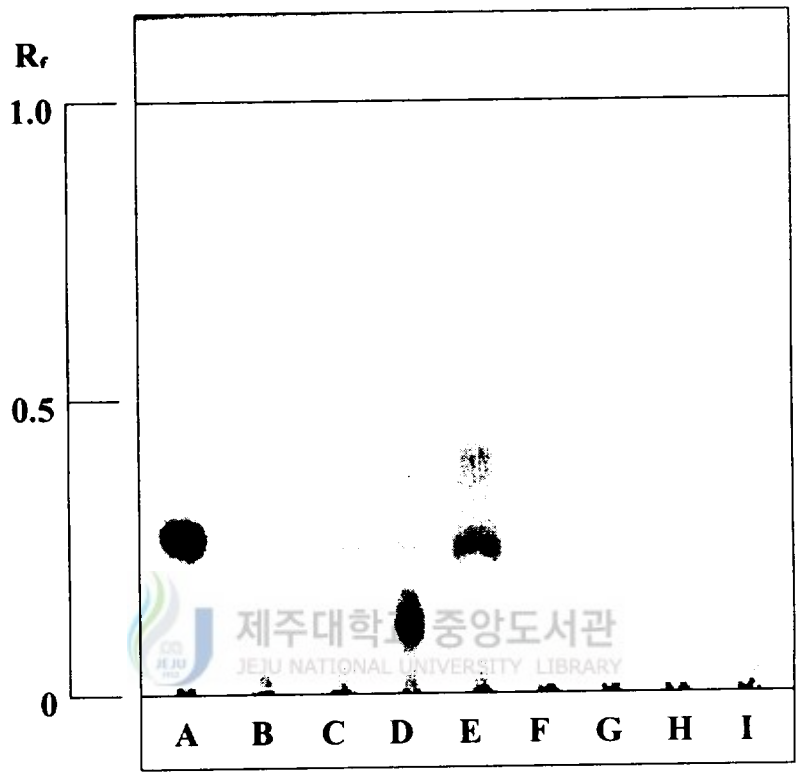
Ⅲ. RESULTS AND DISCUSSION

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

1) TLC analysis of carotenoids in *Citrus* cultivars

Crude carotenoids were extracted from peel and flesh of five citrus fruits grown in Jeju Island and three citrus fruits cultivated in America. Composition of crude carotenoids was analyzed by TLC. Figure 3 shows the TLC chromatograms of a standard β-cryptoxanthin and crude carotenoids extracted from peel of citrus fruits. Crude carotenoids consisted of more than four compounds and contained β-cryptoxanthin with different concentration. The R_f value of β-cryptoxanthin was 0.39. In the peel of citrus fruits, β-cryptoxanthin content was the highest in Satsuma and decreased in order of Seminoru, Suiranuhi, Kiyomi, Navel orange and Valencia orange. Lemon and Grapefruit contained very low concentration of β-cryptoxanthin. In flesh of citrus fruits, the content of β-cryptoxanthin was the highest in Satsuma. Concentration of β-cryptoxanthin was decreased in the order of Siranuhi, Kiyomi, Seminoru, Navel orange and Valencia orange (Figure 4). Among eight citrus fruits, Satsuma contains higher β-cryptoxanthin content than those of others. The carotenoid content extracted from Lemon and Grapefruit was very low compared with other citrus fruits. The content of carotenoids including β-cryptoxanthin was greatly varied according to *Citrus* cultivars.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

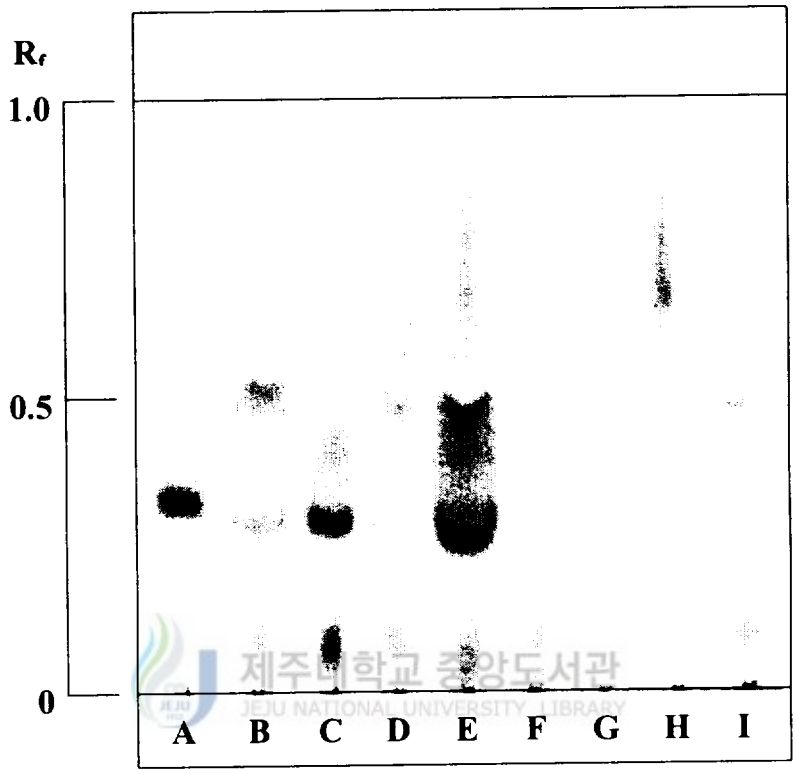


제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Figure 3. TLC chromatograms of standards of β-cryptoxanthin, and carotenoids peel of *Citrus* varieties.

A : Standard of β-cryptoxanthin, B : Kiyomi, C : Siranuhi, D : Seminoru, E : Satsuma, F : Navel orange, G : Lemon, H : Grapefruit, I : Valencia orange

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



제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Figure 4. TLC chromatograms of standards of β-cryptoxanthin, and carotenoids flesh of *Citrus* varieties.

A : Standard of β-cryptoxanthin, B : Kiyomi, C : Siranuhi, D : Seminoru, E : Satsuma, F : Navel orange, G : Lemon, H : Grapefruit, I : Valencia orange

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

2) Analysis of β-cryptoxanthin by HPLC

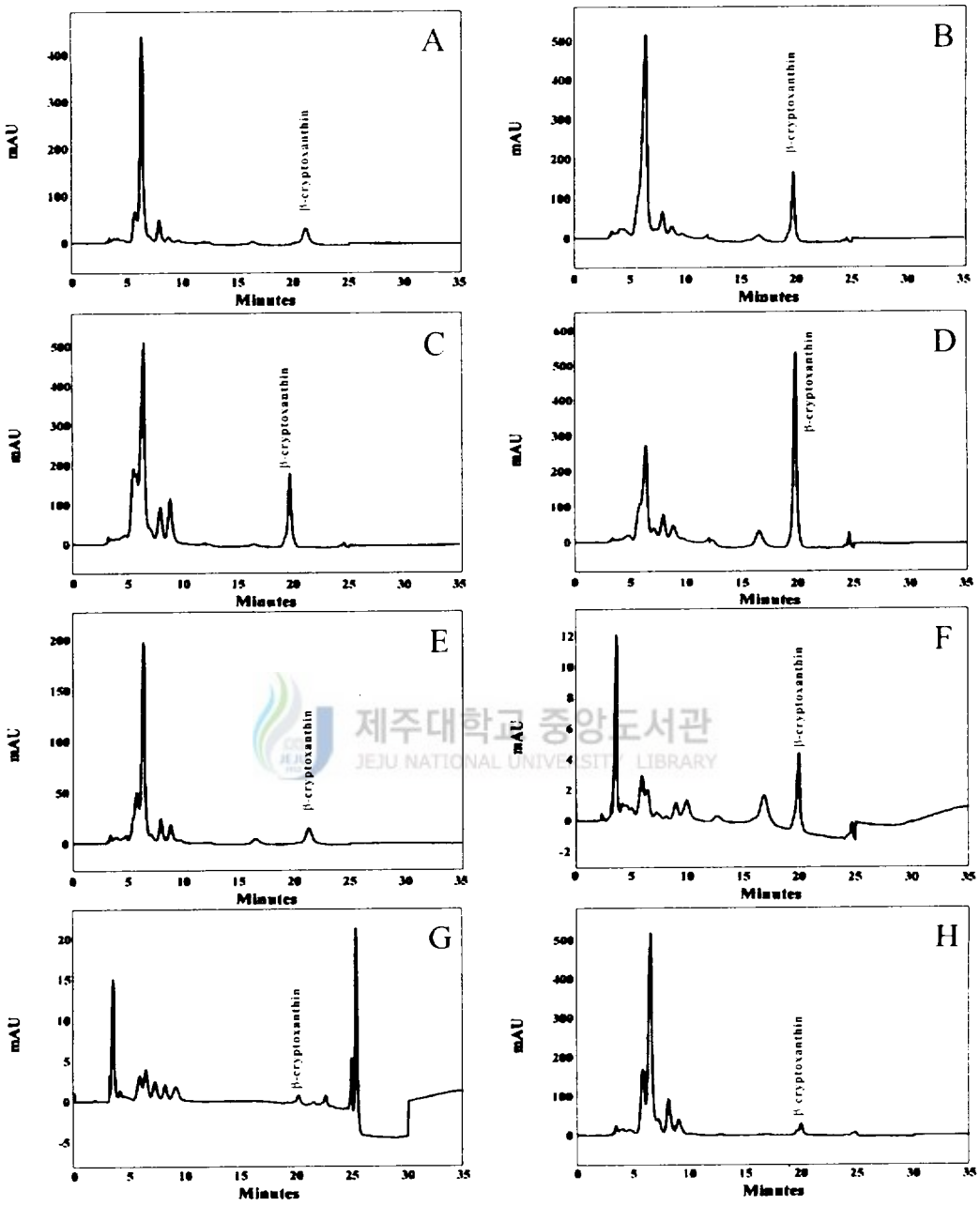
In order to determine the concentration of β-cryptoxanthin from crude carotenoids quantitively, HPLC analysis was performed. The peaks were identified by comparison of the spectra with standard and by the retention time of β-cryptoxanthin. Previously, crude carotenoids were saponified to increase the content of free β-cryptoxanthin (Nogata *et al.*, 1996). Because the yellow pigments of carotenoids absorbs maximally at 450 nm, β-cryptoxanthin can be easily determined using a spectrophotometer in the visible range (Tee and Lim, 1991). Typical chromatograms of carotenoids in citrus fruits are shown in Figure 5 and Figure 6. The carotenoids were fractionated with various peaks. β-Cryptoxanthin and β-carotene as standards indicated 20 min and 25 min of retention time, respectively. Various carotenoids as isomeric pigments were mainly eluted at 5 min of retention time. The peel of Satsuma showed large amount of β-cryptoxanthin, and relatively small amount of other compounds (Figure 7). In addition, the flesh of Kiyomi, Siranuhi, Seminoru and Satsuma included β-cryptoxanthin as a dominant peak (Figure 8). The high concentration of β-cryptoxanthin from Satsuma coincided with the analysis of TLC. The β-cryptoxanthin content in the peel of Lemon and Grapefruit cultivated in America was much smaller than those of other *Citrus* cultivars. Crude extract from both citrus fruits contained low amount of carotenoids pigment including β-cryptoxanthin. The β-cryptoxanthin content from the flesh of Kiyomi, Siranuhi, Seminru and Satsuma cultivated in Jeju island was higher than those of Lemon and Grapefruit and Valencia orange grown in America. In particular, crude carotenoids prepared from Satsuma contained relatively purified β-cryptoxanthin. In general, the peel contained higher concentration of β-cryptoxanthin than that of flesh except for Kiyomi cultivar. The amount of β-cryptoxanthin ranged from 0.3 to 2.1 mg% in the peel of domestic *Citrus* cultivars. The amounts of β-cryptoxanthin were less than 0.1% in the peel of three foreign

