A DISSERTATION
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Cloning of Genes for Carotenoid Biosynthetic Pathway from Rapeseed and Overexpression of Its β-Carotene Hydroxylase in Transgenic Tobacco

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Department of Food Science and Engineering

GRADUATE SCHOOL

CHEJU NATIONAL UNIVERSITY

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ABSTRACT

Plant carotenoids are red, orange, and yellow lipid-soluble pigments embedded in the membrane of chloroplasts and chromoplasts of many flowers, fruits, and the carrot root in late stages of plant development. Carotenoids are essential components of higher plant photosystems, and function as photoprotection, substrates of abscisic acid synthesis, and scanvengers of reactive oxygen.

A rapeseed (*Brassica napus* L.) is a member of the *Brassica* genus and is an indigenous cultivar in Jeju Island. Also it has been cultivated as an oil source and ornamental purpose. The bright-yellow color of rapeseed flower is rich in carotenoids derived from isoprenoids. Rapeseed is an good source of carotenoids and can be used as a model studies on pigmentation and useful compounds. Carotenoids in particular have also been the target of numerous biotechnological efforts with the objective of creating new, or altering the properties of existing, coloring compounds. The focus of this study is to examine the biosynthesis, regulation and contribution of flower coloration of carotenoids in rapeseed.

Understanding of carotenoid biosynthetic pathway in plants has been advanced greatly in the past decade, mainly due to cloning of many of the genes for enzymes involved in the pathway. Total number of 11 genes were cloned in this experiment. They were HMG CoA reductase (HMG-R), 1-deoxy-D-xylulose-5-phosphate synthase (DXPS), Geranylgeranyl pyrophosphate synthase (GGPPS), phytoene synthase (PSY), phytoene desaturase (PDS), lycopene

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β-cyclase (LCY-b), lycopene ε-cyclase (LCY-e), β-carotene hydroxylase (β-CHX), ε-carotene hydroxylase (ε-CHX), zeaxanthin epoxidase (ZEP), and glyceraldehyde 3 phosphate dehydrogenase (GAPDH). To elucidate the regulation steps for carotenoid synthesis in *Brassica napus*, nine genes (*DXPS, GGPS, PSY, PDS, LCY-b, LCY-e, β-CHX, ε-CHX, and ZEP*) participating in xanthophyll accumulation were investigated. The full-length cDNAs of genes for enzymes involved in the carotenoid biosynthetic patyway (*DXPS, PSY, LCY-b, LCY-ε, β-CHX, ε-CHX*) were cloned by RACE, and confirmed by nucleotide sequencing. The predicted amino acid sequences showed 73 to 94% homology with the reported *Arabidopsis* sequences.

In addition, the concentration and composition of carotenoids and the expression of carotenoid biosynthetic genes were investigated in the different tissues of *Brassica napus*. Carotenoids were extracted from leaves, petals, and flower bud of rapeseed and the compounds were analyzed by HPLC. Molecular weights of these compounds were also measured by LC/MS. Thus, identification of carotenoids was conducted. Northern blot analysis revealed differenential expression patterns between the different carotenoid genes. The expression of *PSY* gene was detected in leaves, petals and flower buds. All genes involved in the formation of β-carotene and its derived xanthophylls exhibited a high steady-state mRNA transcript level in leaves and flower buds. The amount of zeaxanthin epoxidase mRNA showed no variation between different tissues.

The important enzymatic reaction steps for xanthophyll biosynthesis are hydroxylation reactions carried out by two types of carotene hydroxylase. One is the β-ring hydroxylation catalyzed by β-*CHX*, which is responsible for the

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formation of zeaxanthin, violaxanthin, and neoxanthin. The other is ε-ring hydroxylation catalyzed by ε-*CHX*, that leads to the formation of lutein. Further modification by zeaxanthin epoxidase produces violaxanthin that is finally converted to neoxanthin by neoxanthin synthase.

This study aimed to show the function and the effects of the constitutive expression of *Br* β-*CHX* gene in transgenic tobacco plants. A full-length cDNA clone encoding β-*CHX* was isolated from *Brassica napus* cv Halla, and the gene was introduced into tobacco (*Nicotana tobaccum* cv. nc) by *Agrobacterium* mediated transformation. The cDNA clone termed *Br* β-*CHX* showed high sequence homology with β-*CHX* genes from various plant species and encoded a protein for 304 amino-acid residues with predicted molecular weight of 33.4 kDa. Fifty of tobacco transformed with β-*CHX* were selected and analyzed. The expression of the *Br* β-*CHX* were identified by genomic DNA PCR, RT- PCR and Northern blot analysis. Also, the changes of carotenoid composition were analyzed by HPLC. Expression of β-CHX showed increasement of violaxanin and neoxanthin. However, contents of lutein and β-carotene had a tendency to decrease in transgenic tobacco. These results suggest that β-CHX is an important enzyme in the down stream regulation in xanthophyll biosynthetic pathway.

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ABBREVIATION

ABA	abscisic acid
β-CHX	β-carotene hydroxylase
ε-CHX	ε-carotene hydroxylase
DDW	double distilled water
DEPC	diethylpyrocarbonate
DXP	1-deoxy-dxylulose 5-phosphate
DXPS	1-deoxy-d-Xylulose 5-Phosphate Synthase
DXR	1-deoxy-d-Xylulose 5-Phosphate reductoisomerase
G-3-P	glycerol-3-phosphate
GGPPS	geranylgeranyl pyrophosphate synthase
GPPS	geranyl pyrophosphate synthase
HMG-CoA	3-hydroxy-3-methylgluatryl coenzyme A
HMGR	HMG-CoA reductase
HMGS	HMG-CoA synthase
HPLC	high-performance liquid chromatography
IPI	isopentenyl diphosphate isomerase
IPP	isopentenyl diphosphate
LC-MS	liquid chromatographymass spectrometry
LCY-b	lycopene β-cyclase
LCY-e	lycopene ε-cyclase
MEP	C-methyl-D-erythritol 4-phosphate
MOPS	morpholinepropanesulfonic acid
MVA	acetate/mevalonate
MVK	MVA kinase
PCR	polymerase chain reaction
PDS	phytoene desaturase
PSY	phytoene synthase
RT-PCR	reverse-transcriptase-PCR
UV	ultraviolet
VED	violaxanthin de-epoxidase
ZDS	ζ-carotene desaturase
ZEP	zeaxanthin epoxidase

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PART I

Cloning of Genes for Carotenoid Biosynthetic Pathway in Rapeseed (*Brassica napus* L.)

I. Introduction

1. Research Background

Carotenoids are usually C_{40} tetraterpenoids built from eight C_5 isoprenoid units and are synthesized by all photosynthetic organisms (plant, algae and cyanobacteria) and some non-photosynthetic bacteria and fungi.

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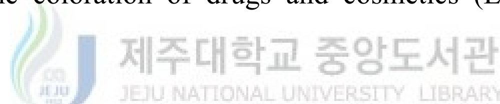
Plant carotenoids are red, orange, and yellow lipid-soluble pigments embedded in the membrane of chloroplasts and chromoplasts of many flowers and fruits and the carrot root in late stages of plant development. In these tissues they accumulate in chromoplasts and render bright yellow, orange or red colours that attract animals which facilitate pollination and seed dispersion.

Carotenoids are essential components of higher plant photosystems, and their composition in photosynthetic plant tissues. Carotenoids are multifunctional compounds serving as structure components of light-harvesting complexs, accessory pigments, substrates of abscisic acid synthesis, and components of photoprotection and scarvengers of singlet oxygen. There are two types of carotenoids-carotenes, which are nonoxygen-containing pigments, and xanthophylls, which contain oxygen in their molecular structure.

Carotenoids making β-carotene with provitamin A activity are essential components

of the human diet, and there are considerable evidences that many carotenoids have anti-cancer activity possibly by serving as antioxidants and free radical scavengers (Demmig-Adams and Adams, 1996; Fraser and Bramley, 2004). A number of plant carotenoids and derived compounds (apocarotenoids) have industrial and nutritional value based on their colorant and aromatic properties, and their ability to act as natural antioxidants that help to prevent some types of human cancer and degenerative diseases. Carotenoids absorbed through the diet, and often metabolized into other compounds, are responsible for the color of familiar animals such as lobster, flamingo, and fish. Currently, the xanthophylls, lutein and its stereoisomer zeaxanthin as well as astaxanthin, are used as neutraceuticals against macular degeneration. Lutein and zeaxanthin are known to play a critical function in maintaining a normal visual function. Lutein is also an important xanthophyll that creates the pigmentation in fish and poultry, and for the coloration of drugs and cosmetics (Eonseon et al., 2003).



Rapeseed (*Brassica* and related species, Brassicaceae) is a member of the *Brassica* genus of plants and is now second largest oilseed crop in the world providing 13% of the world's supply. Canola refers to cultivars of rapeseed that produce seed oils with less than 2% erucic acid (22:1) and meals with less than 30μmol of aliphatic glucosinolateds per gram. The oil from improved varieties of rapeseed has important potential in the human diet as a cooking oil, while the oilmeal can be important source of protein for animals. Specialty canola refers to canola-quality cultivars with improved oil profiles for use in high temperature. Specialty canola cultivars normally produce oils that contain less than 4% linolenic acid (18:3) and/or greater than 70% oleic acid (18:1). Oils from these cultivars have greater temperature stability and improved shelf life.

Rapeseed (*Brassica napus* L.) is an important plant, serving as a source of vegetable oil. It is appealing to health-conscious consumers because it has the lowest saturated fat content of all major edible vegetable oils. Also it has many industrial applications as food and feed

additives, in agricultural, cosmetics, pharmaceuticals, and ornamental applications. The term "industrial rapeseed" refers to rapeseed cultivars that produce oils with 45% or more erucic acid and seed meals that are either high or low in glucosinolates. Cultivars with these characteristics are used primarily for non-edible purposes such as lubricants and hydraulic fluids (Raymer, 2002).

The major groups of pigments, the betalanins, the carotenoids and the anthocyanins are responsible for the attractive natural display of flower colors. Anthocyanins synthesized as part of the flavonoid pathway among the flowering plants, their biosynthesis and regulation are best understood. However, over the past few years, significant progress has been made in undrestanding the biosynthesis and participation of carotenoids derived from isoprenoids in flower pigmentation. These pigments play important ecological functions , for example in the attraction of pollinating animals.

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2. Literature reviews and Objectives

The structures of common plant carotenoids and a simplified biosynthetic pathway are shown in **Fig 1.** In plant, Carotenoids are synthesized within the plastids from the central isoprenoid pathway (Cunningham and Gantt, 1998; Hirschberg, 2001; Laule et al., 2003). Isoprenoids are a large group of compounds which derive from a common building unit, isopentenyl pyrophosphate (IPP). Plant synthesize IPP and dimethylallyl pyrophosphate (DMAPP) by two independent pathway : the mevalonic acid (MVA) pathway, which produces cytosolic IPP, and the plastidial methylerythritol phosphate (MEP) pathway (Eisenreich et al., 2001; Lichtenthaler, 1999)

IPP biosynthesis The classical Ac-MVA pathway involves condensation of three units of acetyl CoA to form 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA), which is

reduced to MVA. The reduction of HMG-CoA to MVA is catalyzed by HMG-CoA reductase (HMGR), a key regulatory enzyme of this pathway that has been extensively studied (Rodwell et al., 2000). The cytosolic pathway, which starts from acetyl-CoA and proceeds through the intermediate mevalonate (MVA), provides the precursors for sterols and ubiquinone. The platidial MVA-independent pathway, which involves a condensation of pyruvate and glyceraldehyde-3-phosphate via 1-deoxy-D-xylulose-5-phosphate as a first intermediate, is used for the synthesis of isoprene, carotenoids, abscisic acid, and the side chains of chlorophylls and plastoquinone (Laule et al., 2003)

The initial reaction of the non-MVA pathway involves the formation of 1-deoxy-D-xylulose 5-phosphate (DX5P) by condensation of (hydroxyethyl) thiamin derived from the decarboxylation of pyruvate with the C1 aldehyde group of D-glyceraldehyde 3-phosphate (Arigoni et al., 1997). The reaction is catalyzed by deoxyxylulose 5-phosphate synthase (DXPS). In plants, it has been shown that 1-deoxy-D-xylulose can be incorporated into carotenoids, plastoquinone and the phytol moiety of chlorophylls.

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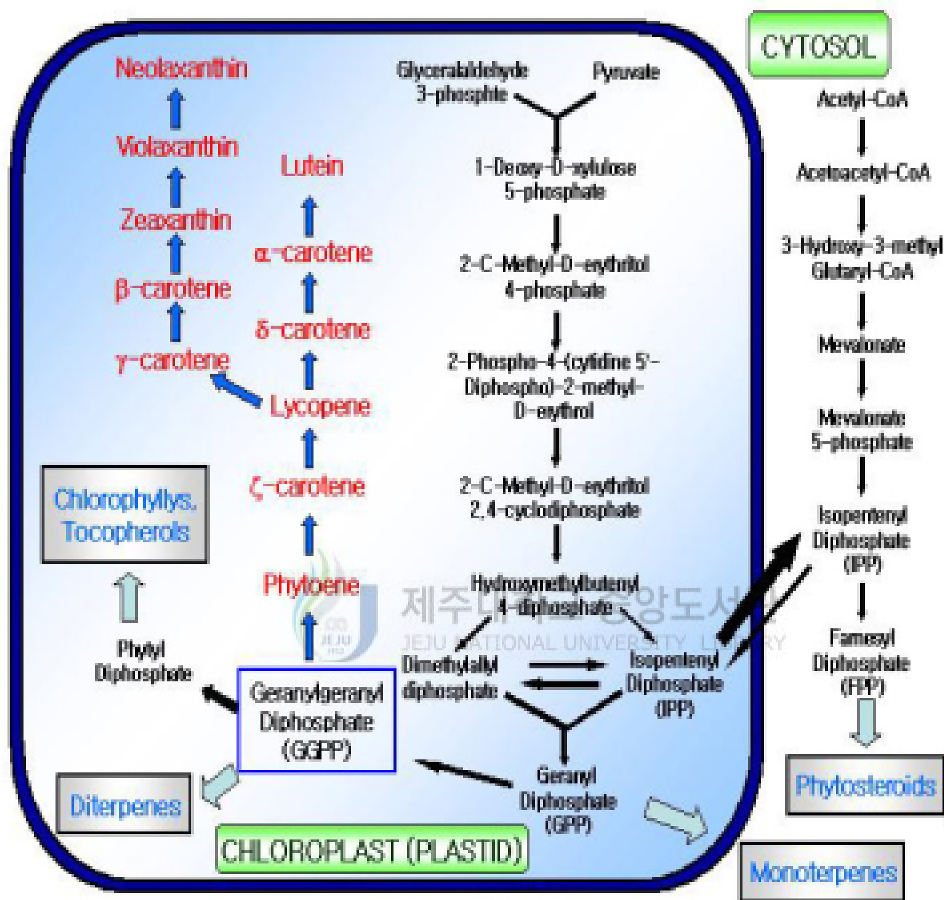

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Fig. 1. Overview of isoprenoid metabolic pathways localized to the cytosol and to plastids in plants (modified by Laule et al., 2003).

Carotenoid biosynthetic genes and enzymes Carotenoids are generally C_{40} terpenoid compounds formed by the condensation of eight isoprene units. Carotenes are hydrocarbons that are either linear or cyclized at one or both ends of the molecule, and xanthophylls are formed by the introduction of various oxygen functions to carotenes (Goodwin, 1980). The most prominent chemical feature of the carotenoids is the polyene chain, consisting of 3~15 conjugated double bonds, which is responsible for the characteristic absorption spectrum and therefore the colour of the carotenoid, and the photochemical properties of the molecule (Britton, 1995).

The committed step to carotenoid synthesis is the formation of the first C_{40} compound phytoene by condensation of the two geranyl geranyl diphosphate (GGDP) by phytoene synthase. Phytoene is subjected to a series of sequential desaturation reactions, by phytoene desaturase (PDS) and the other two separate enzymes, to yield lycopene. Lycopene is then cyclized to β-carotene by two β-carotene cyclization or to α-carotene one β- and one ε-cyclization (**Fig. 2**). The two types of rings are produced by distinct enzymes, the lycopene β-cyclase and lycopene ε-cyclase. The formation of ε-rings and the productions of β-, ε-carotenoids (α-carotenes derivatives) are two of the key differences distinguishing carotenoid biosynthesis in plants from that in cyanobacteria, fungi, and bacteria. α-Carotene serves as the precursor of hydroxylation to lutein, the most abundant carotenoid in green plants. β-Carotene is subjected to a series of hydroxylation and epoxidation reactions to yield the other common leaf xanthophylls; zeaxanthin, anthraxanthin, violaxanthin, and neoxanthin. The latter two are precursors for the synthesis of the hormone abscisic acid (ABA). α- and β-carotene subsequently are further processed to different xanthophylls, such as lutein, violaxanthin, and zeaxanthin. Lutein is a major xanthophyll in the light-harvesting system of most higher plants.

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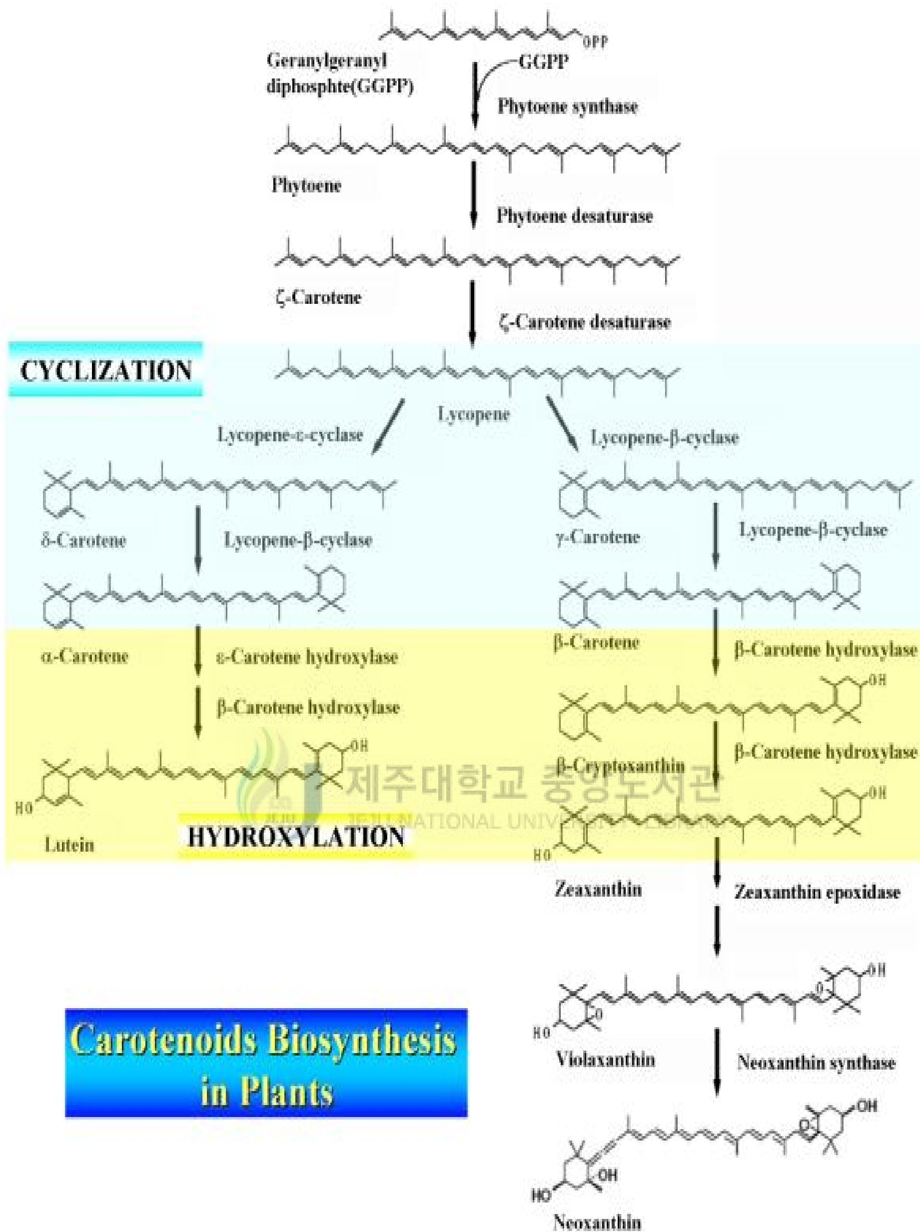

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Fig. 2. Carotenoids biosynthetic pathway in higher plants (modified by Tian et al., 2003).

In the first step of the pathway, the enzyme geranylgeranyl pyrophosphate synthase (GGPPS) catalyzes three successive condensation reactions, the first of them being the condensation of isopentenyl pyrophosphate and dimethylallyl pyrophosphate (DMAPP) to form geranylgeranyl pyrophosphate (GGPP). *GGPPS* gene has been purified from pepper, arabidopsis, and different organism and was shown to catalyze the corresponding reactions in vitro. In addition, GGPP is a precursor of other plant compounds such as chlorophyll, phylloquinones, plastoquinones, taxol, tocopherols, gibberellic acid and diterpenes. Therefore, it is likely that the synthesis of this branch compound is subject to complex regulation and possibly, compartmentalization.

In the next step of carotenoid biosynthesis, the enzyme phytoene synthase (PSY) catalyze the two-step conversion of GGPP into prephytoene pyrophosphate (PPPP) and then into colorless intermediate phytoene. In ripening fruits of tomato, transcription of PSY was found to be up-regulated (Welsch et al., 2000). PSY is a branching enzyme that directs substrates irreversibly to carotenoids. Hence, it has been a target in several genetic manipulation studies. Expression of antisense RNA to the *PSY* gene (*psy1*) of tomato was found to reduce the accumulation of carotenoids in fruits by 97%, without noticeable effect on carotenoids in leaf tissues (Ray et al., 1992). However, the levels of gibberellins and other isoprenoids were perturbed in these plants (Fraser et al., 1995). Overexpression of *PSY* in tomato resulted in carotenoid-rich seed coats, cotyledons, and hypocotyls. However, plants were reduced in stature because of changes in gibberellic acid due to competition for prenyl pyrophosphates by both pathways. This report illustrates how problems arise when interfering with a well-balanced metabolism (Fraser et al., 1995).

However, the genetic manipulation of rapeseed (*B. napus*) using a bacterial *PSY* gene (*crtB*) to increase carotenoid content to high levels was a success. *crtB* was overexpressed in a seed-specific manner and the protein product targetted to the plastid.

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The embryos from the transgenic plants were visibly orange and mature seeds increased by 50-fold in carotenoids (Shewmaker et al., 1999).

The four sequential desaturaions from phytoene to lycopene are catalysed by two related enzymes in plants: PDS and ZDS. This enzymes create additional double bonds in the C_{40} backbone of phytoene, thus converting this colourless carotenoid into a coloured compound, lycopene. The tomato PDS cDNA was cloned by a similar approach and its identity was confirmed by expression in *Escherichia coli*, which resulted in the formation of ζ-carotene (Pecker et al., 1992). cDNA encoding *ZDS* have also been identified in *Arabidopsis* and pepper. Both these *PDS* and *ZDS* clones isolated so far in plants have N-terminal transit peptides sequences for plastid targetting.