제주대학교 중앙도서관 JEJU NATIONAL UNIVERSITY LIBRARY

Citrus cultivars. In addition, the β-cryptoxanthin in flesh of domestic *Citrus* cultivars also were higher than those of foreign *Citrus* cultivars. The amounts of β-cryptoxanthin are 2.178 ± 0.008 mg% and 0.663 ± 0.002 mg% in peel and flesh of Satsuma, respectively. Hart and Scott reported that the β-cryptoxanthin content of Satsumas fruits contained 1.18 mg% (Hart and Scott, 1995). The concentration of β-cryptoxanthin in Lemon and Grapefruit was considerably less than those found in other *Citrus* cultivars and contained below 0.1 mg% in both peel and flesh. The β-cryptoxanthin of Valencia orange was only about 0.1 mg% in both peel and flesh. Hart and Scott reported that Valencia orange contained higher content of β-cryptoxanthin (Hart and Scott, 1995). It implies that same *Citrus* cultivars may contain different concentration of β-cryptoxanthin. In previous analysis of β-cryptoxanthin from other *Citrus* cultivars, Miyagawa wase showed 5.26 mg% in peel and 0.78 mg% in flesh (Whang and Yoon, 1995). Byungkyool and Dongjeongkyool contained 1.66 m% and 0.88 mg% in peel, respectively (Ko *et al.*, 2000). Pupin et al. have reported that β-cryptoxanthin content of Brazilian orange juice (*Citrus sinensis*) ranged from 0.10 to 0.46 mg/L, and β-carotene content ranged from 0.10 to 0.53 mg/L, showing 70% of recovery level (Pupin *et al.*, 1999). It implies that some pigments can be destroyed or modified during the extraction and isolation of carotenoids from citrus fruits.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

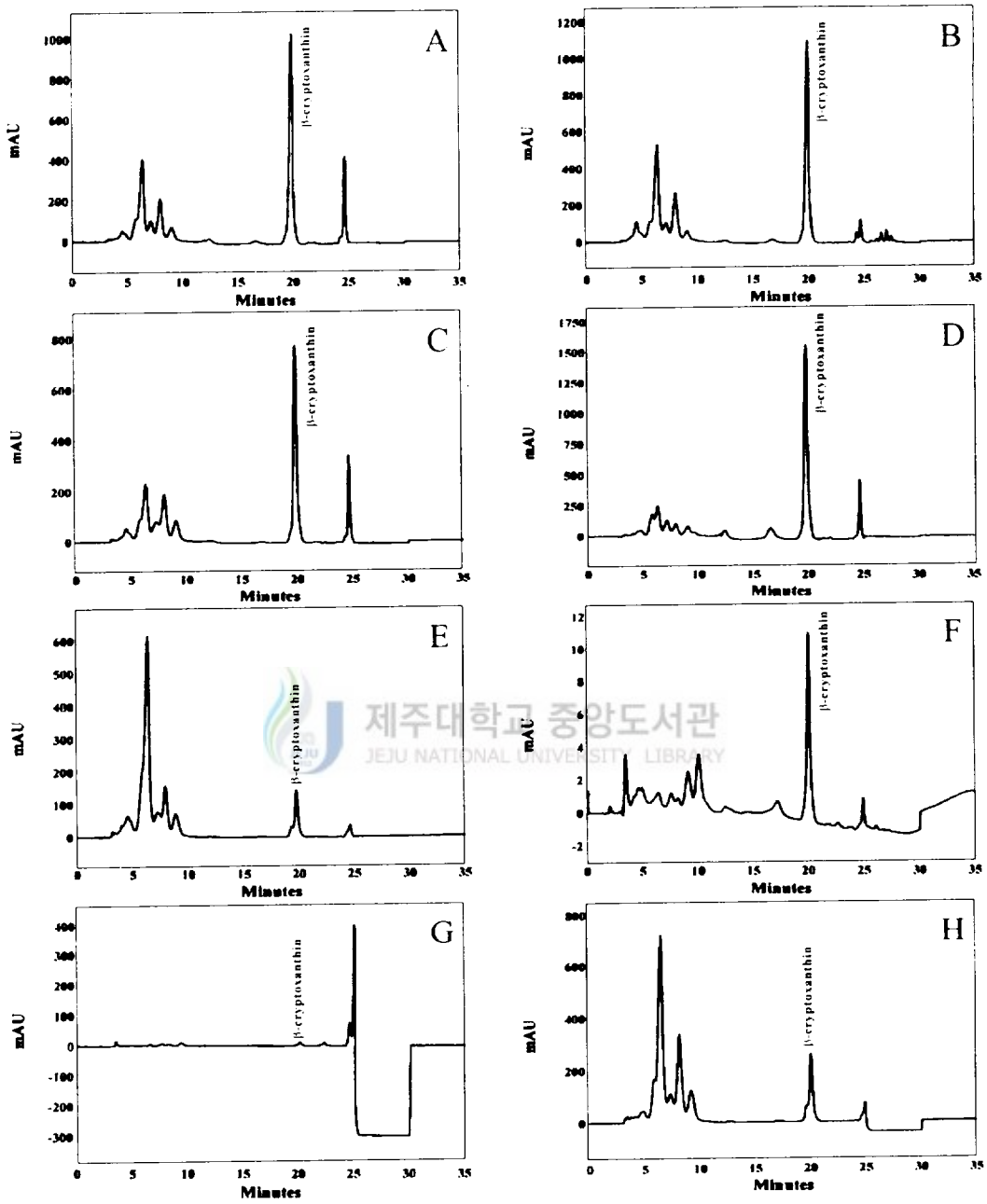
In general, carotenoids have numerous biological properties (Slattery *et al.*, 2000) and are known to play specific roles in mammalian tissues (Tee and Lim, 1991). Sumida et al. have reported that β-cryptoxanthin prepared from Satsuma Mandarin (*Citrus unshiu* Marc.) juice showed the inhibitory effect on azoxymethan-induced aberrant crypt foci (ACF) (Sumida *et al.*, 1999). Therefore, Satsuma cultivar will be an important source of biologically active pigments as well as basic material to breed new variety of *Citrus* by genetic engineering.



제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Figure 5. HPLC elution profiles of carotenoid pigments extracted from peel of *Citrus* varieties.

A : Peel of Kiyomi, B : Peel of Siranuhi, C : Peel of Seminoru, D : Peel of Satsuma, E : Peel of Navel orange, F : Peel of Lemon, G : Peel of Grapefruit, H : Peel of Valencia orange



제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Figure 6. HPLC elution profiles of carotenoid pigments extracted from flesh of *Citrus* varieties.

A : flesh of Kiyomi, B : flesh of Siranuhi, C : flesh of Seminoru, D : flesh of Satsuma, E : flesh of Navel orange, F : flesh of Lemon, G : flesh of Grapefruit, H : flesh of Valencia orange

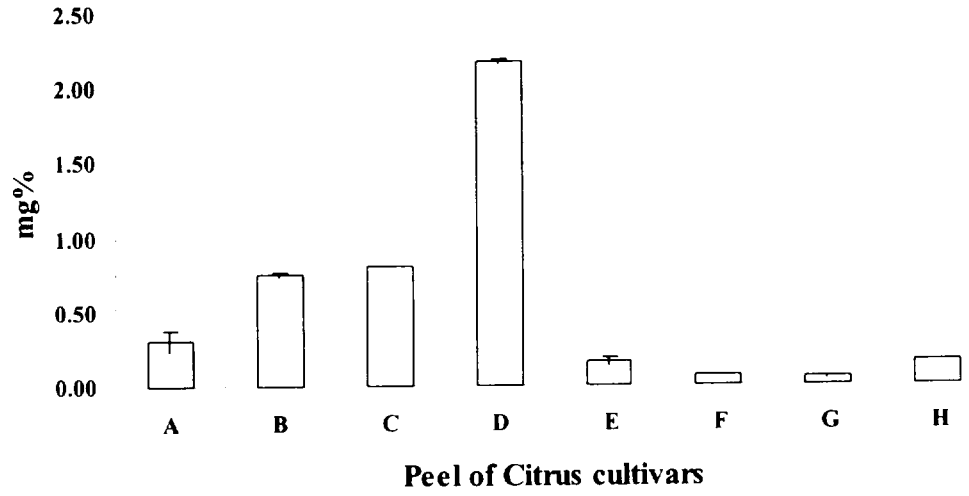


Figure 7. β-Cryptoxanthin content from peel of *Citrus* cultivars.

A : Kiyomi, B : Siranuhi, C : Seminoru, D : Satsuma, E : Navel orange, F : Lemon, G : Grapefruit, H : Valencia orange

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

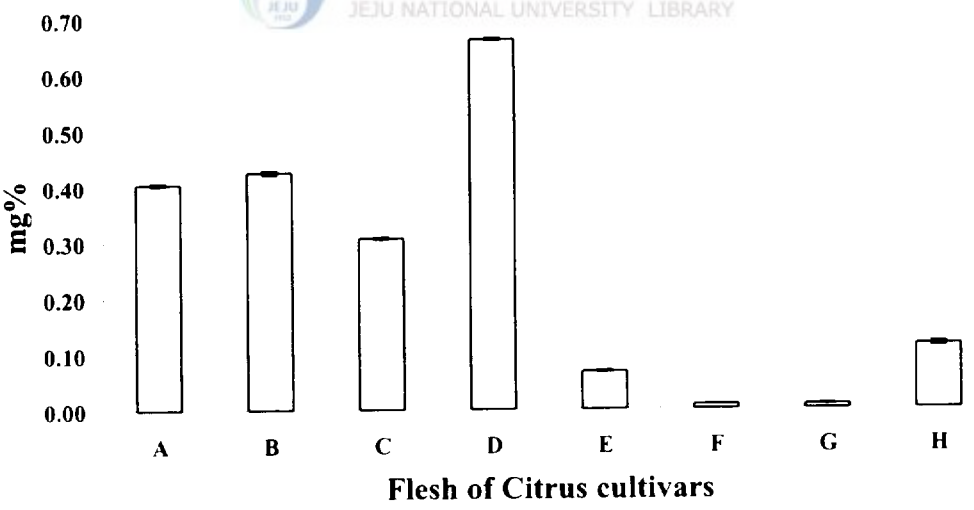


Figure 8. β-Cryptoxanthin content from flesh of *Citrus* cultivars.

A : Kiyomi, B : Siranuhi, C : Seminoru, D : Satsuma, E : Navel orange, F : Lemon, G : Grapefruit, H : Valencia orange

Table 5. Taxonomic names of *Citrus* and Fortunella.

Common name	Taxonomic name
Kiyomi	*Citrus* Tangor
Siranuhi	*Citrus sp.*
Seminoru	*Citrus* Tangelo
Satsuma	*Citrus unshiu* Marcovitch
Navel orange	*Citrus sinensis* Osbeck var. basiliensis Tanaka
Lemon	*Citrus limon*
Grapefruit	*Citrus paradisi*
Valencia orange	*Citrus sinensis* Osbeck
Fortunella	*Fortunella margarita* (Lour.) Swingle

제주대학교 중앙도서관

Table 6. The land of origin of *Citrus* and Fortunella.

Cultivars	The land of origin
Kiyomi	Jungmun Seogwipo-si, Jeju, Korea
Siranuhi	Topyong, Seogwipo-si, Jeju, Korea
Seminoru	Korea
Satsuma	Jungmun Seogwipo-si, Jeju, Korea
Navel orange	Wimi, Namjejugun, Jeju, Korea
Lemon	America
Grapefruit	America
Valencia orange	America
Fortunella	Jeju, Korea

2. Cloning and Expression Analysis of *Ggps, Pds, Zds* and *Lcy* Gene

To study of the *Ggps, Pds, Zds* and *Lcy* showing fruit specificity of expression, the experimental scheme (Figure 9) 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 10). Eight kinds of degenerate oligonucleotides (Table 7) were designed from the sequence of the highly conserved regions of the known *Ggps, Pds, Zds* and *Lcy* genes. The oligonucleotides were used as primers for PCR reaction to amplify a partial *Ggps, Pds, Zds* and *Lcy* cDNA fragment from mRNA of *Citrus* fruit. About 500bp for *Ggps, Pds, Zds* and *Lcy* PCR products were amplified, respectively. The DNA fragments were cloned into pGEM-T® vector (Figure 11 and 12) and sequenced using T7 and T3 primers.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

2) Isolation of cDNA clone

(1) Ggps

By using degenerate primers (GGPS5 and GGPS3), about 500bp cDNA fragment encoding Ggps was amplified by PCR reaction. Fifteen positive clone were isolated out of 600,000 plaques (Figure 11). We screened full cDNAs from the *Citrus* fruit cDNA library and isolated a cDNA clone, which is called Ggps (Figure 13A). We could not isolate any clones having different sequences from *Ggps*, from the *Citrus* leaf cDNA library as well as the fruit library.