Manipulation of desaturation activity in plants also produced transgenic plants with increased β-carotene contents. *Erwinia uredovora* PDS gene (*crtI*) was introduced into tomato under the control of CaMV 35S pormoter by *Agrobacterium*-mediated transformation. β-Carotene in crtI-transformed tomato fruit increased three-fold, up to 45% of the total carotenoid content. Since the end-product by CRT1 activity is lycopene, an increase was reported in lycopene (Misawa et al., 1993).

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The ends of the linear carotenoid lycopene can be cyclized and various oxygen functions introduced to form the xanthophylls. The cyclzation of lycopene is a crucial branching point in this pathway, yielding α-carotene with one ε-ring and one β-ring and β-carotene with two β-rings, in which two cyclases, namely, lycopene β-cyclase (LCY-b) and lycopene ε-cyclase (LCY-e), are responsible for these reactions (Cunningham et al., 1996).

Lycopene β-cyclase catalyses the formation of the bi-cyclic β-carotene from

lycopene in plants and cyanobacteria. This enzyme introduces two β-rings at the ends of the linear lycopene molecule. cDNA encoding lycopene β-cyclase has been cloned from *C. annuum* and its corresponding gene was found to be constitutively expressed during fruit development (Hugueney et al., 1995). The cDNA which encodes lycopene β-cyclase (*crtL*) was cloned from tomato and tobacco and functionally expressed in *E. coli* (Pecker et al., 1996). mRNA levels of CRTL were found to decrease in ripening fruits of tomato at breaker stage. This transcriptional down-regulation of lycopene β -cyclase leads to accumulation of lycopene in tomato during ripening.

Overexpression of lycopene β-cyclase in a fruit- specific manner, was performed in tomato. Fruits of three transformants showed a significant increase in β-carotene content and displayed different colour phenotypes, from orange to red, depending on the lycopene/β-carotene ratio (Rosati et al., 2000).

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Xanthophyll biosynthetic genes Xanthophyll are oxygenated carotenoids that perform a variety of critical roles in photosystem structure and assembly, light harvesting, and photoprotection (Demming-Adams et al., 1996; Horton et al., 1996). The xanthophyll content of photosynthetic plant tissues is highlyly conserved through evolution, with lutein, β-carotene, neoxanthin, and violaxanthin. Zeaxanthin and antheraxanthin accumulate to high levels in response to strong light stress. Lutein and zeaxanthin are xanthophylls that are derived from α-carotene and β-carotene, respectively.

α-Carotene is converted into lutein by sequential hydroxylations, which are catalyzed by ε-ring hydroxylase and β-ring hydroxylase. β-Carotene is converted to zeaxanthin by two-step hydroxylation, which is catalyzed by hydroxylase.

Zeaxanthin is widespread in bacteria, fungi, and plants. β-CHX has been cloned and characterized from all three phyla. All are nonheme di-iron oxidases that contain conserved His motifs required for activity (Bouvier et al., 1998). However, enzymes

from the three phyla have low protein identity and are thought to have evolved independently, but they efficiently catalyze the hydroxylation of both β-rings of β-carotene to form zeaxanthin (Misawa et al., 1990; Hundle et al., 1993; Bouvier et al., 1998; Masamoto et al., 1998).

The formation of lutein from α-carotene requires the action of the second hydroxylase, the ε-CHX, which has not been cloned from any organism, but it has been identified genetically in *Arabidopsis* (Pogson et al., 1996). Two genes that encode β-*CHX* (β-*CHX* 1 and 2) are present in the *Arabidopsis* genome (Sun et al., 1996; Tian and DellaPenna, 2001). The predicted mature proteins share 81% protein identity and are expressed coordinately. When expressed in vitro, both β-CHX are highly acive toward β-rings and function poorly with ε-ring-containing substrates. Mutational studies also have identified the LUTEIN1 (LUT1) locus as being essential for the hydroxylation of ε-rings in *Arabidopsis*. Plants homozygous for the LUT1 mutation showed 80% reduction in lutein levels and accumulation the immediate monohydroxy precursor zeinoxanthin. This means LUT1 defines a novel class of carotenoid hydroxylase enzymes. More recently, the β-*CHX* 1 gene was constitutively overexpressed in both the sense and antisense orientation in leaf tissue of *Arabidopsis*. Overexpression resulted in a twofold increase in xanthophyll cycle carotenoids (Violaxanthin +Antheraxanthin + Zeaxanthin) without affecting lutein levels (Davison et al., 2002). Expression of a β-*CHX 1* antisense construct reduced the levels of violaxanthin and neoxanthin in leaf tissue without affecting lutein levels. However it was unclear that which of the closely related *β-CHX* genes were affected and to what levels (Rissler and Pogson, 2001).

Furthermore, zeaxanthin is converted to violaxanthin via antheraxanthin by zeaxanthhin epoxidase (ZEP). Zeaxanthin can be synthesized in two ways by hydroxylation of β-carotene or by deepoxidation of violaxanthin. In the first case, zeaxanthin formation is catalyzed by the enzyme β-carotene hydroxylase using β

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-carotene as substrate. In the second case, violaxanthin is converted to zeaxanthin by the enzyme zeaxanthin deepoxidase under low-light conditions (Woitsch and Romer, 2003).

Carotenoids biosynthesis and its regulation have been reported in various plant species, such as *Arabidopsis* (Pogson et al., 1996; Park et al., 2002), tomato (Giuliano et al., 1993; Fraser et al., 1994; Ronen et al., 1999; Isaacson et al., 2002), pepper (*Capsicum annuum*), (Bouvier et al., 1996, 1998), tobacco (*Nicotiana tabacum*), Busch et al., 2002) and algae (Steinbrenner and Linden, 2001).

Some of the plant-originated genes involved in plant carotenoids biosynthesis gene are given in **Table 1.** Other enzymes involved in the conversion of β-carotene and α -carotene to xanthophyll pigments, such as hydroxylases, epoxidase, de-epoxidase, and epoxy-carotenoid cleavage enzymes, have been studied and their cDNAs or genomic sequences have been identified (Cunningham, 1998).

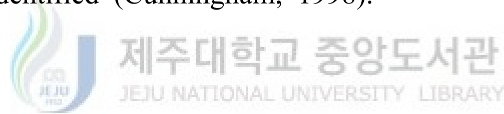

Table 1. Carotenoid biosynthetic genes cloned in plants

Enzymes	Plant source	Gene	Clone type	GenBank accession number
GGPPS	*A. thaliana*	*GGPS2*	cDNA	U44876
		GGPS2	cDNA	U44877
	Catharanthus roseus	*GGPS*	Genomic	X92893
	Capsicum annum	*GGPS*	cDNA	P80042
	Nicotiana tabacum	*GGPS1*	cDNA	AB041632
		GGPS2	cDNA	AB041633
PSY	*A. thaliana*	*PSY*	cDNA	AY056287
	C. annum	*PSY*	cDNA	X68017
	Helianthus annus	*PSY*	cDNA	AJ308385
		PSY	cDNA	AJ304825
	L. esculentum	*PSY1*	Genomic	X60441
		PSY1	cDNA	M84744
	Narcissus psedonarcissus	*PSY*	cDNA	X78814
PDS	*C. annum*	*PDS*	cDNA	X68058
	Glycine max	*PDS*	cDNA	M64704
	Hordeum vulgare	*PDS*	cDNA	AY062039
	L. esculentum	*PDS*	Genomic	U64919
	Zea mays	*PDS*	cDNA	U37285
ZDS	*L. esculentum*	*ZDS*	cDNA	AF195507
	Z. mays	*ZDS*	cDNA	AF047490
LCY-b	*A. thaliana*	*LCY-b*	Genomic	AF117256
		LCY-b	cDNA	AY091396
	L. esculentum	*LCY-b*	cDNA	AF254793
		LCY-b	cDNA	X86452
	N. pseudonarcissus	*LCY-b*	cDNA	X98796
	Z. mays	*PS-1*	Genomic	AY206862

(continued)

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Table 1. (Continued)

Enzymes	Plant source	Gene	Clone type	GeneBank accession number
LCY-e	*Arabidopsis*	*Lcy-e*	cDNA	U50738
	Tomato	*Lcy-e*	cDNA	Y14387
	Potato	*Lcy-e*	cDNA	AF321537
	Citrus maxima	*Lcy-e*	cDNA	AY994158
	Citrus sinensis	*Lcy-e*	cDNA	AF450280
	Citrus Paradisi	*Lcy-e*	cDNA	AF486650
β-CHX	Pepper	*Bch*	cDNA	Y09225
	Pepper	*Bch2*	cDNA	Y09722
	Arabidopsis	*Chyb1*	cDNA	U58919
	Tomato	*CrtR-b1*	cDNA	Y14809
	Tomato	*CrtR-b2*	cDNA	Y14810
	Citrus maxima		cDNA	DQ002893
	Citrus sinensis		cDNA	AY623047
	Citrus unshiu	*CHX 2*	cDNA	AF315289
ZEP	Pepper	*ZEP*	cDNA	X91491
	Arabidopsis	*ZEP*	cDNA	AF281655
	Tobacco	*ZEP*	cDNA	X95732
	Tomato	*ZEP*	cDNA	Z83835
	Citrus sinensis	*ZEP*	cDNA	AF437874
	Citrus unshiu	*ZEP*	cDNA	AB114654
VDE	Tobacco	*Vde1*	cDNA	U34817
	Arabidopsis	*Vde1*	cDNA	U44133
	Lettuce	*Vde1*	cDNA	U31462
	Citrus sinensis	*Vde1*	cDNA	AF444297

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Regulation of carotenoid biosynthetic pathway Mevalonic acid (MVA) derived cytosolic precursors might contribute to the production of carotenoids only during specific developmental stages such as in etiolated seedlings, wherease plant carotenoids synthesized in chloroplasts and chromoplasts are derived mostly from the MEP pathway (Lichtenethaler, 1999; Rodriguez-Concepdion et al., 2004).

In the MEP pathway, only 1-deoxy-D-xylulose 5-phosphate synthase (DXPS) appears to be limiting for carotenoid biosynthesis during tomato fruit ripening (Lois et al., 2000). The limiting nature of the reaction catalysed by DXPS for the biosynthesis of plastidial isoprenoids was also confirmed in photosynthesis tissues by constitutive overexpression of the corresponding gene in *Arabidopsis* (Estevez et al., 2001).

Bramley (2002) reviewed carotenoids biosynthesis and regulation during ripening and development in tomato fruit. During tomato fruit ripening, the expression of *PSY* and *PDS* increased (Giuliano et al., 1993; Fraser et al., 1994; Ronen et al., 1999; Isaacson et al., 2002), wherease the expression of both *LCY-b* and *LCY-e* disappeared (Pecker et al., 1996; Ronen et al., 1999), leading to massive accumulation of lycopene.

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In satsuma mandarin, the gene expression of *PSY* increased in the peel and juice sacs with the onset of coloration (Ikoma et al., 2001; Kim et al., 2001) wherease the gene expression of *PDS* and β-carotene hydroxylase remained constant once fruit was fully developed (Kita et al., 2001).

During flower development, the total carotenoid pathway is upregulated at the transcriptional level. Enhanced expression of the *PSY* gene is a major factor. This has been demonstrated for petals from tomato (Giuliano et al., 1993), marigold (Moechs et al., 2001) and *Gentiana lutea* (Zhu et al., 2002)

Objectives of this study Undrestanding of the carotenoid biosynthetic pathway in plants has been advanced greatly in the past decade, mainly due to cloning of many of the genes for enzymes involved in the pathway. Also many plant isoprenoids have been shown to have industrial and medical importance. The β-carotene and α-tocopherol are both basic nutrients required for the maintenance of human health. Industrial uses of isoprenoids include products such as colorants, fragrances, and flavorings. A detailed understanding of isoprenoid biosynthetic pathways and their regulation are essential to fully exploit these and future uses of isoprenoids.

Rapeseed is an good source of carotenoids and can be used as a model studies on pigmentation and useful compounds. Carotenoids in particular have also been the target of numerous biotechnological efforts with the objective of creating new, or altering the properties of existing, coloring compounds. The focus of this study is to examine the biosynthesis, regulation and contribution of flower coloration of carotenoids in rapeseed.

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To elucidate the regulation steps for carotenoid synthesis in *Brassica napus*, nine genes (*DXPS, GGPPS, PSY, PDS, LCY-b, LCY-e, β-CHX, ε-CHX,* and *ZEP*) participating in xanthophyll accumulation were investigated. The cDNAs related to linear carotenoid biosynthesis (*PSY* and *PDS*), the cDNAs related to cyclization (*LCY-b* and *LCY-e*) and the cDNAs related to hydorxylation and epoxidation (*β-CHX, ε-CHX*, and *ZEP*) were isolated from *B. napus* leaves and petals.

In this work, the cloning and expression analysis of many of genes required for carotenoid biosynthesis in rapeseed are described. This study is to provide some insight into the organization and regulation of carotenoid biosynthesis in rapeseeds. In addition, the concentration and composition of carotenoids were investigated in the different tissues of *Brassica napus*. Carotenoids were extracted from rapeseed and were analyzed by HPLC. Molecular weights of these compounds were also measured by LC/MS. Thus, identification of carotenoids was conducted.

II. Materials and Methods

1. Plant materials

Rapeseed (*Brassica napus cv.* Halla) were provided by the Mokpo Experimental Station of National Institute of Crop Science. Seeds were sown into pots and plants were grown under common culture condition in the greenhouse. Mature leaves, fully developed flowers, and immature green flower buds were harvested and used for RNA isolation and carotenoid analysis in six months after seedling.

2. Bacterial strains and plasmids

E.coli XL1-Blue was used as a host for subcloning. The F' in XL1-Blue allows blue/white screening on X-gal.

pGEM-T easy (Promega, USA) and yT&A (Intron, Korea) that are T/A-type PCR cloning vectors were used for cloning of polymerase chain reaction (PCR) product. Forex-T vector (Takara.co.kr) were used for protein expression in *E. coli*.

3. Cloning of carotenoid biosynthetic genes

3-1. Isolation of total RNA from plant

Total RNA was isolated from different tissues including leaves, petals and flower buds of the rapeseed. The plant tissues frozen in liquid N_2 were homogenized using mortar and pestle and RNAs were extracted using the TRIzol Reagent (Mol. Bio. Co. Ltd) according to the manufacturer's instruction.

Reverse transcription was carried out using M-MuLV reverse transcriptase (MBI). A 20 ㎕

reaction volume was used for 1-5μg of total RNA.

3-2. Polymerase chain reaction (PCR) amplification

PCRs were carried out using Taq DNA polymerase (Takara, Japan). Thermal cycling was performed using following program in the Peltier Thermal Cycler-200 (MJ Research, USA) .

Degenerate primers were designed by using CODEHOP program (http://bioinformatics.weizmann.ac.il/blocks/codehop.html) comparing three protein sequences of *Arabidopsis*. Template was performed with degenerated primers that corresponded to the conserved amino acid sequence of individual carotenogenic enzymes.

3-3. Generation of full-length cDNA

Full-length cDNAs were generated by RACE (Rapid Amplification of cDNA Ends). And their products were characterized by partial sequencing. 5' and 3' RACEs were performed using Gene Racer Kit (Invitrogen) according to the supplier's instruction. The each PCR product containing full-length cDNA was cloned into the pGEM-T easy (Promega, USA) and the resulting fragments were sequenced and assembled according to the overlapping sequences.

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A full-length cDNA of the β-*CHX* gene was isolated by RT-PCR with primers (**Table 2**). These primers were designed on the basis of the conserved regions of the previously reported β-*CHX* sequence (Accession No. AY545229).

The rapeseed genes, *HMG-R, DXPS, GGPS, PSY, PDS, LYC-b, LYC-e, ε-CHX,* and *ZEP* were cloned by amplification of a partial coding sequence using degenerate primer in **Table 3.**

Nucleotide and amino acid sequences analyzed with NCBI (http://www.ncbi.nlm.nih.gov/BLAST/), ExPASY (http://us.expasy.org/tools), and CAP3 sequence assembly program (http://pbil.univ-lyon.fr/cap3.php).

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

Table 2 . Primer sequences for cloning of carotenoid biosynthetic genes

Primer	Primer sequence (5'-3')	Tm (℃)	Expected PCR product (bp)
Brβ-*CHX*-UP(SN1)	ATGGCGGCAGGTCTCTCAACCAC	65.1	900
Brβ-*CHX*-DN(EN901)	TCAAGAGCTCGAACTCTTTTTGTA	54.2	
Br-*GGPS*-UP	ATGGCTTCTTCAGTGACTCTAGG	53.3	1200
Br-*GGPS*-DN	TTTCAGTTCTGTCTATTGGCAATG	54.9	
Brβ-*CHX*-UP(SN498)*	TCACATCACAAACCAAGAGAAGGC	59	150
Brβ-*CHX*-DN(EN633)*	GGCGCCAAAGCAGAGACCAGG	64.7	
Br*GAPDH*-UP(P177)*	CGTTTGGTGGCTAGAGTTATCC	60	177
Br*GAPDH*-DN(P177)*	CTTCTCGCCAAAGAGAAGTGTT	58	
Br*GAPDH*-UP(cytosol)	ATGGCTGACAAGAAGATTAAGATC	52.5	900
Br*GAPDH*-DN(cytosol)	TTAGGCCTTTGACATGTGAATGATC	58	

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β-CHX, β-carotene hydroxylase; *GGPS*, geranylgeranyl diphosphate synthase; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase; *, Real time RT-PCR primers

Table 3. Primer sequences for degenerate PCR

Primer	Primer sequence (5'-3')	Expected PCR size (bp)
DXPsyn DE-UP (S250)	GCCCACCCGCCACCYTNGAYGGNCC	960
DXPsyn DE-DN (E570)	TCGAGGGGGACGCCYTTRTTNCC	
Br-PSY DE-UP	GAGGTGTGCGCCGAGTAYGCNAARAC	450
Br-PSY DE-DN	CGGTGCCGGCCACRTARTARCA	
Br-PDS DE-UP2	CGAGATGCTGACCTGGCCNGARAA	900
Br-PDS DE-DN2	GGAGGCCAGGTACTTCTGCTTNGTRTARTC	
Br-LCY-b cyc DE-UP	ATGGACACCCTGCTGAARACNCCNAA	750
Br-LCY-b cyc DE-DN	TGAACACCATCTTGTTCACGTCRAANGCRTG	
Br-ε-hyd DE-UP (P313)	ATCTCCCTCCTCTTTCTCGTCT	340
Br-ε-hyd DE-DN (P313)	GTAGATGGGTCCGTACTCGTTC	
Br-ZEP DE-UP1	GCTTCGACGTGCTGGTGTTYGARAARGA	870
Br-ZEP DE-DN1	GGAGTCCTCGATGGCCATRCANCC	

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Y, C/T; N, A/G/C/T; R, A/C

4. Northern blot analysis

Northern blot analysis was performed against total RNA preparations. Ten micrograms of total RNA was separated on a 1.2% denaturing agarose gel, and then transferred to positively charged nylon membrane (Schleicher & Schull, USA). According to the instruction manual (BD Bioscience, USA), hybridization was carried out using α-^{32}P labeled DNA probe that was made by PCR (Sambrook & Russel, 2001) as previously mentioned in 'DNA isolation PCR amplification' section. The membrane was washed using standard saline solution and exposed to X-ray film at -70℃.

5. Real-time RT-PCR assay

β-*CHX* mRNA levels were quantified by real-time PCR by using *Br-GAPDH* mRNA levels for normalization (Tian et al., 2003). Primers for probe preparation of β-*CHX* were as follows: the forward, 5'-TCA CAT CAC AAA CCA AGA GAA GGC-3' and the reverse, 5-GGC GCC AAA GCA GAG ACC AGG-3' (**Table 3**). The relative quantity of the transcripts was calculated by using the comparative threshold (C_T) method.

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The frozen material was immediately processed for RNA extraction by Trizol reagent and DNase treatment and first- strand cDNA synthesis followed by M-MuLV reverse transcriptase (MBI). The cDNA material was stored at -20℃.

Real-time quantitative PCR was performed with a MJ-PTC 200 aparatus. For each combination of primer pair and cDNA sample the following master mix was prepared fresh on ice : 10 ㎕ of 2 × master mix, 1 ㎕ of cDNA sample , 4 ㎕ of DDW and 1 ㎕ of primer pairs. A master mix for each primer set was prepared such that each well contained the following: 2× SYBR Green master mix (FINNZYMES). Primer and sample combination were run in triplicate in each real-time PCR test. The qPCR cycling regimen was 15 min at 95℃, 45 iterations of a three-step temperature series (30s at 95℃, 30s at the optimal annealing

temperature for each pair of primers, 30s at 72℃). Fluorescence measurements were made recorded at each 30s extension step. For each reaction tube a PCR cycle threshold (Ct) was defined as the cycle value at which the second derivative of the growth function of Sybr green fluorescence was maximal. For relative quantitation of expression levels, the value of Ct for each of the target amplified products in each experimental condition were determined. The $2^{-\Delta\Delta Ct}$ method is a convenient way to analyze the relative changes in gene expression from real-time quantitative RT-PCR experiments. Data analysis was performed using $2^{-\Delta\Delta Ct}$ method.

Following amplificatin, samples were slowly heated in order to detect the loss in fluorescence that occurs at the melting temperature, which is characterized by a specific melting peak for each PCR product. The sharp and fully overlapping melting peak provides the specific sequence confirmation for the amplified PCR product with each dsDNA product.

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6. Carotenoid extraction and HPLC analysis

Carotenoids were extracted from leaves and different stages as follows. For the isolation of crude carotenoids, approroximately 1g of leaves and flowers were extracted three times with 50 ㎖ extraction solvent (MeOH : Ethyl acetate= 1: 1) containing 0.1% butylated hydroxy toluene. The extracts were evaporated to dryness. Carotenoid extracts were separated on a C_{18} reverse phase column (3.9 × 30cm, particle size 10μm) at 1 ㎖/min(waters chromatography). Carotenoids were seperated by HPLC with a linear gradient beginning with a 100% eluate A (80% of acetonitrile, 15% of methanol, and 5% of ethylacetate to 100%) and eluate B at 15min (60% of acetonitrile, 25% of methanol, and 15% of ethylacetate). Spectra were recorded using photodiode array detector (UV 6000LP) and the absorbance of the carotenoid extracts was determined at 450nm **(Table 4)**. The standard β-carotene and lutein were purchased from Sigma Chemicals.

Table 4. Operation condition of HPLC for carotenoids analysis

Instrument	P4000 gradient pump, UV6000LP detector, SCM1000vacuum degasser, As1000 auto smpler with column oven
Column:	Waters PAH C18 5㎛ 4.6×250mm column
Mobile phase #A	ACN : MeOH : ethyl acetate (80:15:5, v/v)
Mobile phase #B	ACN : MeOH : ethyl acetate (60:25:15, v/v)
Gradient :	1% to 99% #A and 99% to 1% #B mobile phase with a linear for 0 to 60min
Flow rate :	1㎖/min
Wavelength	450nm
Other conditions	- Sample injection : 20㎕
	- Equlibration time : 3.0min
	- Run time : 60 min
	- Column temperature : ambient

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

LC/MS analysis

The molecular mass was determined using electrospray ionization mass spectrometry on a Quattro microTM API mass spectrometer (Waters) The analytical column was a MSC18 column (4.67 × 50mm, 5㎕) (XTerra, Waters, Ireland). The optimized mobile phase was methanol- ethyl acetate (50/50; v/v) , and the flow rate was kept at 0.25㎖/min. The fraction of carotenoids collected from microbore HPLC was analyzed by injection of 10㎕. The spectral analysis was done in a positive ion mode at a capillary voltage of +3.0kV, a cone voltage of 30V and at a source temperature of 120℃. The molecular weight of the carotenoid was determined using the maximum entropy deconsolution algorithm (MaxEnt) to transform the range of 500/700 m/z to give a true mass scale spectrum.