The length of the Ggps cDNA clone is 1965 bp. The *Ggps* cDNA contains 1248 bp coding region, 477 bp 5′ UTR (untranslated region), and 240 bp 3′ UTR,

which has an uninterrupted open reading frame deriving 45.9kDa polypeptide (Figure 13).

(2) Pds

About 500bp cDNA fragment encoding Pds was amplified by PCR reaction with degenerate primers (PDS5 and PDSF3). Three positive clone were isolated out of 600,000 plaques (Figure 11). We screened full cDNAs from the *Citrus* fruit cDNA library and isolated a cDNA clone, which is called *Pds*. We didn't any isoform from the *Citrus* leaf and fruit cDNA libraries.

The *Pds* cDNA contains 1918 bp coding region, 150 bp 5' UTR, and 111 bp 3' UTR, which has an uninterrupted open reading frame deriving 54.3kDa polypeptide (Figure 13).

(3) Zds

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

About 500bp cDNA fragment encoding Zds was amplified by PCR reaction with degenerate primers (ZDS5 and ZDS3). Fifteen positive clone were isolated out of 600,000 plaques (Figure 12). We screened full cDNAs from the *Citrus* fruit cDNA library and isolated a cDNA clone, which is called *Zds*. We didn't any isoform from the *Citrus* leaf and fruit cDNA libraries.

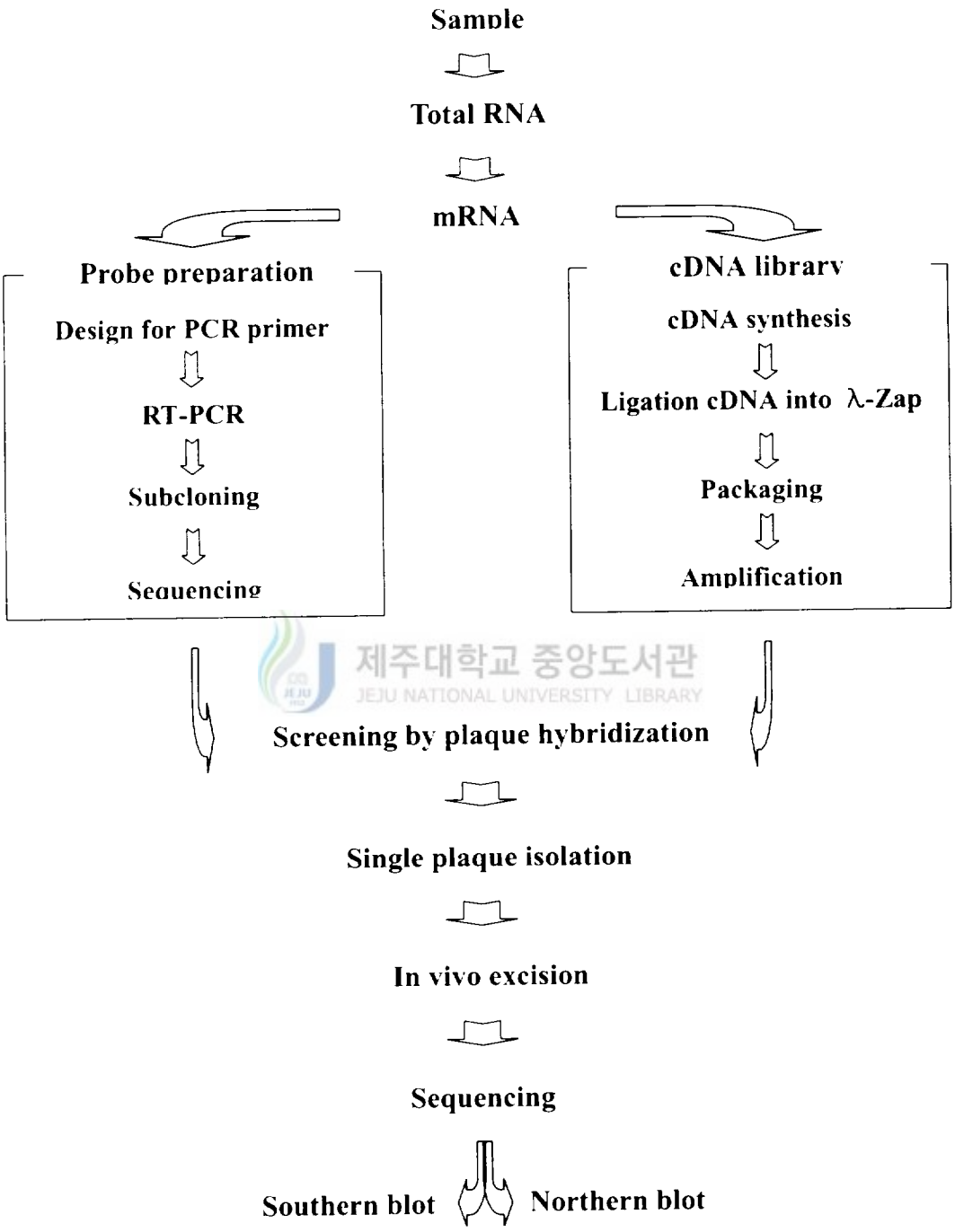
The length of the *Zds* cDNA clone is 2064 bp. The Zds cDNA contains 1710bp coding region, 113 bp 5' UTR, and 241 bp 3' UTR, which has an uninterrupted open reading frame deriving 61.4kDa polypeptide (Figure 13).

(4) Lcy

By using degenerate primers (LCY5 and LCY3), about 500bp cDNA fragment encoding Lcy was amplified by PCR reaction. Ten positive colne were isolated out of 600,000 plaque (Figure 12). We screened full cDNAs from the *Citrus* fruit cDNA library and isolated a cDNA clone, which is called *Lcy*. We didn't any isoform from the *Citrus* leaf and fruit cDNA libraries.

The *Lcy* cDNA contains 1882 bp coding region, 212 bp 5' UTR, and 98 bp 3' UTR, which has an uninterrupted open reading frame deriving 54.3kDa polypeptide (Figure 13).

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY



제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Figure 9. Flow chart for experimental scheme.

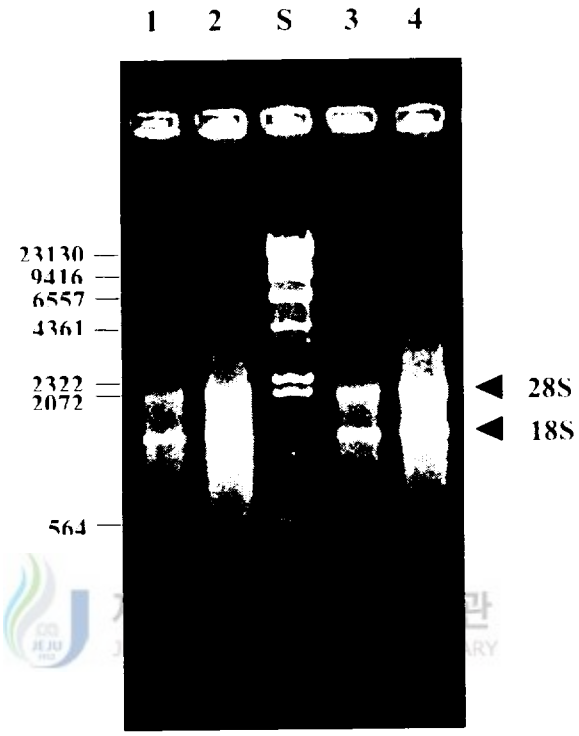
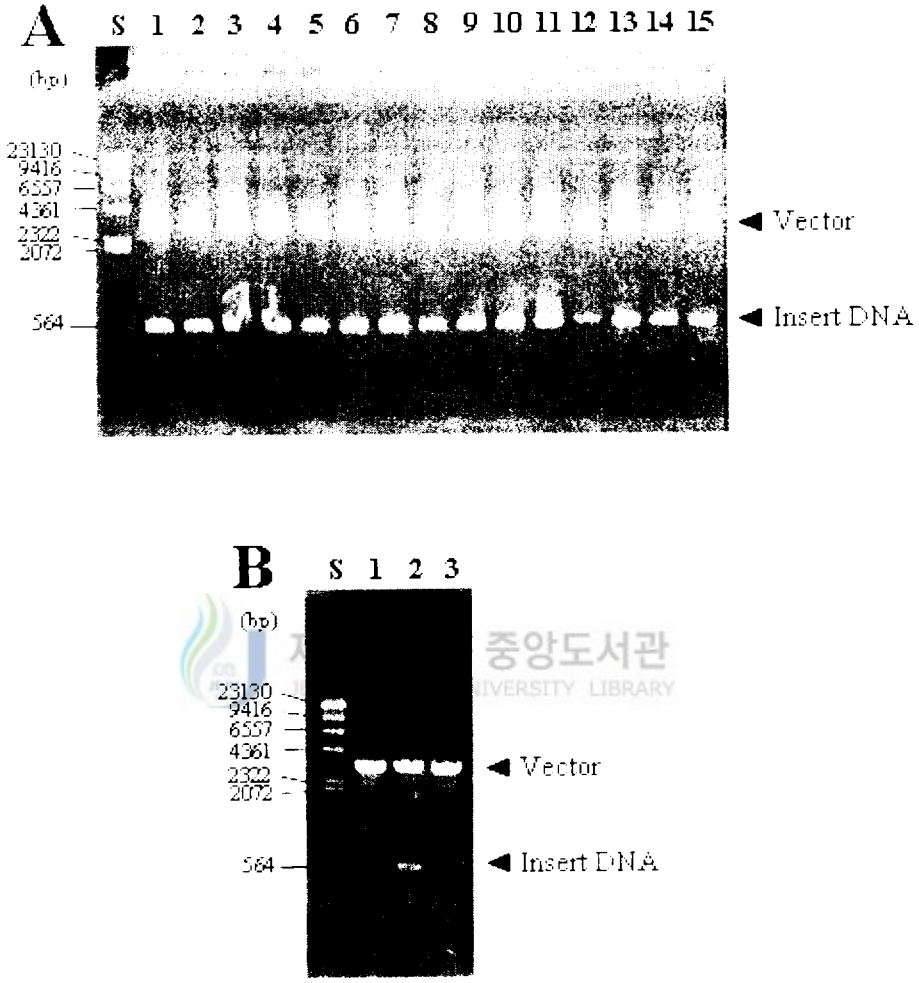


Figure 10. Total RNA extracted from *Citrus* fruits.



중앙도서관

Figure 11. *Ggps* (A) and *Pds* (B) cDNA fragments subcloning. The PCR products of *Ggps* and *Pds* were subcloned into pGEM-T vector. The plasmids for Ggps (A) and Pds (B) digested with *EcoR*I, and then electrophoresed onto agarose gel. S (bp) represents the molecular standards of λ/*Hin*dIII.

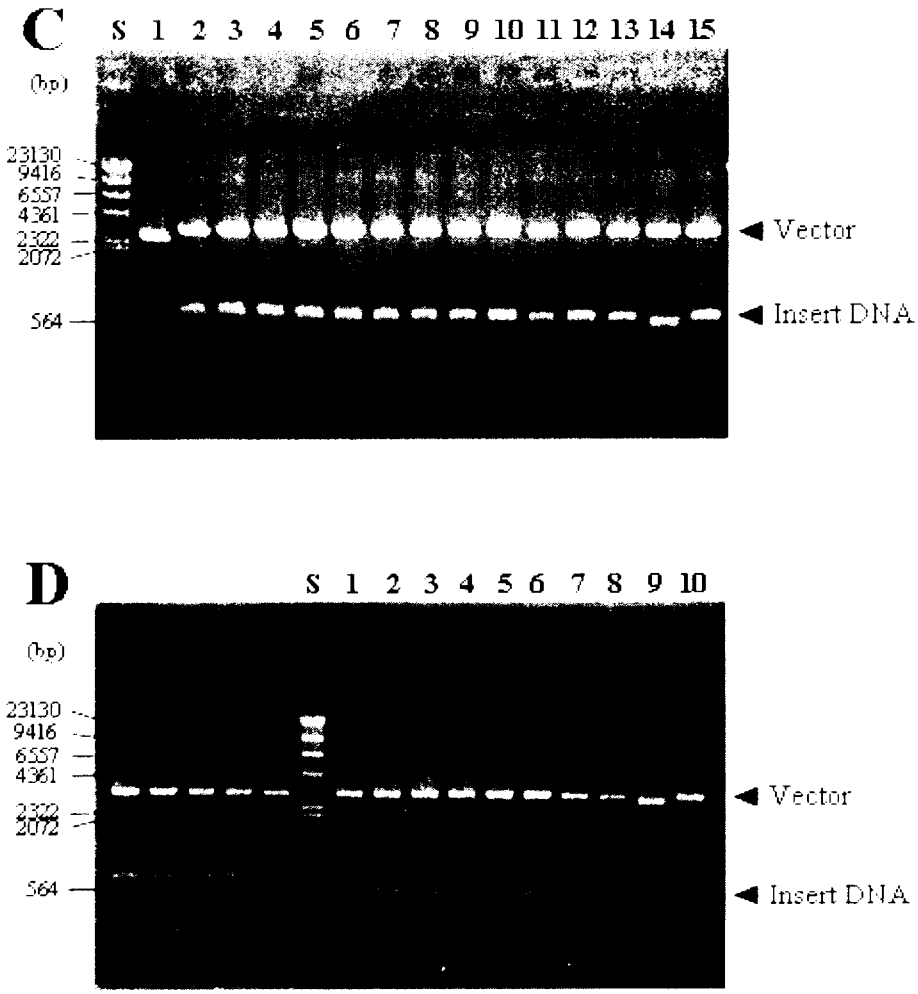


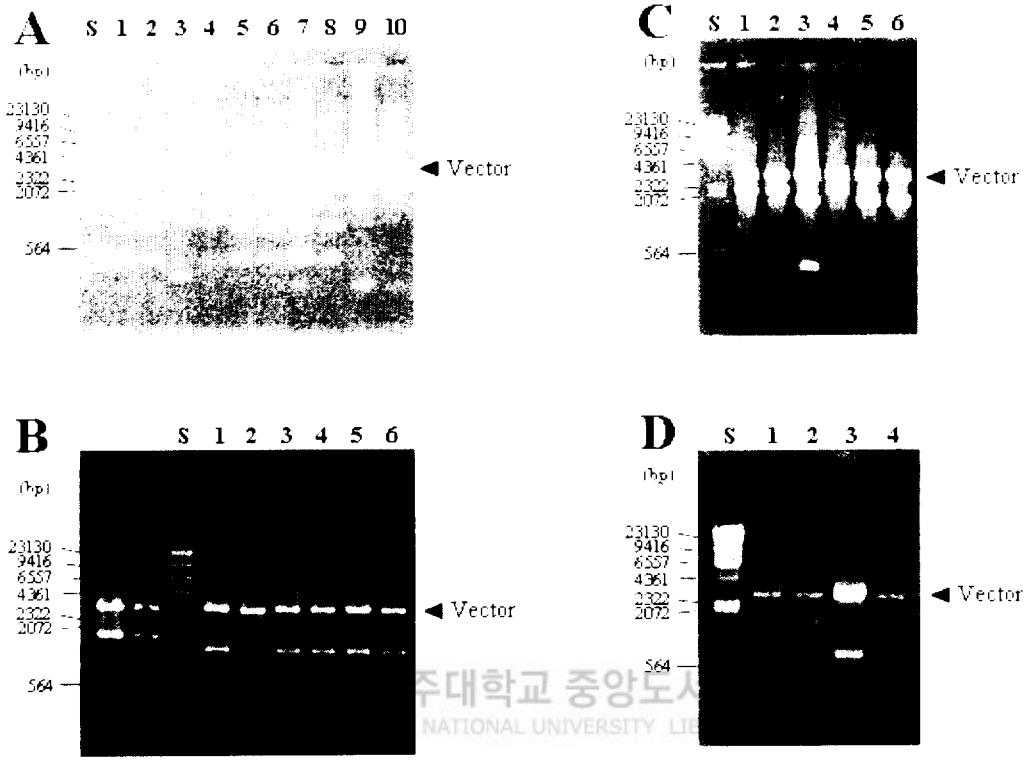
Figure 12. *Zds* (A) and *Lcy* (B) cDNA fragments subcloning. The PCR products of *Zds* and *Lcy* were subcloned into pGEM-T vector. The plasmids for Zds (C) and Lcy (D) digested with *EcoR*I, and then electrophoresed onto agarose gel. S (bp) represents the molecular standards of λ/*Hin*dIII.

Table 7. The nucleotide sequences of the regions highly conserved among plant *Ggps*, *Pds*, *Zds* and *Lcy* genes. On the basis of these regions, degenerated oligonucleotides were synthesized and used for PCR amplification.

Primers	Sequence	Usage
GGPS5	5′-GAYGAYYTNCCYTGYATGGA-3′	PCR
GGPS3	5′-AYRTCATCNAYHACYTGAWA-3′	PCR
PDS5	5′-GTTTGGTCTTCAGTTTGATA-3′	PCR
PDSF3	5′-TAGAGTGCTCCTTCCACTGC-3′	PCR
ZDS5	5′-GAYGTTTAYTTRAGYGGTCC-3′	PCR
ZDS3	5′-GTCTTYTGATCRGGYCTGAA-3′	PCR
LCY5	5′-GGTGGCGGCCCGGCTGGGCT-3′	PCR
LCY3	5′-TCCAATCCATGAAAACCATC-3′	PCR

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Mixed Base	Code Name	Mixed Base	Code Name	Mixed Base	Code Name	Mixed Base	Code Name
A+G	R	C+T	Y	A+G+C+T	N	A+T+C	H
A+T	W	G+T	K	G+A+C	V	G+C	S
G+A+T	D	A+C	M	G+T+C	B		



주대학교 중앙도서
NATIONAL UNIVERSITY LIB

Figure 13. Agarose gel (1%) electrophoresis of *Eco*RI and *Xho*I digestion products of *in vivo* excised phagemid from containing *Ggps* (A), *Pds* (B), *Zds* (C) and *Lcy* (D) 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.

Figure 14. Nucleotide sequence and deduced amino acid sequence for the *Ggps* cDNA clones isolated from *Citrus* fruits. The sequence was registered in GenBank (GenBank accession number AY166797).

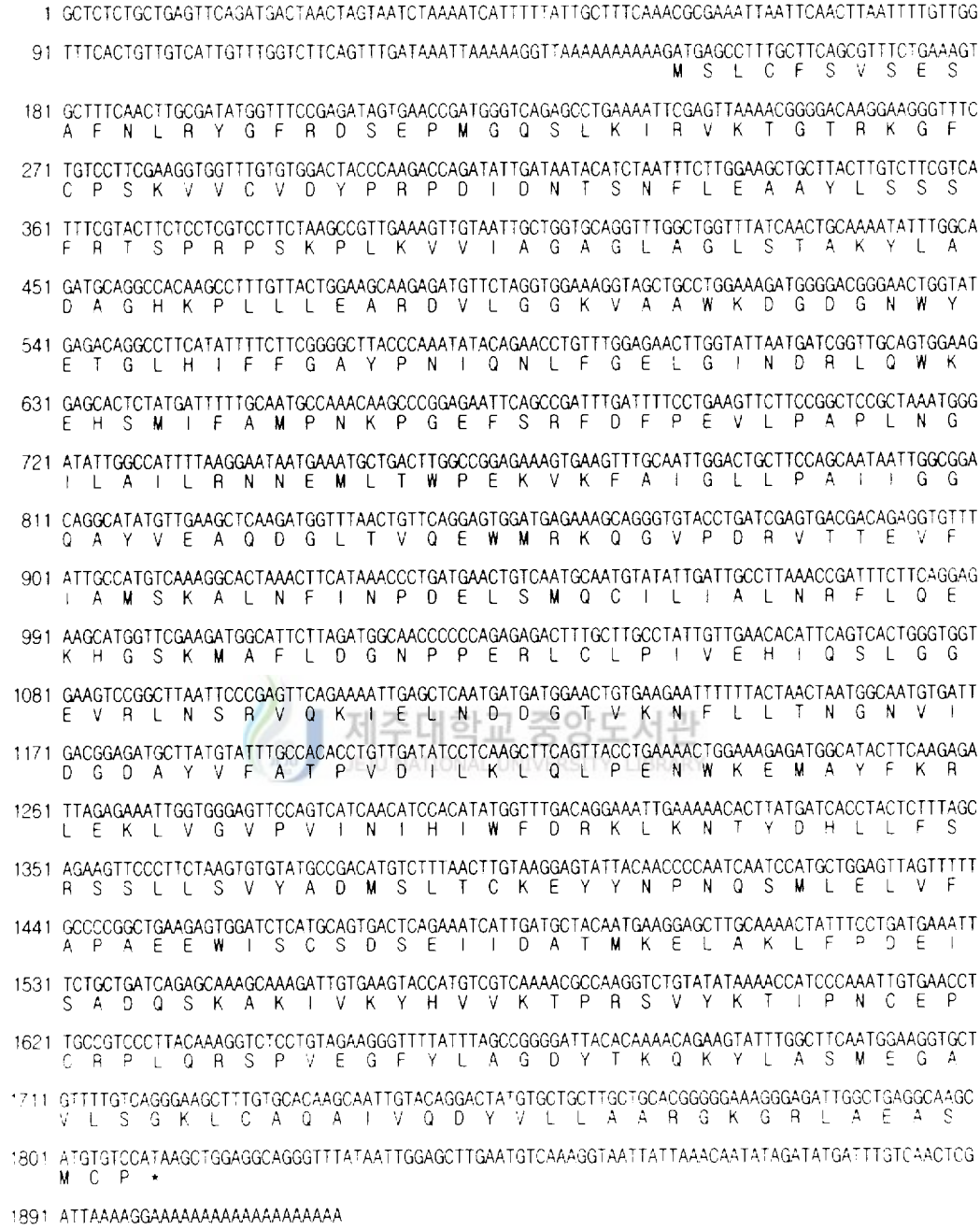


Figure 15. Nucleotide sequence and deduced amino acid sequence for the *Pds* cDNA clones isolated from *Citrus* fruits. The sequence was registered as GenBank accession number AB046992 (92%).