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III. Results

1. Isolation and identification of rapeseed carotenoid biosynthetic genes

Figure 1 presents the carotenoid biosynthetic pathway, and enzymes in plants, leading from glyceraldehyde-3-phosphate and pyruvate to lutein and zeaxanthin, violaxanthin and neoxanthin. The genes cloned from leaves of *B. napus* were listed in **Table 5.** Phytoene synthase, phytoene desaturase, lycopene β-cyclase, lycopene ε-cyclase, β-carotene hydroxylase, and ε-carotene hydroxylase are specific genes in the carotenoid pathway. Additional cDNAs cloned include HMGR, 1-deoxy-D-xylulose synthase (*DXS*), and *GGPS* that catalyze steps of the central isoprenoid pathway common to the synthesis of carotenoids and other plastid-derived terpenoids such as tocopherols, plastoquinone, and chlorophylls.

In general, the genes were highly homologous to the corresponding *Arabidopsis* genes at the protein level with the identity ranging between 73% and 94 %. **Table 5.** lists characteristics of the isolated rapeseed cDNAs.

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On the basis of the conserved amino acid sequences among plant species in carotenoid biosynthetic genes, degenerate primers were designed for each of *HMG-R, DXS, GGPS , PSY, PDS, LYC-b, LYC-ε, β-CHX, ε-CHX, ZEP* and *GAPDH* (**Table 3**). Reverse transcription PCR was performed using total RNA from leaves and flowers of *B. napus*.

Table 5. Isolation and characterization of genes involved in carotenoid biosynthesis

cDNA	Nucleotide (bp)	Amino acid	% Protein identity with *Arabidopsis* homologue	Accession number
HMGR	768 bp	256	93	NP177775
DXPS	2148 bp	720	94	Q38854
GGPPS	1200 bp	328	78	NP195399
PSY	1458 bp	402	86	AAM62787
PDS	930 bp	300	92	NP193157
LCY-b	1800 bp	498	90	NP187634
LCY-e	1800 bp	535	85	NP200513
β-*CHX*	912 bp	304	85	NP194300
ε-*CHX*	2060 bp	545	73	NP190881
ZEP	900 bp	314	94	AAD42899

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Abbreviations : HMG-R, 3-hydroxy-3-methylglutaryl coenzyme A reductase; *DXPS*, 1-deoxy-D-xylulose-5-phosphate synthase; *GGPPS*, geranylgeranyl diphosphate synthase; *PSY*, phytoene synthase; *PDS*, phytoene desaturase; *LCY-b*, β-ring cyclase; *LCY-e*, ε-ring cyclase; β-*CHX*, β-carotene hydroxylase; ε-*CHX*, ε-carotene hydroxylase; *ZEP*, zeaxanthin epoxidase, respectively.

Isolation of hydroxymethylglutaryl-CoA reductase (*HMGR*) gene

Br-HMGR (3-hydroxy-3-methylglutaryl-Coenzyme A reductase) was 768bp in length. This partial gene showed that sequence identity was 98% with HMGR1 from *Raphanus sativus*. The deduced amino acid sequences of clone *Br-HMGR* was compared with other plant *HMGR* and showed high sequence similarity to those of *Arabidopsis thaliana* (93%) *Arabidopsis thaliana* (93%), and *Morus alba* (83%).

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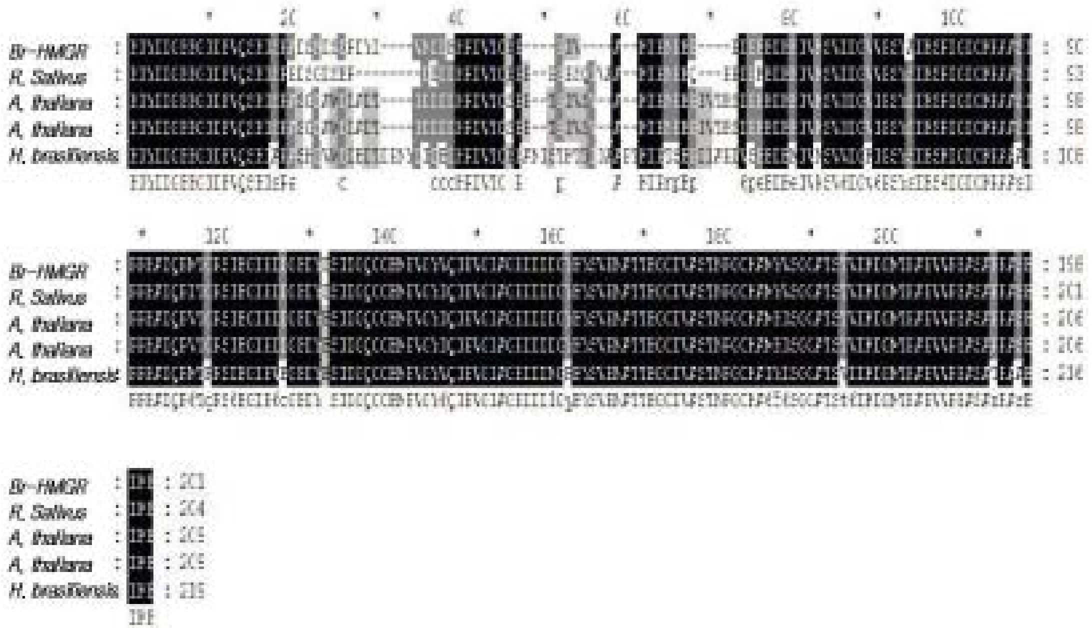

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Fig. 3. Alignment of deduced amino acid sequences of partial *Br-HMGR* with other plant *HMGR* genes. The amino acid sequences of HMGR from *Raphanus sativus(CAA48611),* *Arabidopsis thaliana* (NP177775), *Arabidopsis thaliana* (AAA32814), *Morus alba* (AAD03789.1), and *Hevea brasiliensis* (AAU08214) are shown. The alignment was created with GeneDoc program. Gaps in the sequences are indicated by dashes. Numbers of nucleotides are indicated on the right margin.

Isolation of 1-deoxy-D-xylulose 5-phosphate synthase (*DXPS*) gene

A gene for 1-deoxy-D-xylulose 5-phosphate synthase was isolated by RACE. Its open reading frame was 2148bp in length. Analysis of the cDNA of *Br-DXPS* revealed that it has similarity to 1-deoxy-D-xylulose 5-phosphate synthase gene of *Arabidopsis* (**Fig. 4**). The deduced amino acid sequence of the *Br-DXPS* has high similarity to those of *Arabidopsis thaliana* (94%), *Lycopersicon esculentum* (82%), *Pueraria montana var. lobata* (87%), *Medicago truncatula* (85%), and *Capsicum annuum* (83%).

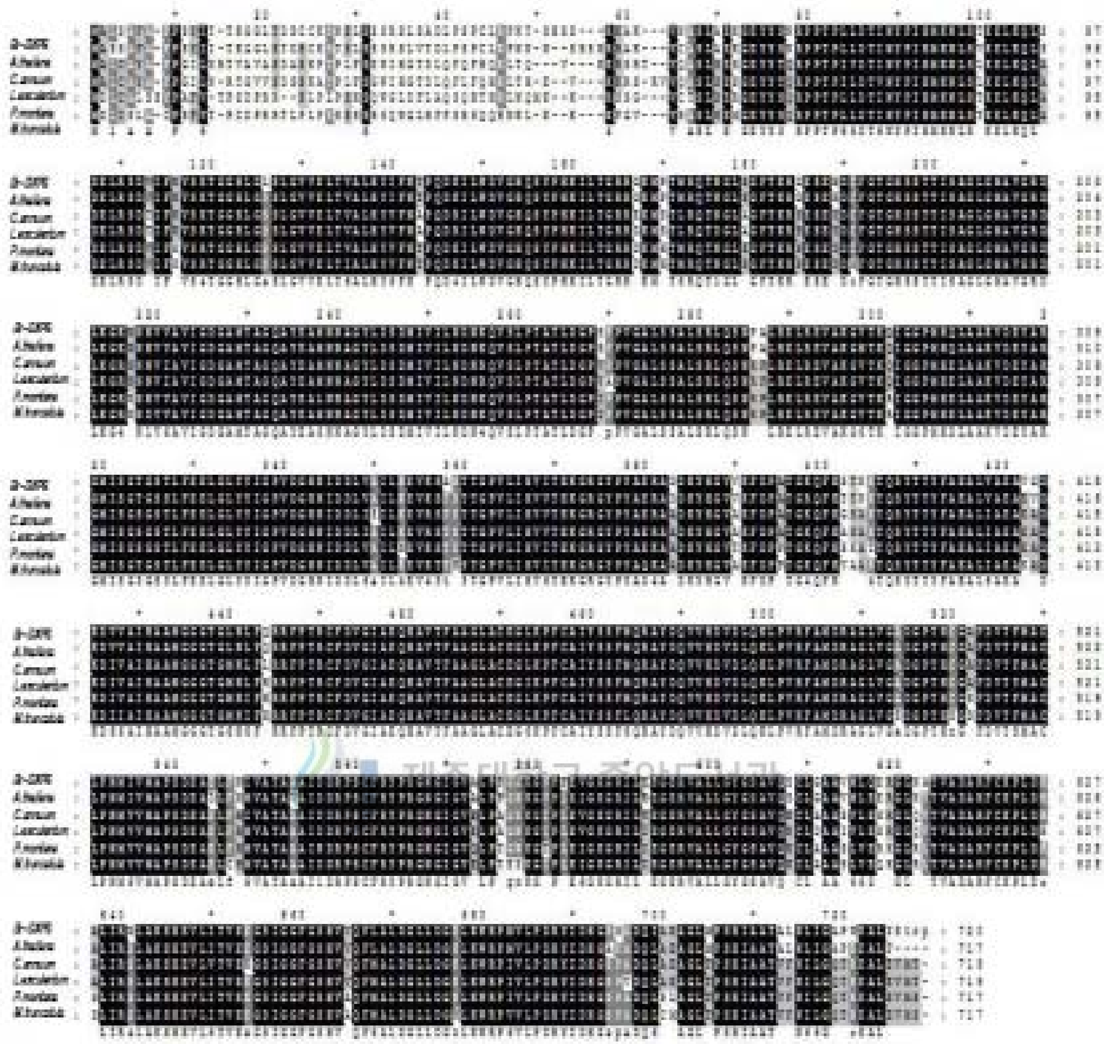

Fig. 4. Alignment of deduced amino acid sequences of *Br-DXPS* with other plant *DXPS* genes. The amino acid sequences of the *DXS* from *Arabidopsis thaliana* (Q38854), *Lycopersicon esculentum* (AAD38941), *Pueraria montana var. lobata* (AAQ84169.1), *Medicago truncatula* (CAD22530.1), and *Capsicum annuum* (CAA75778.1) are shown. The alignment was created with GeneDoc program. Gaps in the sequences are indicated by dashes. Numbers of nucleotides are indicated on the right margin.

Isolation of geranylgeranyl diphosphate synthase (*GGPS*) gene

A cDNA of *Br-GGPS* was 1200bp with its open reading frame. Its deduced amino acid sequences showed 77% similarity to that of *Sinapis alba* and 78% similarity to that of *Arabidopsis thaliana* (Fig. 5).