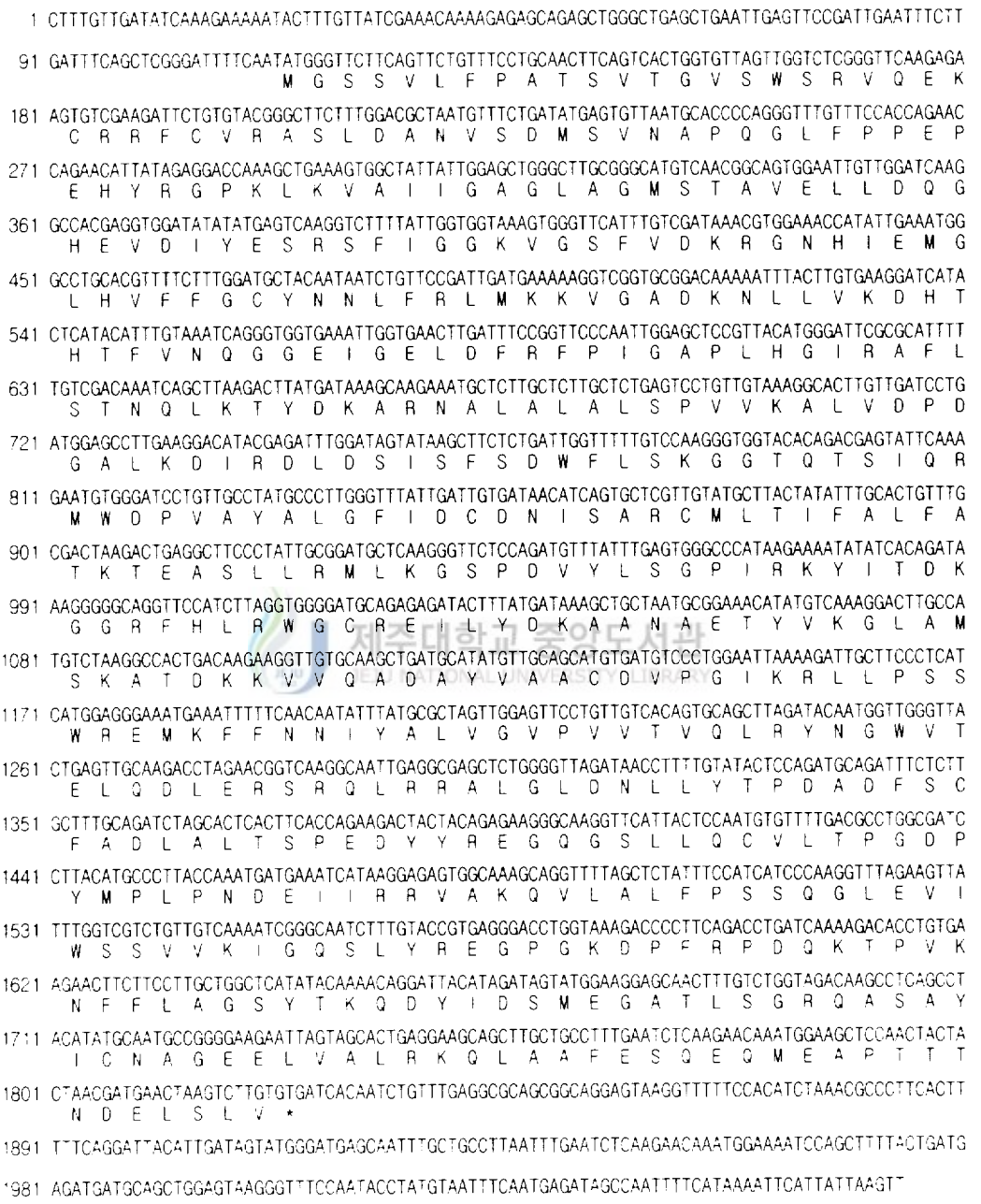


Figure 16. Nucleotide sequence and deduced amino acid sequence for the *Zds* cDNA clones isolated from *Citrus* fruits. The sequence was registered as GenBank accession number AB072343 (85%).

Figure 17. Nucleotide sequence and deduced amino acid sequence for the *Lcy* cDNA clones isolated from *Citrus* fruits. The sequence was registered in Genbank (Genbank accession number AY166796).

3) Comparison of the deduced amino acid sequence of *Ggps* and *Lcy* with polypeptides known *Ggps* and *Lcy*

(1) Ggps

The deduced amino acid sequences were compared between *Citrus* and other organisms. The sequence of *Ggps* has the highest homology with oak (79%) (GenBank accession number AJ298245). The Ggps sequence has a high degree of similarity with various species of plants (19-79%), fungi (14-18%), and bacteria (5-20%) (Figure 18). The phylogenetic dendrogram showed similar patterns, which indicated that Ggps isolated from fruits can be categorized into one group (Figure 20). Therefore, these results suggested an evolutionary link among the fruit-producing plants.

(2) Lcy

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

The deduced amino acid sequences were compared between *Citrus* and other organisms. The sequence of *Lcy* has the highest homology with tabacco (83%) (GenBank accession number X81787). Its sequence has a high degree of similarity with various species of plants (29-83%), but not with bacteria (15-34%) (Figure 19). The phylogenetic dendrogram showed similar patterns, which indicated that *Lcy* isolated from fruits can be categorized into one group (Figure 21). Therefore, these results suggested an evolutionary link among the fruit-producing plants.

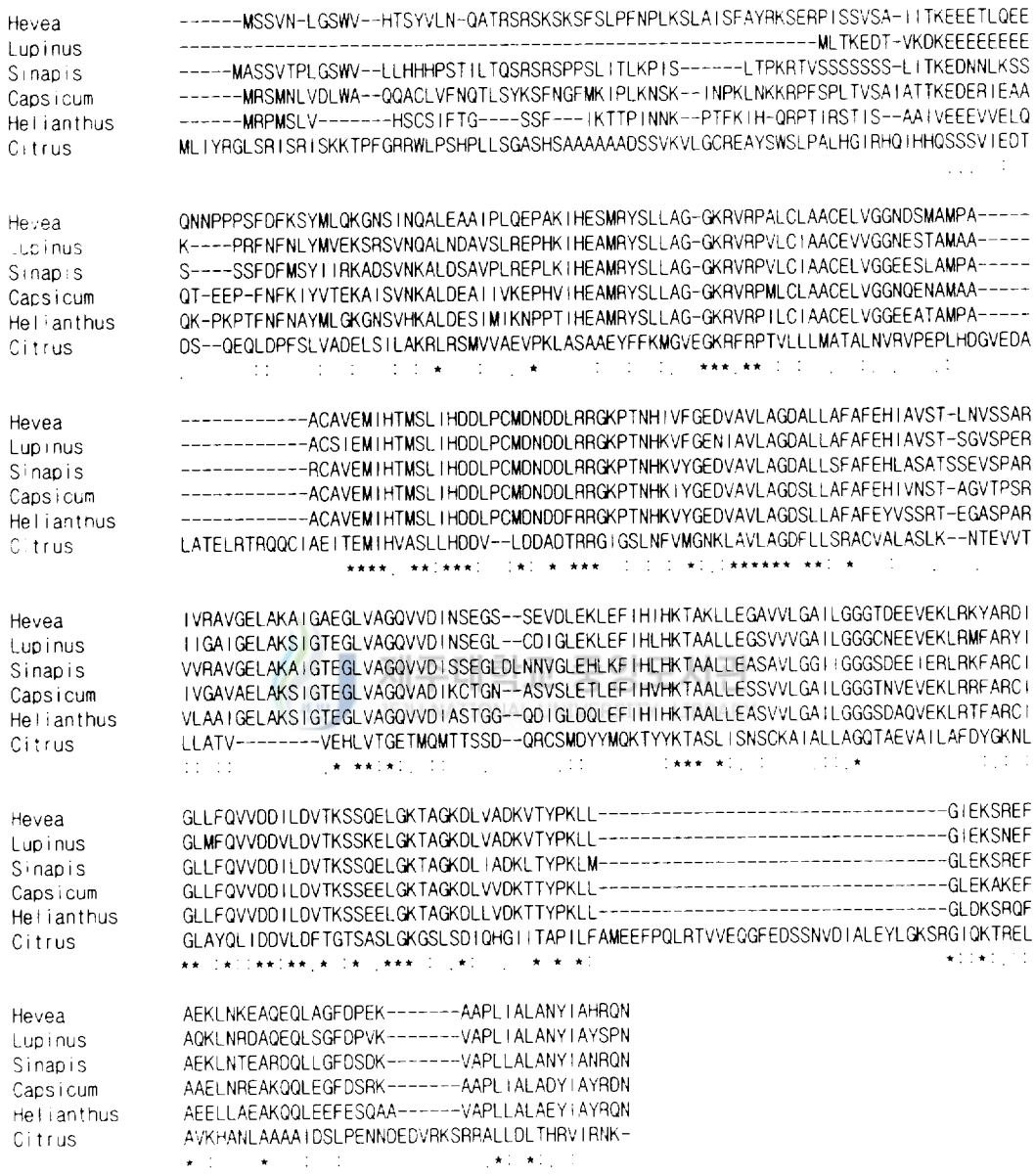


Figure 18. Alignment of the deduced amino acid sequences of *Ggps* isolated from various species. Multiple sequence alignment was done with cDNA encoding Ggps, including Hevea (AB055496), Sinapis (X98795), Lupinus (U15778), Capsicum (X80267) and Helianthus (AF020041)

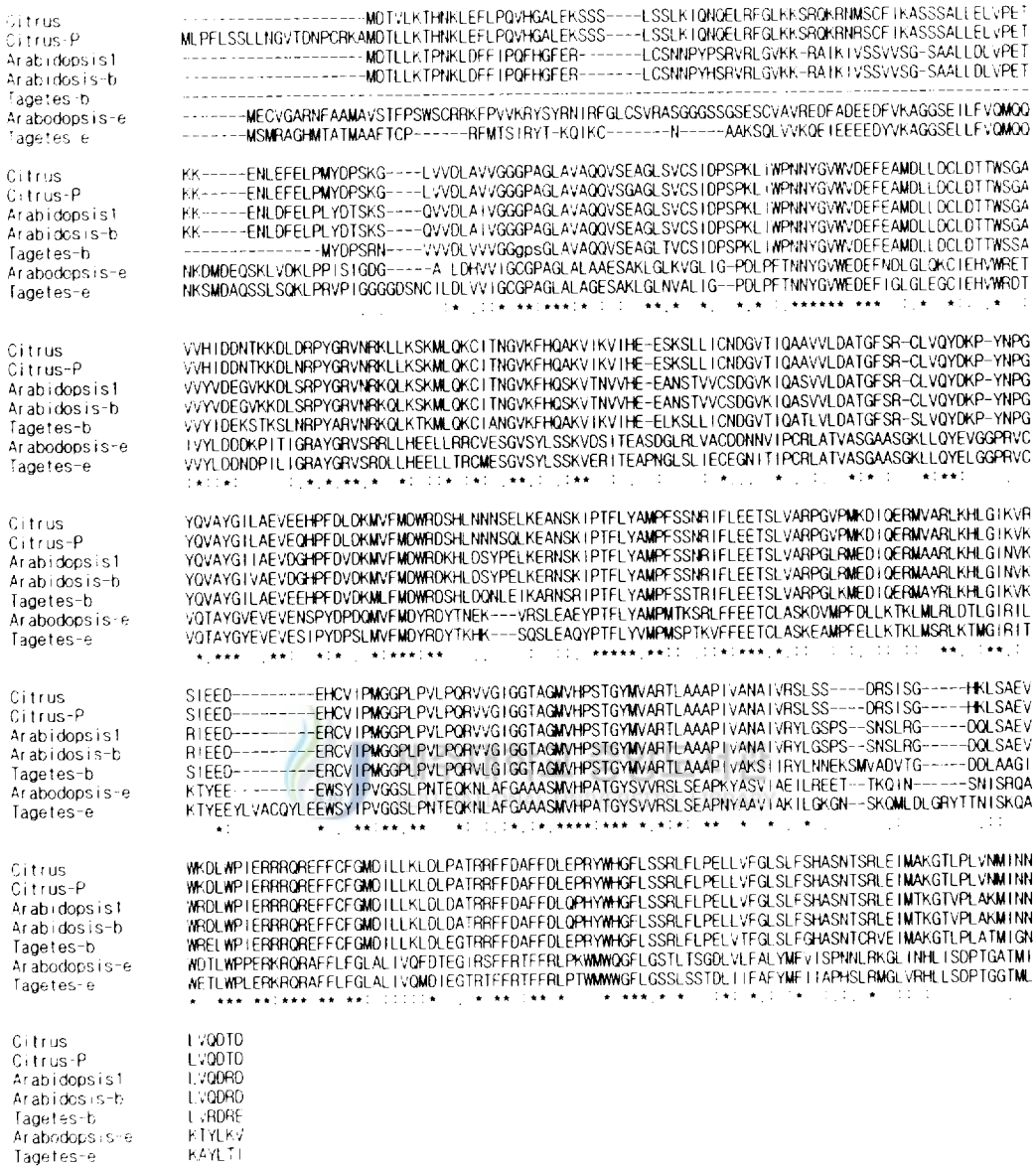
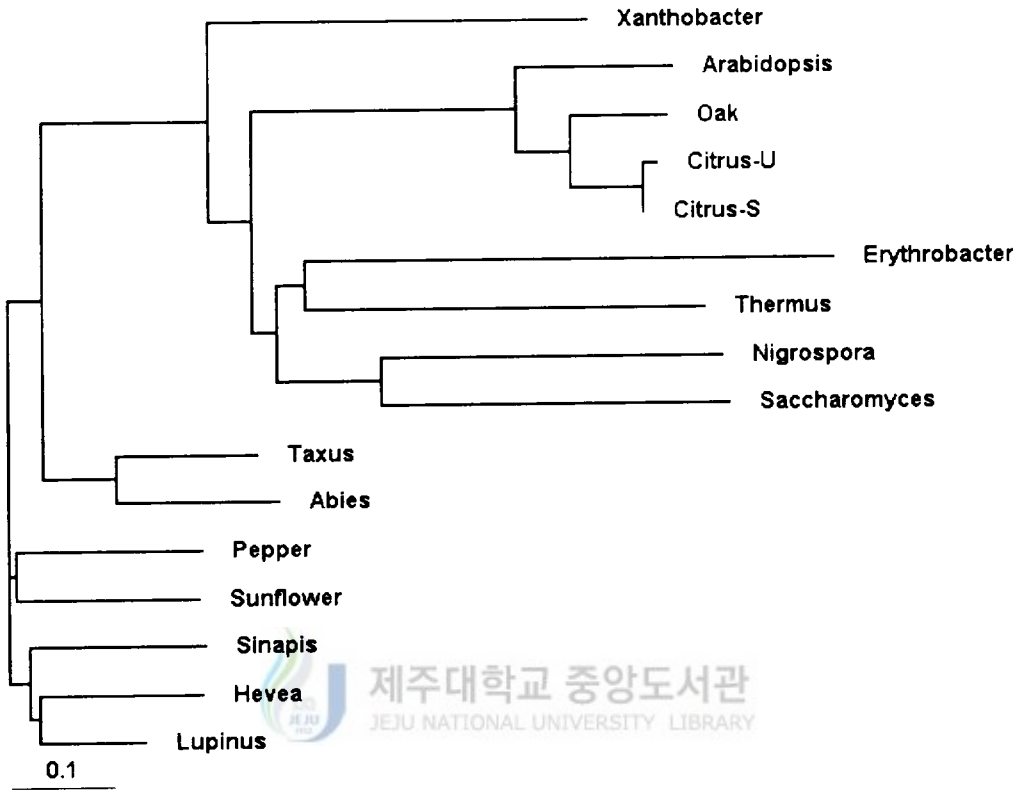
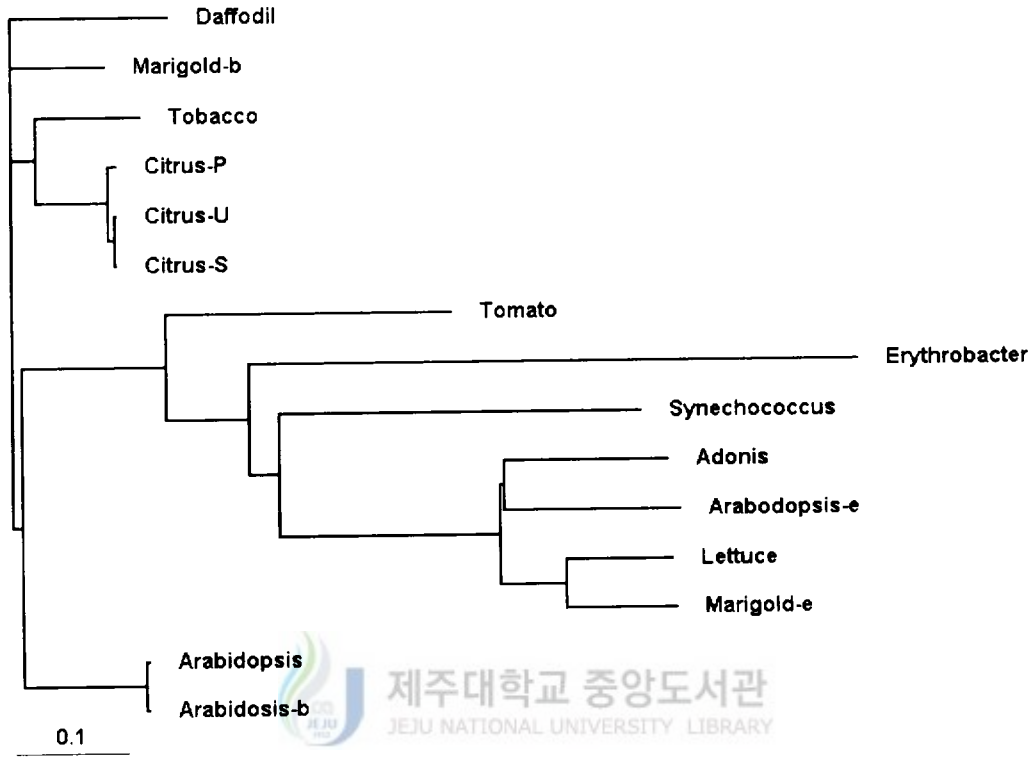


Figure 19. Alignment of the deduced amino acid sequences of *Lcy* isolated from various species. Multiple sequence alignment was done with cDNA encoding LCT, including Citrus-P (AF152246), Arabidopsis1 (L40176), Arabidopsis-b (ATU50739), Arabidopsis-e (ATU50738), Tagetes-b (AY099484) and Tagetes-e (AY099485). 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 1.8) (Thompson *et al.*, 1994). An asterisk indicates identical amino acid residues. The amino acid residues showing similar character are represented by a dot.



제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Figure 20. Phylogenetic relationship of the *Citrus Ggps* to *Ggps* of other various species. The *Ggps* cDNA sequences used for amino acid translation and GenBank accession numbers are: Citrus-U , Xanthobacter (AF408847), Arabidopsis (Y17376), Oak (AJ28245), Citrus-S (AJ243739), Erythrobacter (D83513), Thermus (D87817), Nigrospora (AB037600), Saccharomyces (U31632), Taxus (AF081514), Abies (AF425235), Pepper (X80267), Sunflower (AF020041), Sinapis (X98795), Hevea (AB055496) and Lupinus (U15778). Phylogenetic analysis is based on the deduced amino acid sequences of *Ggps* from various species. The tree was generated by Clustal X (version 1.8) and TreeView (version 1.8).



제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Figure 21. Phylogenetic relationship of the *Citrus Lcy* to *Lcy* of other various species. The *Lcy* cDNA sequences used for amino acid translation and GenBank accession numbers are: Citrus-U, Daffodil (X98796), Marigold-b (AY099484), Tobacco (X81787), Citrus-P (AF152246), Citrus-S (AF240787), Tomato (AF254793), Erythrobacter (D83513), Synechococcus (X74599), Adonis (AF321535), Arabidopsis-e (U50738), Lettuce (AF321538), Marigold-e (AY099485), Arabidopsis (L40176) and Arabidopsis-b (U50739). Phylogenetic analysis is based on the deduced amino acid sequences of *Lcy* from various species. The tree was generated by Clustal X (version 1.8) and TreeView (version 1.8).

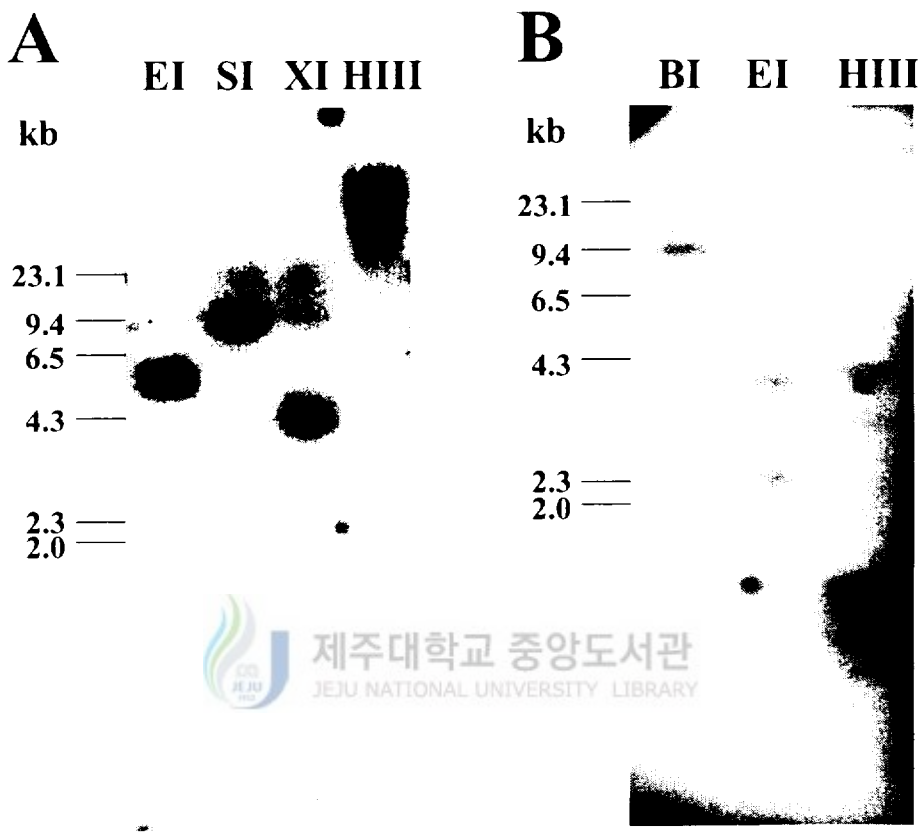
4) Genomic Southern analysis

The genomic DNA isolated from *Citrus* leaves was digested with *BamHI, EcoRI, SacI, Xba*I and *Hind*III, and then separated on 0.7% agarose gels. As shown in Figure 22 and Figure 23, the digested DNA fragments were hybridized with the *Ggps, Pds, Zds* and *Lcy* cDNA probe and washed at high stringency (0.1 x SSPE and 0.1% SDS, 68℃). The genomic blot pattern showed 1 to 5 bands in each DNA sample, which indicated that *Ggps, Pds, Zds* and *Lcy* is present as low copy in *Citrus* genome.

5) Expression pattern in *Citrus*

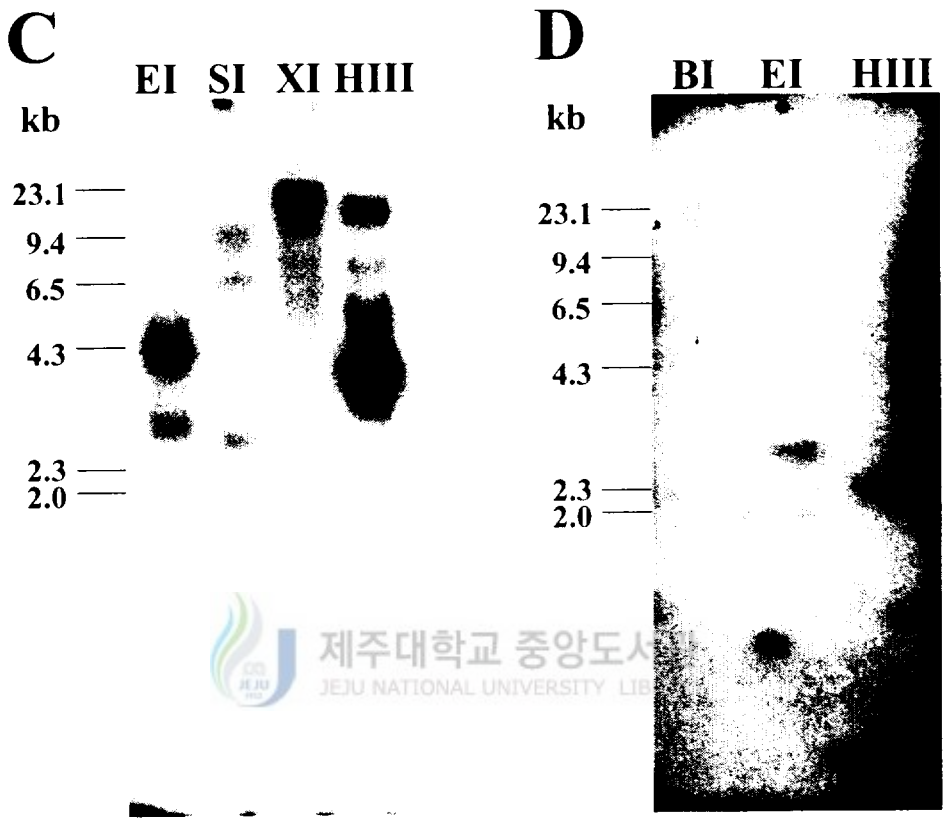
We investigated the expression patterns of *Citrus Ggps, Zds and Lcy* gene in the process of fruit and leaf development (Figure 24). *Citrus* fruits and leaves were harvested at five stages determined by maturity and internal fruit color, and size of fruit. Fruit tissues were partitioned into flesh and peel, except for the mini-green fruit of 25 DAF.