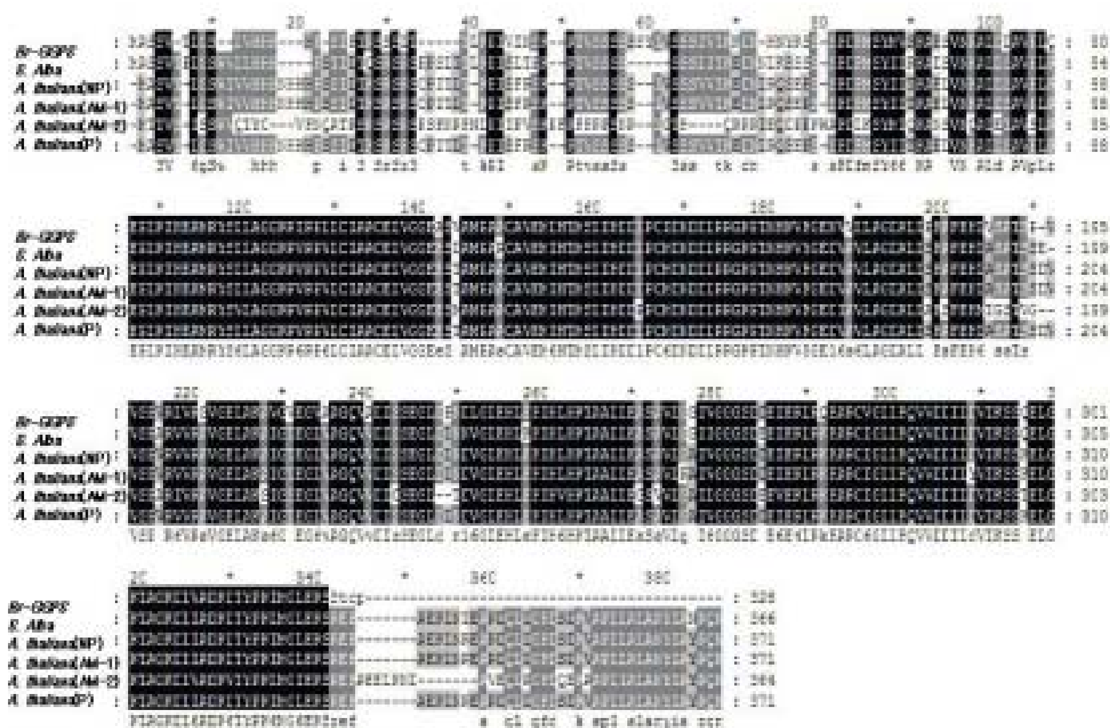

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Fig. 5. Alignment of deduced amino acid sequences of *Br-GGPS* with other plant *GGPS* genes. The amino acid sequences of Br-*GGPS* with other plant *GGPS* genes from *Sinapis alba* (CAA67330), *Arabidopsis thaliana* (NP195399), *Arabidopsis thaliana* (AM21638), and *Arabidopsis thaliana* (P34802) are shown. The alignment was created with GeneDoc program. Gaps in the sequences are indicated by dashes. Numbers of nucleotides are indicated on the right margin.

Cloning of phytoene synthase (*PSY*) gene

To isolate full-length cDNA, rapid amplified cDNA ends (RACEs) were carried out. Gene specific primers were synthesized from partial cDNA sequence fo phytoene synthase. 5' and 3' RACE products were directly inserted to pGEM-T easy vector and the sequences of their 5' and 3' ends were confirmed (**Fig. 6**). Full-length cDNAs having full-length open reading frame (ORF) were generated by end-to -end PCR, and its full sequence confirmed (**Fig. 7**).

The total length of the *Br-PSY* cDNA consists of 1349bp and 402 amino acids with a calculated mass of 44.2 kDa. The deduced amino acid sequence was compared with those of *Arabidopsis thaliana* (86%), *Citrus sinensis* (83%), and *Momordica charantia var. abbreviata* (80%) (**Fig. 8**). Unusually, three different types of *PSY* genes from leaves and petals of *B. napus* were isolated (**Fig. 9**).

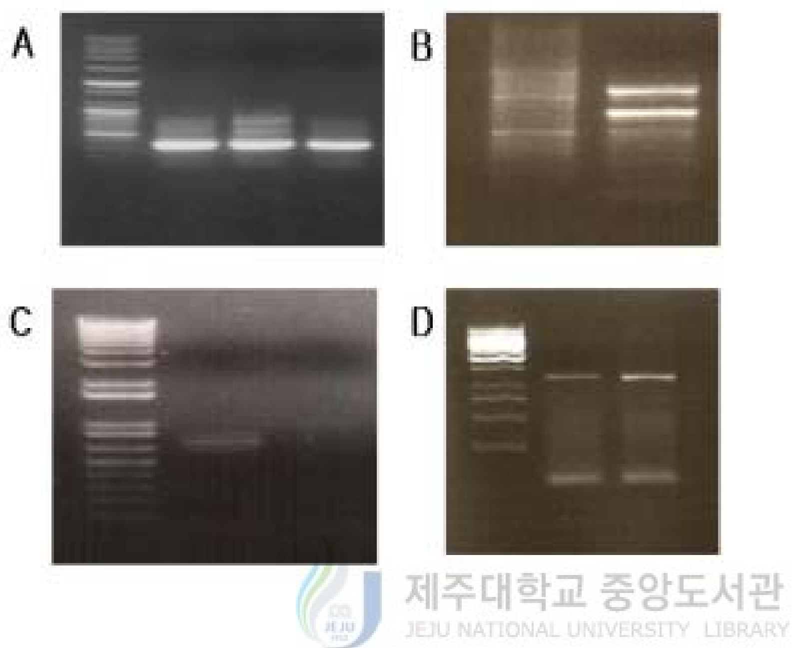

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Fig. 6. Agarose gel showing the products of degenerate PCR (A), 5' RACE (B) , 3' RACE (C), and full sequence (D) from *B. napus*.

```
  1 ATGTCTTCTG TAGCAGTGTT ATGGGTTGCT CCCTCTTCTC CAATTCCAGA
     M  S  S  V  A  V  L  W  V  A  P  S  S  P  I  P  D

 51 CCCAATGAAC AATCCTGGGT TAATAAGAGT TCTAGAATCT TCTAGATTGC
     P  M  N  N  P  G  L  I  R  V  L  E  S  S  R  L  L

101 TTTCTCCTTG TCTGAATCAG AGACTCGACA CTGGCAAGAG GAGTTCTTCT
     S  P  C  L  N  Q  R  L  D  T  G  K  R  S  S  S

151 TCTTCTGTAA TGAGCTGCAG AAGAAGATTA AGTAGTGCTG TCTCTTCAAG
     S  S  V  M  S  C  R  R  R  L  S  S  A  V  S  S  S

201 TTTAGTAGCA AGTCCTGCTG CAGGAGAGAT ATCCCTCTCA TCTGAAGAGA
     L  V  A  S  P  A  A  G  E  I  S  L  S  S  E  E  K

251 AGGTTTACAA CGTTGTGCTG AAACAAGCAG CTCTGGTTAA CAAACAGCTT
     V  Y  N  V  V  L  K  Q  A  A  L  V  N  K  Q  L

301 AGGGACCTTG ATGATCTGAA GAAGCCACAG GATATTGTTC TTCCTGGGAC
     R  D  L  D  D  L  K  K  P  Q  D  I  V  L  P  G  T

351 TACTACTGGG AGTTTGAGTT TGTTGGGTGA AGCTTATGAT CGATGCGGCG
     T  T  G  S  L  S  L  L  G  E  A  Y  D  R  C  G  E

401 AAGTTTGTGC TGAATATGCT AAAACGTTTT ATCTCGGAAC TTTGCTTATG
     V  C  A  E  Y  A  K  T  F  Y  L  G  T  L  L  M

451 ACACCTGAGA GGCGAAAGGC CATTTGGGCT ATCTATGTTT GGTGTAGAAG
     T  P  E  R  R  K  A  I  W  A  I  Y  V  W  C  R  R

501 AACTGATGAA CTCGTAGATG GGCCTAATGC ATCACACATA ACACCCATGG
     T  D  E  L  V  D  G  P  N  A  S  H  I  T  P  M  A

551 CGTTAGATAG ATGGGAAGCA AGGTTAGAAG ATCTTTTCCG TGGCCGTCCT
     L  D  R  W  E  A  R  L  E  D  L  F  R  G  R  P

601 TTCGATATGC TTGACGCTGC TCTAGCTGAT ACAGTTGCTA GATACCCCGT
     F  D  M  L  D  A  A  L  A  D  T  V  A  R  Y  P  V

651 CGATGTTCAG CCATTTAGAG ACATGGTAGA AGGAATGAGA ATGGATCTGA
     D  V  Q  P  F  R  D  M  V  E  G  M  R  M  D  L  R

701 GGAAGTCTAG ATACAAGAAC TTTGATGATC TCTACCTTTA CTGCTACTAT
     K  S  R  Y  K  N  F  D  D  L  Y  L  Y  C  Y  Y

751 GTAGCCGGAA CCGTCGGTTT GATGAGCGTT CCGGTTATGG GGATCGATCC
     V  A  G  T  V  G  L  M  S  V  P  V  M  G  I  D  P

801 CAAGTCCAAA GCAACGACAG AGAGTGTTTA CAACGCTGCC TTGGCTCTCG
     K  S  K  A  T  T  E  S  V  Y  N  A  A  L  A  L  G

851 GTATAGCCAA TCAGCTTACC AACATACTCA GAGACGTTGG CGAAGATGCG
     I  A  N  Q  L  T  N  I  L  R  D  V  G  E  D  A

901 AGAAGAGGAA GGGTTTATCT GCCACAAGAT GAGCTAGCTC AGGCTGGTCT
     R  R  G  R  V  Y  L  P  Q  D  E  L  A  Q  A  G  L
```

(Continued)

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```
 951 CTCAGATGAA GACATATTCG CTGGAAAAGT CACTGATAAA TGGAGAAACT
       S  D  E   D  I  F  A   G  K  V   T  D  K   W  R  N  F