In summary, we isolated a cDNA clone encoding *Ggps, Pds, Zds* and Lcy, and investigated the expression patterns during fruit and leaf development in *Citrus*, which is a nonclimacteric fruit-producing woody plant.



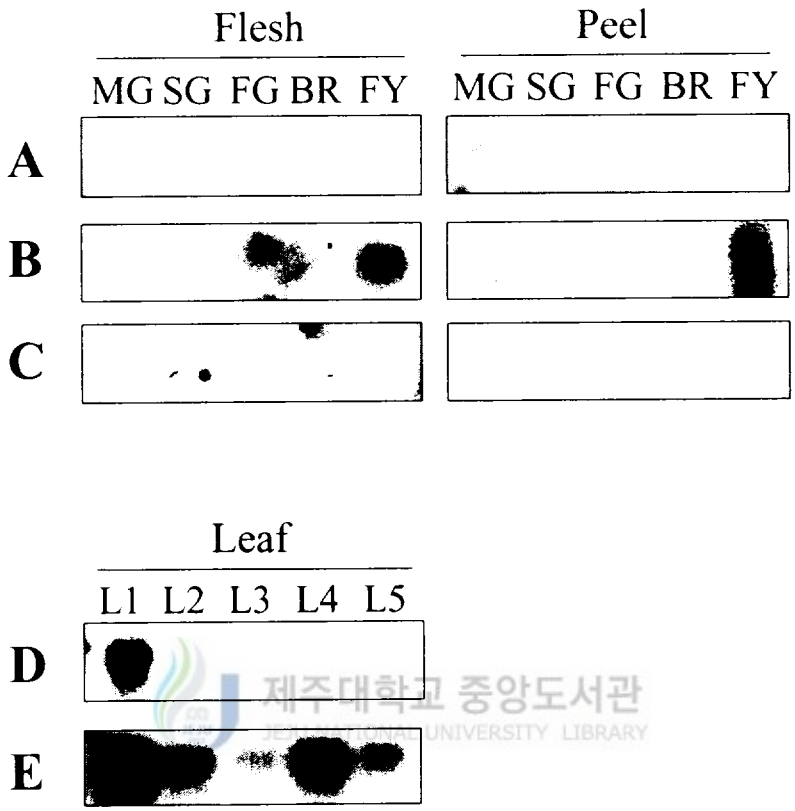
제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Figure 22. Genomic blot analysis of *Ggps* (A) and *Pds* (B). (A) Genomic DNA (10 ㎍) was digested with *Eco*RI (EI), *Sac*I (SI), *Xba*I (XI) and *Hin*dIII (HI) for each DNA sample. (B) Genomic DNA (10 ㎍) was digested with *Bam*HI (BI), *Eco*RI (EI) and *Hin*dIII (HI) for each DNA sample. Size markers (kb) are indicated on the left.



제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Figure 23. Genomic blot analysis of *Zds* (A) and *Lcy* (B). (A) Genomic DNA (10 ㎍) was digested with *Eco*RI (EI), *Sac*I (SI), *Xba*I (XI) and *Hin*dIII (HI) for each DNA sample. (B) Genomic DNA (10 ㎍) was digested with *Bam*HI (BI), *Eco*RI (EI) and *Hin*dIII (HI) for each DNA sample. Size markers (kb) are indicated on the left.



제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Figure 24. Northern blot analysis of *Ggps* (A, D), *Zds* (B, E) and *Lcy* (C) gene expression in five developmental stages of flesh and peel of fruits, and leaves of *Citrus*. Total RNA (20 ㎍/lane) was separated on a 1.2 % formaldehyde gel, transferred to Hybond N membrane (Amersham Life Science, Amersham, UK), and hybridized with *Ggps*, *Zds* and *Lcy* cDNA probes.

SUMMARY (국문요약)

감귤 중 과육에는 carotenoide 생합성 중에 나타나는 β-carotene 과 ζ-carotene, β-cryptoxanthin 이 많고, 과피에는 cryptoxanthin 과 antheraxanthin, violaxanthin 이 많이 축적되어 있다. 따라서 carotenoid 생합성에 관여하는 Ggps 와 Pds, Zds, Lcy 의 유전자 발현이 β-cryptoxanthin 를 비롯한 carogenoid 의 축적과 밀접하게 관련되어 있을 것으로 생각되어진다. 그러므로, *Ggps* 와 *Pds, Zds, Lcy* 를 암호화하는 cDNA 의 발현양상의 특성을 연구하기 위해 감귤과육, 과피 및 잎의 library 로부터 분리하였다.

국내산 감귤과 수입산 감귤의 과피와 과육으로부터 β-cryptoxanthin의 함량을 측정하였다. 추출한 색소농축물은 TLC (Thin-layer Chromatography) 분석에 의해 확인되었다. 감귤의 β-cryptoxanthin 의 함량분석은 HPLC (High Performance Liquid Chromatography) 방법으로 수행하였다. 과피와 과육 중 β-cryptoxanthin의 함량은 Satsuma (*Citrus unshiu* Marcovitch)가 다른 감귤보다 높게 나타났다. Kiyomi (*Citrus* Tangor) 그리고 Siranuhi (*Citrus sp.*), Seminoru (*Citrus* Tangelo), Satsuma (*Citrus unshiu* Marcovitch), Navel orange (*Citrus sinensis* Osbeck var. basiliensis Tanaka), Lemon (*Citrus limon*), Grapefruit (*Citrus paradisi*), Valencia orange (*Citrus sinensis* Osbeck) 의 과피 내의 β-cryptoxanthin의 함량은 각각 0.315±0.066 그리고 0.749±0.011, 0.811±0.002, 2.178±0.008, 0.164±0.0026, 0.066±0.001, 0.048±0.001, 0.164±0.001 mg%이었다. 또한 Kiyomi (*Citrus* Tangor) 그리고 Siranuhi (*Citrus sp.*), Seminoru (*Citrus* Tangelo), Satsuma (*Citrus unshiu* Marcovitch), Navel orange (*Citrus sinensis* Osbeck var. basiliensis Tanaka), Lemon (*Citrus limon*), Grapefruit (*Citrus paradisi*), Valencia orange (*Citrus sinensis* Osbeck) 의 과육 내의 β-cryptoxanthin의 함량은 각각 0.405±0.003 그리고 0.427±0.004, 0.307±0.002, 0.663±0.002, 0.068±0.002, 0.008, 0.007±0.001, 0.114±0.004 mg%이었다.

β -cryptoxanthin 생합성은 여러 유전자의 발현에 의해 조절되지만, 본 연구에서는 이들 유전자 중, carotenoid 생합성의 첫단계인 geranylgeranyl pyrophosphate 의 생합성에 관여하는 geranylgeranyl diphosphate synthase (Ggps) 그리고 phytoene 에서 ζ -carotene 으로의 생합성에 관여하는 phytoene desaturase (Pds), ζ -carotene 에서 lycopene 으로의 생합성에 관여하는 ζ -carotene desaturase (Zds), lycopene 에서 carotene 으로의 생합성에 관여하는 lycopene cyclase (Lcy) 유전자를 감귤로부터 분리하였다.

감귤 (*Citrus unshiu* Marc.) 의 과피와 과육의 cDNA library 에서 Ggps 를 암호화하는 cDNA 클론을 분리하였다. 염기서열분석과 유연관계조사를 통해 이 클론은 과실수의 Ggps 와 유사한 서열특성을 가지고 있음을 알 수 있었다. Ggps 클론은 426 개 아미노산을 이루는 한 개의 ORF 를 포함하고 있었다.

그리고, 감귤로부터 Pds 와 Zds 에 관한 연구를 위해 먼저 과육과 과피, 잎 cDNA library 로부터 cDNA 클론들을 분리하였다. 염기서열분석과 유연관계조사를 통해 이 클론들이 기존에 감귤로부터 밝혀진 Pds 와 Zds 임을 확인하였다. Pds 는 553 개의 아미노산을 이루는 한 개의 ORF 를 포함하고 있었고, Zds 는 570 개의 아미노산을 이루는 한 개의 ORF 를 포함하고 있었다.

마찬가지로, 감귤로부터 Lcy 를 암호화하는 cDNA 클론을 분리하기 위해 감귤 과피 그리고 과육, 잎 cDNA library 로부터 cDNA 클론을 분리하였다. 염기서열분석과 유연관계조사를 통해 이 클론은 과실수의 Lcy 와 유사한 서열특성을 가지고 있음을 알 수 있었다. Lcy 클론은 504 개 아미노산을 이루는 한 개의 ORF 를 포함하고 있었다.

제주대학교 중앙도서관

REFERENCES

Albrecht, M., Klein, A., Hugueney, P., Sandmann, G., Kuntz, M. 1995. Molecular cloning and functional expression in *E. coli* of a novel plant enzyme mediating ζ-carotene desaturation. *FEBS*. 372:199-202

Baldwin, E. A. 1993. Citrus fruit. In: Seymour, G. B., Taylor, J. E., Tucker, G. A. (eds) Biochemistry of Fruit Ripening. Chapman & Hall, New York.

Bouvier, F., Hugueney, P., d'Harlingue, A., Kuntz, M., Camara, B. 1994. Xanthophyll biosynthesis in chromoplasts: Isolation and molecular cloning of an enzyme catalyzing the conversion of 5,6-epoxycarotenoid into ketocarotenoid. *Plant J.* 6:45-54

Breitenbach, J., Kuntz, M., Takaichi, S. and Sandmann, G. 1999. Catalytic properties of an expressed and purified higher plant type ζ -carotene desaturase from *Capsicum annuum*. *Eur. J. Biochem.* 265:376-383

Burke, C. and Croteau, R. 2002. Geranyl diphosphate synthase from Abies grandis: cDNA isolation, functional expression, and characterization. *Arch. Biochem. Biophys.* 405:130-136

Cooper, D. A., Eldridge, A. L. and Peters, J. C. Dietary carotenoids and certain cancers, heart disease, and age-related macular degeneration: A reviews of recent research. Nutr. Rev. 57: 201-214 (1999)

Cunningham, Jr. F. X. and Gantt, E. 1998. Genes and enzymes of carotenoid biosynthesis in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 49:557-583

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Cunningham, Jr. F. X., Pogson, B., Sun, Z., McDonald, K. A., DellaPenna, D. and Gantt, E. 1996. Functional analysis of the β and ε lycopene cyclase enzymes of arabidopsis reveals a mechanism for control of cyclic carotenoid formation. *Plant Cell.* 8:1613-1626

Dogbo, O. and Camara, B. 1987. Purification of isopentenyl pyrophosphate isomerase and geranylgerayl pyrophosphate synthase from *Capsicum* chromoplasts by affinity chromatography. *Biochim. Biophys. Acta.* 920:140-148

Dorgan, J. F., Swanson, C. A., Potischman, N., Miller, R., Schussler, N. and Stephenson, H. E. Relation of serum carotenoids, retinol, α -tocopherol, and selenium with breast cancer risk: results from a prospective study in Colombia, Missouri U.S.A. Cancer Causes Control 9: 89-97 (1998)

Giuliano, G., Bartley, G. E. and Scolnik, P. A. 1993. Regulation of carotenoid biosynthesis during tomato development. Plant Cell. 5:379-387

Hable, W. E., Oishi, K. K. and Schumaker, K. S. 1998. *Viviparous-5* encodes phytoene desaturase, an enzyme essential for abscisic acid (ABA) accumulation and seed development in maize. Mol. Gen. Genet. 257:167-176

Hart, D. J. and Scott, K. J. Development and evaluation of an HPLC method for the analysis of carotenoids in foods, and the measurement of the carotenoid content of vegetables and fruits commonly consumed in the UK. Food Chemistry 54: 101-111 (1995)

Hirschberg, J. 2001. Carotenoid biosynthesis in flowering plants. *Curr Opin Plant Biol.* 4:210-218

Hugueney, P., Romer, S., Kuntz, M., Camara, B. 1992. Characterization and molecular cloning of a flavoprotein catalyzing the synthesis of phytofluene and *zeta*-carotene in *Capsicum* chromoplasts. *Eur. J. Biochem.* 209:399-407