1001 TCATGAAAAT GCAGCTTAAG CGAGCAAGAA TGTTCTTTGA TGAAGCTGAG
        M  K  M   Q  L  K   R  A  R  M   F  F  D   E  A  E

1051 AAAGGTGTTA CTGAGCTTGA CGCAGCTAGC AGATGGCCGG TATGGGCATC
     K  G  V  T   E  L  D   A  A  S   R  W  P  V   W  A  S

1101 GCTCCTATTG TACAGGAGGA TATTGGACGA GATTGGAGCG AATGATTGCA
      L  L  L   Y  R  R  I   L  D  E   I  G  A   N  D  C  N

1151 ACAACTTTAC GAAGAGAGCT TATGTTGGGA AAGCGAAGAA GATTGCAGCT
        N  F  T   K  R  A   Y  V  G  K   A  K  K   I  A  A

1201 CTCCCATTAG CTTATGCTAA ATCAATACTA AAGGCTTCAA GTTCAAGATG
     L  P  L  A   Y  A  K   S  I  L   K  A  S  S   S  R  *

1251 AAATCACTAG TGAATTCGCG GCCGCCTGCA GGTCGACCAT ATGGGAGAGC

1301 TCCCAACGCG TTGGATGCAT AGCTTGAGTA TTCTATAGTG TCACCTAAAT

1351 AGCT
```

Fig. 7. Nucleotide sequence of *Br-psy* and its deduced amino acid sequence. PCR amplified cDNAs were separated on a 1% agarose gel.

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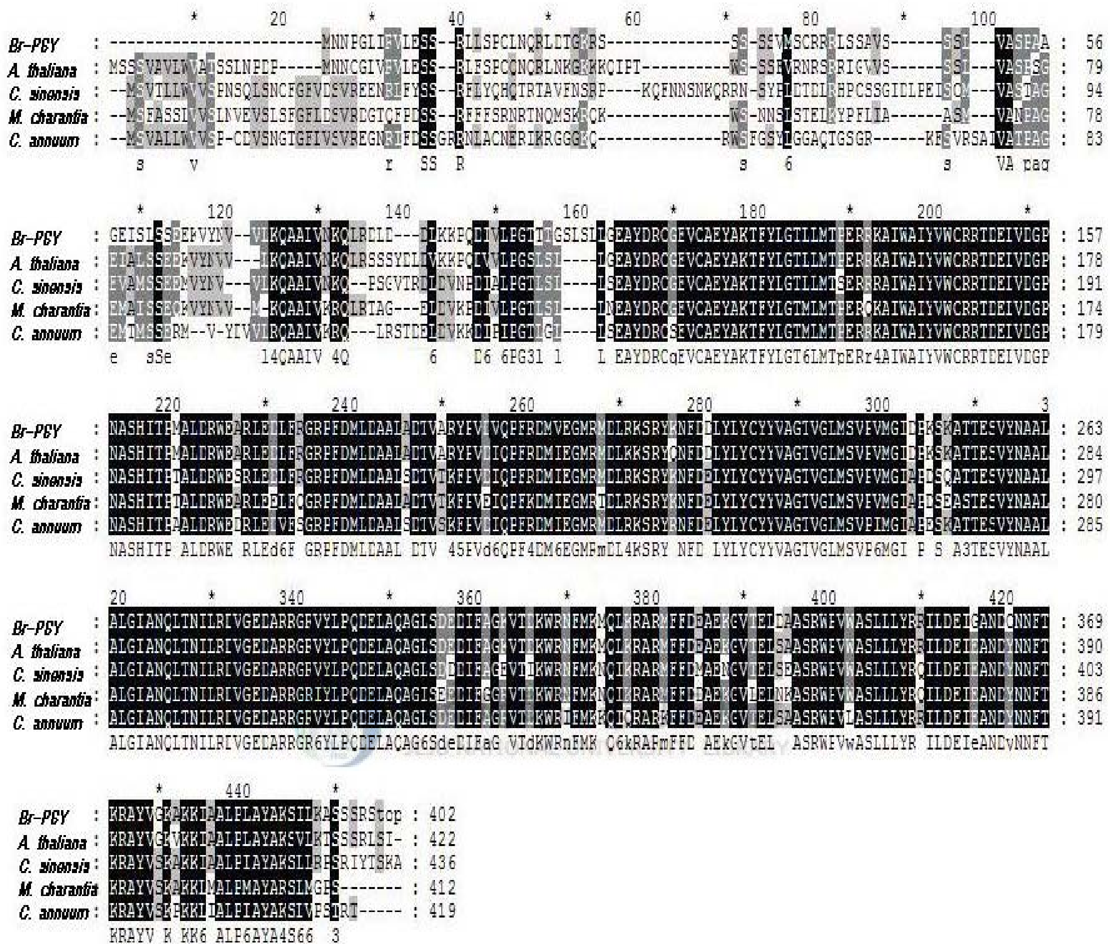

Fig. 8. Multiple alignment of predicted *Br-PSY* protein sequence with homologous sequence of *Arabidopsis* and other proteins. The deduced amino acid of PSY from *Arabidopsis thaliana* (AAB65697), *Citrus sinensis* (ABB72444), *Momordica charantia var.* abbreviata (AAR86104) and *Capsicum annuum* (CAA48155) are shown. Numbers indicate the position of amino acid residues. Conserved residues are highlighted (in black when present in all sequences). The alignment was created with GeneDoc program. Gaps in the sequences are indicated by dashes. Numbers of nucleotides are indicated on the right margin.

```
                     *        20         *        40         *        60         *        80
ISO-PSY : MSSVAVLWVASSSPNPDPMNTCGLVRAVESSRVLSRCQNQRMDNGRRKQTTKTWTSSSSSSLMSYRR---NVVSSSVVA- :  76
Petal   : MSSVAVLWVAPS-PNPDPMNTCGLVRALESSRVLSRCQ--RMSNGRRKQTTTTWSSS---VMRRERR-SSVVSSSLVVN- :  72
Yu      : MSSVAVLWVAPSSPIPDPMNNPGLIRVLESSRLLSPCLNQRLDTGKR--------SSSSSVMSCRRRLSSAVSSSLV-AS :  71
          MSSVAVLWVApSsPnPDPMNtcGL6Ra6ESSR6LSrCqnqR6dnG4Rkqtt tw SSsssv6  rRR ss VsSSlVva

                     *       100         *       120         *       140         *       160
ISO-PSY : SHAGEIALSSEEKVYNVVLRQAALVNKQLRSTSPELDDVKKPRDIVLPG----SLSLLGEAYDRCGEVCAEYAKTFYLGT : 152
Petal   : -PAGEMTLSSEEKVYNVVLSQAALVNEQLRSTSPVLDDVKKPQDTVRPG----SLSLLGEAYDRCGEVCAEYAKTFYLGT : 147
Yu      : PAAGEISLSSEEKVYNVVLKQAALVNKQLR----DLDDLKKPQDIVLPGTTTGSLSLLGEAYDRCGEVCAEYAKTFYLGT : 147
            AGE6 LSSEEKVYNVVL QAALVNkQLRstsp LDD6KKPqDiVlPG    SLSLLGEAYDRCGEVCAEYAKTFYLGT

                     *       180         *       200         *       220         *       240
ISO-PSY : LLMTPERRKAIWAIYVWCRRTDELVDGPNASHITPMALDRWEARLEDLFRGRPFDMLDAALADTVARYPVDIQPFRDMIE : 232
Petal   : LLMTPERRKAIWAIYVWCRRTDELVDGPNASHITPMALDRWEARLEDLYRGRAFDMLDAALADTVTRYPVDIQPFRDMIE : 227
Yu      : LLMTPERRKAIWAIYVWCRRTDELVDGPNASHITPMALDRWEARLEDLFRGRPFDMLDAALADTVARYPVDVQPFRDMVE : 227
          LLMTPERRKAIWAIYVWCRRTDELVDGPNASHITPMALDRWEARLEDL5RGRpFDMLDAALADTVaRYPVD6QPFRDM6E

                     *       260         *       280         *       300         *       320
ISO-PSY : GMRMDLRKSRYKNFDDLYLYCYYVAGTVGLMSVPVMGIDPKSKATTESVYNAALALGIANQLTNILRGVGEDARRGRVYL : 312
Petal   : GMRMDLRKSRYNNFDELYLYCYYVAGTVGLMSVPVMGIDPKSKATTESVYNAALALGIANQLTNILRDVGEDARRGRVYL : 307
Yu      : GMRMDLRKSRYKNFDDLYLYCYYVAGTVGLMSVPVMGIDPKSKATTESVYNAALALGIANQLTNILRDVGEDARRGRVYL : 307
          GMRMDLRKSRYkNFDdLYLYCYYVAGTVGLMSVPVMGIDPKSKATTESVYNAALALGIANQLTNILRdVGEDARRGRVYL

                     *       340         *       360         *       380         *       400
ISO-PSY : PQDELAQAGLSDEDIFAGKVTDKWRNFMKMQLKRARMFFDEAEKGVTELDAASRWPVWASLLLYRRILDEIEANDCNNFT : 392
Petal   : PQDELAQAGLSDEDIFAGKVTDKWRNFMRMQLKRARMFFDEAEKGVTELDAASRWPVWASLLLYRRILDEIEANDYNNFT : 387
Yu      : PQDELAQAGLSDEDIFAGKVTDKWRNFMKMQLKRARMFFDEAEKGVTELDAASRWPVWASLLLYRRILDEIGANDCNNFT : 387
          PQDELAQAGLSDEDIFAGKVTDKWRNFM4MQLKRARMFFDEAEKGVTELDAASRWPVWASLLLYRRILDEIeANDcNNFT