Ko, K-C., Kim, C-S., Lee, N. H., Lee, S-P. and Moon, D-K. Determination of β-cryptoxanthin in peel and flesh of citrus fruits produced in Cheju Island. Food Sci. Biotechnol. 9: 288-291 (2000)

Kuntz, M., Romer, S., Suire, C., Hugueney, P., Weil, J. H., et al. 1992. Identification of a cDNA for the plastid-located geranylgeranyl pyrophosphate synthase from *Capsicum annuum*: correlative increase in enzyme activity and transcript level during fruit ripening. *Plant J.* 2:25-34

Lagarde, D. and Vermaas, W. 1999. The zeaxanthin biosynthesis enzyme β-carotene hydroxylase is involved in myxoxanthophyll synthesis in *Synechocystis* sp. PCC 6803. *FEBS Letters.* 454:247-251

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Lintig, J. V., Welsch, R., Bonk, M., Giuliano, G., Batschauer, A. and Kleing, H. 1997. Light-dependent regulation of carotenoid biosynthesis occurs at the level of phytoene synthase expression and is mediated by phytochrome in *Sinapis alba* and *Arabidopsis thaliana* seedlings. *Plant J.* 12(3):625-634

Matsumura, H., Takeyama, H., Kusakabe, E., Burgess, J. G., Matsunaga, T. 1997. Cloning, sequencing and expressing the carotenoid biosynthesis genes, lycopene cyclase and phytoene desaturase, from the aerobic photosynthetic bacterium *Erythrobacter longus* sp. strain Och101 in *Escherichia coli*. *Gene.* 189:169-174

Nishino, H., Tokuda, H., and Yano, M. Anti-tumor promoting effect of cryptoxanthin, a natural carotenoid. 1998. The fourth joint meeting conference of the American association of cancer research and the Japanese cancer association, innovative approaches to the prevention, diagnosis, and therapy of cancer

Nishino, H., Tokuda, H., Murakoshi, M., Satomi, Y., Masuda, M., Onozuka, M., Yamaguchi, S., Takayasu, J., Tsuruta, J., Okuda, M., Khachik, F., Narisawa, T., Takasuka, N. and Yano, M. 2000. Cancer prevention by natural carotenoids. Biofactors 13: 89-94

Nogata Y., Yoza, K. I., Kusumoto, K. I., Kohyama, N., Sekiya, K. and Ohta, H. 1996. Screening for inhibitory activity of citrus fruit extracts against platelet cyclooxygenase and lipoxygenase. J. Agric. Food Chem. 44: 725-729

Pupin, A.M., Dennis, M.J. and Toledo, M.C.F. 1999. HPLC analysis of carotenoids in orange juice. Food Chemistry 64: 269-275

Ronen, G., Cohen, M., Zamir, D. and Hirschberg, J. 1999. Regulation of carotenoid biosynthesis during tomato fruit development: expression of the gene for lycopene epsilon-cyclase is down-regulated during ripening and is elevated in the mutant *Delta. Plant J.* 17(4):341-351

Sambrook, J., Fritsch, E. F. and Maniatis, T. 1989. "Molecular Cloning: A Laboratory Manual", 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, Chap. 8.

Sanger, F., Nicklen, S. and Coulson, R. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Nat'l. Acad. Sci. USA* 74, 5463-5467

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Sitthithaworn, W., Kojima, N., Viroonchatapan, E., Suh, D. Y., Iwanami, N., Hayashi, T., Noji, M., Saito, K., Niwa, Y. and Sankawa, U. 2001. Geranylgeranyl diphosphate synthase from *Scoparia dulcis* and *Croton sublyratus*. Plastid localization and conversion to a farnesyl diphosphate synthase by mutagenesis. *Chem. Pharm. Bull.* 49(2):197-202

Slattery, M.L., Benson, J., Curtin, K., Ma, K.-N., Schaeffer, D., and Potter, J.D. 2000. Carotenoids and colon cancer. Am. J. Clin. Nutr. 71: 575-582

Sumida, T., Azuma, Y., Ogawa, H. and Tanaka, T. 1999. Inhibitory effects of β-cryptoxanthin rich powder prepared Satsuma Mandarin (*Citrus Unshiu* Marc.) juice on azoxymethane-induced aberrant crypt foci of rats. Nippon Shokuhin Kagaku Kogaku Kaishi. 46: 473-479

Sun, Z., Gantt, E. and Cunningham, Jr. F. X. 1996. Cloning and functional analysis of the β-catotene hydroxylase of *Arabidopsis thaliana*. *J. Biol. Chem.* 271(40):24349-24352

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Tee, E. S., and Lim, C. L. 1991 The analysis of carotenoids and retinoids: A review. Food Chemistry 41: 147-193

Verwoerd, T. C., Dekker, B. M. and Hoekema, A. 1989. A small-scale procedure for the rapid isolation of plant RNAs. *Nucleic Acids Res.* 17:2362

Wang, K. and Ohnuma, S. I. 1999. Chain-length determination mechnism of isoprenyl diphosphate synthases and implications for molecular evolution. *Trends Biochem. Sci.* 24(11):445-451

Whang, H-J. and Yoon, K-R. 1995. Carotenoid pigment of citrus fruits cultivated in Korea. Korean J. Food Sci. Technol. 27: 950-957

Zhu, C., Yamamura, S., Koiwa, H., Nishihara, M., and Sandmann, G. 2002. cDNA cloning and expression of carotenogenic genes during flower development in Gentiana lutea. *Plant Mol. Biol.* 48:277-285

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

ACKNOWLEDGEMENT

과학이라는 분야에 발을 들여놓은지 벌써 6년이 지났습니다.

너무나 빠른 시간이었습니다. 하지만, 많은 일이 있었습니다. 혼자서는 무엇을 어떻게 해야 할지 모르는 제가 이렇게 제 이름으로 논문을 쓰리라고는 이전에는 상상도 할 수 없었습니다. 이 모든 것이 하나님께서 저를 과학분야에 인도해 주신 덕분인 것 같습니다.

대학에 들어왔을 때, 서울에서 홀로 제주에 와서 무엇을 해야 할지, 어떻게 지내야 할지 막막했습니다. 외가댁이 제주도에 있다 하더라도, 가족과 떨어져서 홀로 지내야 한다는 생각이 저를 움츠려 들게 했습니다. 그런 저를 일찍이 유전공학 실험실로 불러주신 지도교수님이신 김찬식 교수님께 너무나도 감사를 드립니다. 교수님께서 저를 불러주셨기에 과학에 조금이나마 쉽게 접근할 수 있었고, 실험실에 속하게 됨으로써 혼자라는 생각이 제 머리에서 사라졌습니다. 실험실에 들어와서 고세광 선배와 고경철 선배를 만나서 대학생활을 배우고, 함께 생활을 하면서 마치 친형들처럼 따르게 되었습니다. 그리고 저와 같이 들어온 권민이도 절 친형처럼 따라주어서 생활이 더욱 즐거웠습니다.

실험실에서 생활을 하면서, 학과 교수님들께서 저를 알아보시는 것이 저에게는 너무나 즐거운 일이었습니다. 토양학을 가르쳐주신 현해남 교수님, 식품공학을 가르쳐주신 고정삼 교수님, 유기화학을 가르쳐주신 류기중 교수님, 비료학을 가르쳐주신 유장걸 교수님, 미생물학을 가르쳐주신 강순선 교수님께 깊은 감사를 드립니다. 그리고 학부 1,

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

2 학년때 조교선생님이 셨던 강봉천 선생님과 형수님께도 학과 학생 이상으로 대해주셔서 정말 감사합니다.

실험실에서 생활하다보니 학부 동기들보다 다른 실험실 대학원생 선배님들과 학과 선배를 먼저 알게 되었습니다. 그것이 저에게는 행운이었다고 생각합니다. 내성적인 성격이라서 학과 활동에는 많이 참여를 하지 못했는데, 다른 실험실 대학원 선배님들과 선배님들께서 마치 같은 실험실 식구처럼 대해 주셔서 저에게 큰 힘이 됐습니다. 식품공학 실험실, 유기화학 실험실, 비료학 실험실, 토양학 실험실 선배님들께 감사를 드립니다.

학과 활동에 거의 참여를 하지 않고, 실험실 생활만을 하고 있는 저에게 아무런 내색하지 않은 학부 졸업동기인 94 학번 선배님들께도 감사를 드립니다. 같은 학년 막내인 제가 학과 활동에 아무런 일도 하지 않고 있어도 웃으며 절 챙겨주시고, 막내 동생처럼 여겨주셔서 제가 학과 생활을 편하게 할 수 있었습니다.

학부과정을 졸업하고, 대학원에 진학을 해서 다시 김찬식 교수님의 지도를 받게 되면서 특별한 혜택을 받게 되었습니다. 대학원 생활의 대부분을 대전 한국과학기술원에 와서 생활하게 되었습니다. 저에게 있어서는 엄청난 행운이었습니다. 1 년 반이상을 과기원에서 지낼 수 있도록 허락해주신 생물과학과 식물분자생물학 실험실의 정원일 교수님께 깊은 감사를 드립니다. 그리고 저를 지도해 주신 김인중 박사님께도 감사를 드립니다. 아무것도 모르는 저를 데리고, 많은 것을 가르쳐주시고, 편히 생활할 수 있도록 지도해 주셔서 감사합니다. 그리고 장예리 형수님과 예인이에게도 감사를 드립니다.

제주대학교 중앙도서관

과기원 실험실에서 바쁘게 생활을 하면서 과학이라는 분야에 더욱 매력을 느낄 수 있었습니다. 그리고 실험실에서 보여주신 선후배님들의 생활은 제 자신을 반성하게 하는 계기가 되었습니다. 자유로우면서도 자신이 하는 일은 확실히 하고, 자기 발전을 위해서 노력하는 모습이 너무나도 좋았습니다. 지금은 미국에 계시지만 항상 새로운 것을 찾고, 연구를 하시는 권창섭박사님, 큰 형님 같으신 박정일 선배님, 생명공학연구원에 계시는 노승재 선배님, 사물놀이를 좋아하시는 박정무 선배님, 소녀 같은 이영경 선배님, 탱고를 즐기시는 오동하 선배님, 농구를 좋아하는 이우용, 조용한 윤준선, 항상 바쁜 약사 정종현, 또래 친구로 생활에 활력을 주고 지금은 은행에 취직한 친구 주정은, 애경에 다니는 김준, 식물생명공학회 사무보랴 실험도우랴 바쁜 박은애, 모두에게 깊은 감사를 드립니다.

과기원에 와 있다는 핑계로 도움도 못주고, 너무나 많은 것을 떠넘기고 왔는데도 확실하게 해주고 있는 유전공학 실험실 친구 허지만과 막내 김유왕에게도 감사를 드립니다. 소속만 대학원생인 저를 챙겨주시는 고석형 조교선생님께도 감사를 드립니다. 친 동생처럼 때론 친구처럼 대해주고 따라준 정희성, 정승원, 오은혜, 강혜림, 현재희에게도 감사를 드립니다. 제주도에서 생활을 잘 하도록 돌봐주신 외할머니, 외할아버지께 감사를 드리고, 이모와 이모부님께도 감사를 드립니다.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

제가 쓴 논문을 보여드릴 수는 없지만, 항상 제 곁에 계시고, 언제나 웃는 얼굴로 절 보시는 하늘에 계시는 어머니께 깊은 감사를 드립니다. 어머니의 정성과 응원이 없었다면 지금 이런 글을 쓸 수 없었을 것입니다. 맏아들로써 제대로 효도도 못했는데 언제나 챙겨주시고, 걱정해주시고, 많은 것을 저와 의논해 주신 아버지께도 깊은 감사를 드립니다. 좋은 논문을 쓸 수 있도록 힘이 되어준 저희 식구에게 깊은 감사를 드립니다.

지금까지 도와주신 모든 분들에게 깊은 감사를 드립니다.

2002 년 12 월 17 일 모두 깊은 잠에 든 새벽에....

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

"가라사대 너희 믿음이 적은 연고니라 진실로 너희에게 이르노니 너희가 만일 믿음이 한 겨자씨 만큼만 있으면 이 산을 명하여 여기서 저기로 옮기라 하여도 옮길 것이요 또 너희가 못할 것이 없으리라 (마태복음 17 장 20 절)"