                     *       420         *
ISO-PSY : KRAYVGKAKKIAALPLAYAKSILKASSSRStop-- : 425
Petal   : KRAYVGKVKKIAALPLAYAKSVLKASSSRStop-- : 420
Yu      : KRAYVGKAKKIAALPLAYAKSILKASSSRStop-- : 420
          KRAYVGKaKKIAALPLAYAKS6LKASSSRSTOP
```

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Fig. 9. The alignment of deduced amino acid sequences of *phytoene synthase* gene isolated from leaves and petals in *B. napus*.

ISO-PSY : isotype of *phytoene synthase* gene isolated from leaves;

Petal : *phytoene synthase* gene isolated from petal;

Yu : *phytoene synthase* gene isolated from leaves.

Cloning of *phytoene desaturases* (*PDS*) *gene*

Part of *Br-PDS* was cloned and it was 900bp-long. This partial gene showed that sequence identity was 92% of *Arabidopsis thaliana*. The deduced amino acid sequence was compared with those of *Chrysanthemum morifolium* (88%), *Tagetes erecta* (88%), *Citrus paradisi* (87%), and *Momordica charantia var. abbreviata* (87%), and their degrees of homology were shown in parenthesis (Fig. 10).

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Fig. 10. Multiple alignment of predicted partial *Br-PDS* protein sequence with homologous sequence of *Arabidopsis* and other proteins. The deduced amino acid of PDS from *Arabidopsis thaliana* (NP_193157.1). *Chrysanthemum morifolium* (88%, BAE79552.1), *Tagetes erecta* (AAG10426.1), *Citrus paradisi* (AAK51545.1), and *Momordica charantia var. abbreviata* (AAR86105.1) are shown. Numbers indicate the position of amino acid residues. Conserved residues are highlighted (in black when present in all sequences). The alignment was created with GeneDoc program. Gaps in the sequences are indicated by dashes. Numbers of nucleotides are indicated on the right margin.

Cloning of *Lycopene β-cyclase* (*LCY-b*) gene

To isolate full-length cDNA, rapid amplified cDNA ends (RACEs) were carried out (Fig. 11). The total length of the *Br-LCY-b* cDNA consists of 1654 bp corresponding to 501 amino acids with a calculated mass of 55.1kDa (**Fig. 12**) The deduced amino acid sequence was compared with those of *Arabidopsis thaliana* (90%), *Carica papaya* (78%), *Daucus carota subsp. sativus* (78%), *Capsicum annuum* (78%), and *Citrus maxima* (79%) (**Fig. 13**).

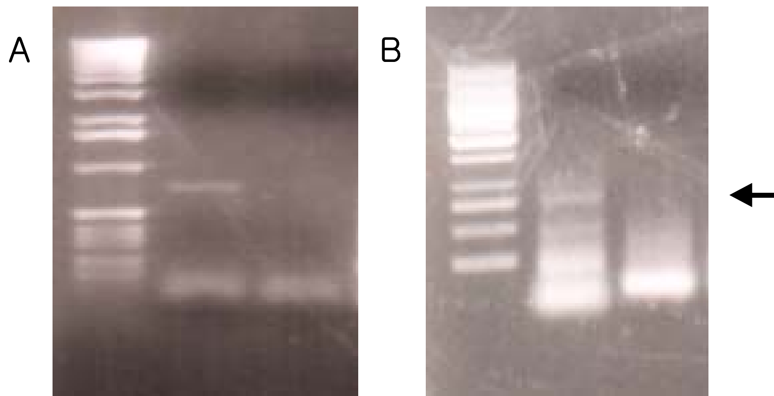

Fig. 11. Agarose gel showing the products of degenerate PCR and 3' RACE.
A: *LCY-b* product by degenerate primer ; B: 3' RACE product by specific primer

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   1 ATGGACACCC TGCTGAAGAC GCCCAACAAG CTCGAATTTT TCATCCCTCA
     M  D  T  L   L  K  T   P  N  K   L  E  F  F   I  P  Q
  51 GTTTCATGGG TTTGAGAGAT TAACCAGTAA CGGTCCAAAT CCTTCAAGGG
      F  H  G   F  E  R  L   T  S  N   G  P  N   P  S  R  V
 101 TTAAACTTTG TGCCAAGAAA AGAGCAATCA AAGTTGGTTC TAGAAGTAGT
       K  L  C   A  K  K   R  A  I  K   V  G  S   R  S  S
 151 ACTAGTAGTA GTGCTCTTCT GGATCTTGTT CCTGAAACCA AGAAGGAGAA
     T  S  S  S   A  L  L   D  L  V   P  E  T  K   K  E  N
 201 TCTCGACTTC GACCTTCCCT TATACGACAC TTCCCTGAAC AAAGTCGTTG
      L  D  F   D  L  P  L   Y  D  T   S  L  N   K  V  V  D
 251 ATCTAGCTAT CGTCGGTGGC GGTCCAGCCG GTTTAGCCGT GGCTCAGCAG
       L  A  I   V  G  G   G  P  A  G   L  A  V   A  Q  Q
 301 GTCTCCGAGG CTGGACTCTC AGTTTGCTCC ATCGACCCTT CTCCCAAGCT
     V  S  E  A   G  L  S   V  C  S   I  D  P  S   P  K  L
 351 CATCTGGCCT AACAACTACG GAGTTTGGGT CGACGAGTTC GAAGCCATGG
      I  W  P   N  N  Y  G   V  W  V   D  E  F   E  A  M  D
 401 ACTTGCTCGA CTGCCTCGAC ACCACCTGGT CCGGTGCCGT TGTCTATATC
       L  L  D   C  L  D   T  T  W  S   G  A  V   V  Y  I
 451 AACGACGGTG CAGAGAAGGA TCTAAGCAGG CCTTACGGGA GAGTCAACCG
     N  D  G  A   E  K  D   L  S  R   P  Y  G  R   V  N  R
 501 TAAACAGCTC AAATCCAAGA TGCTTCAGAA ATGCATCACC AACGGTGTTA
      K  Q  L   K  S  K  M   L  Q  K   C  I  T   N  G  V  M
 551 TGTTTCATCA GTCGAAGGTG ATCAACATGG TTCATGAAGA TGCCAACTCC
       F  H  Q   S  K  V   I  N  M  V   H  E  D   A  N  S
 601 ACTGTGGTTT GCATTGACGG TGTGAAGATT CAGGCTTCTG TTGTTCTTGA
     T  V  V  C   I  D  G   V  K  I   Q  A  S  V   V  L  D
 651 TGCTACTGGT TTCTCCCGGT GCTTGGTTCA GTATGACAAA CCGTATAACC
      A  T  G   F  S  R  C   L  V  Q   Y  D  K   P  Y  N  P
 701 CTGGCTATCA AGTAGCTTAT GGGATCGTGG CTGAGGTTGA TGGCCACCCG
       G  Y  Q   V  A  Y   G  I  V  A   E  V  D   G  H  P
 751 TTTGATGTGG ACAAGATGGT GTTCATGGAC TGGAGAGATA AGCATCTTGA
     F  D  V  D   K  M  V   F  M  D   W  R  D  K   H  L  D
 801 TCCGTACCCT GAGGTTAAAG AACGGAACAG CAAGATCCCT ACGTTCTTGT
      P  Y  P   E  V  K  E   R  N  S   K  I  P   T  F  L  Y
 851 ACGCGATGCC GTTTTCTTCA AACAGAATAT TTCTTGAGGA GACGTCTCTT
       A  M  P   F  S  S   N  R  I  F   L  E  E   T  S  L
 901 GTGGCTAGAC CAGGTCTGAA AATGGAAGAT ATCCAAGAGA GAATGGTCGC
     V  A  R  P   G  L  K   M  E  D   I  Q  E  R   M  V  A
 951 GAGGCTGAAA CACTTGGGGA TCAATGTGAA GCGTATTGAG GAAGACGAGC
      R  L  K   H  L  G  I   N  V  K   R  I  E   E  D  E  R
1001 GTTGTGTGAT CCCTATGGGA GGTCCTCTAC CTGTCTTGCC TCAAAGAGTT
       C  V  I   P  M  G   G  P  L  P   V  L  P   Q  R  V

1051 GTTGGCATTG GCGGTACGGC GGGGATGGTT CATCCTTCGA CTGGTTACAT
     V  G  I  G   G  T  A   G  M  V   H  P  S  T   G  Y  M
1101 GGTGGCTAGG ACTCTTGCAG CTGCTCCAAT AGTTGCAAAC GCTATAGTGA
      V  A  R   T  L  A  A   A  P  I   V  A  N   A  I  V  R
1151 GGTACCTTGG TTCTACTAAA GGAGATGAGC TCTCGGCTGA GGTGTGGAGA
       Y  L  G   S  T  K   G  D  E  L   S  A  E   V  W  R
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1201 GACTTGTGGC CTATTGAAAG GCGGAGACAG AGGGAGTTCT TCTGTTTTGG
     D  L  W  P   I  E  R   R  R  Q   R  E  F  F   C  F  G
1251 GATGGATATA TTGCTGAAGC TTGATTTGGA TGCTACTAGG AGGTTCTTTG
      M  D  I   L  L  K  L   D  L  D   A  T  R   R  F  F  D
1301 ATGCGTTCTT TGACCTGGAA CCGCGTTACT GGCATGGCTT CTTGTCGTCG
       A  F  F   D  L  E   P  R  Y  W   H  G  F   L  S  S
      I  R  E   K  V  Q  F   R  T  V   P  M  A   E  Q  R  R
1351 AGGCTGTTTC TCCCGGACTT GGTGTTCTTC GGGTTGTCGC TCTTCTCGCA
     R  L  F  L   P  D  L   V  F  F   G  L  S  L   F  S  H
1401 TGCTTCTAAT ACCTCGAGGT TGGAGATCAT GACTAAAGGA ACTGTTCCTC
      A  S  N   T  S  R  L   E  I  M   T  K  G   T  V  P  L
1451 TTGCTAAGAT GATCAACAAT TTGGTAAAAG ATAGAGACTA AGGACTGCAA
       A  K  M   I  N  N   L  V  K  D   R  D  *
1501 CTGCATCCTC TTGGATAACT GTGTGTATAA TCATCATAAC ATGATTTCTT
1551 GTGAGGATAT AAGAATCTTT TAGTAGTTTA TTATTTTTTT TCATCAAAGT
1601 TAAGATACTT TTAAAGTGTT AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA
1651 AAAA
```

Fig. 12. Nucleotide sequence of *Br LCY-b* and its deduced amino acid sequence. Numbering begins at the first nucleotide of sequence.

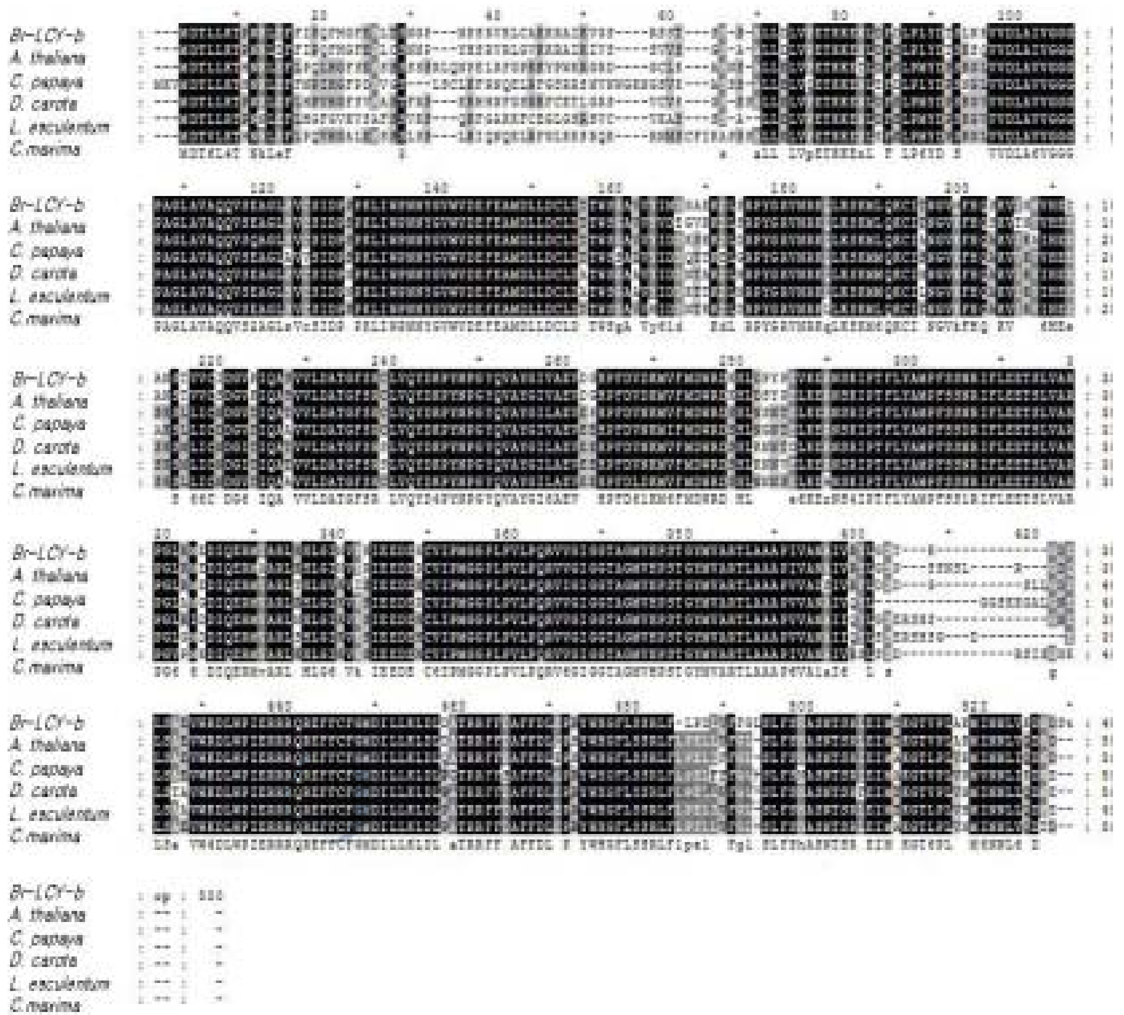

Fig. 13. Multiple alignment of predicted *Br-LYC-β* protein sequence with homologous sequence of *Arabidopsis* and other proteins. The deduced amino acid sequence of LCY-b from *Arabidopsis thaliana* (NP_187634.1), *Carica papaya* (ABD91578.1), *Daucus carota subsp. sativus* (ABB52071.1), *Capsicum annuum* (CAA60119.1), *Lycopersicon esculentum* (S66350), and *Citrus maxima* (AAR89632.1) are shown. Numbers indicate the position of amino acid residues. Conserved residues are highlighted (in black when present in all sequences). The alignment was created with GeneDoc program. Gaps in the sequences are indicated by dashes. Numbers of nucleotides are indicated on the right margin.

Cloning of *Lycopene ε-cyclase* (*LCY-e*) gene

Lycopene ε-cyclase gene was cloned on the basis of EST database of NCBI. The cDNA for *LCY-e* has been cloned and characterized from *Arabidopsis thaliana* (Cunningham et al., 1996). Nucleotide sequence analysis of the 1800bp revealed an open reading frame of 535 codons, potentially coding for a polypeptide of a calculated molecular mass of 58.8kDa. The amino acid sequence of the putative *LCY-e* from *B. napus* is 85 % identical to its homologous protein from *Arabidopsis* and 73% identical to the *Citrus unshiu* lycopene ε-cyclase (**Fig. 14**).

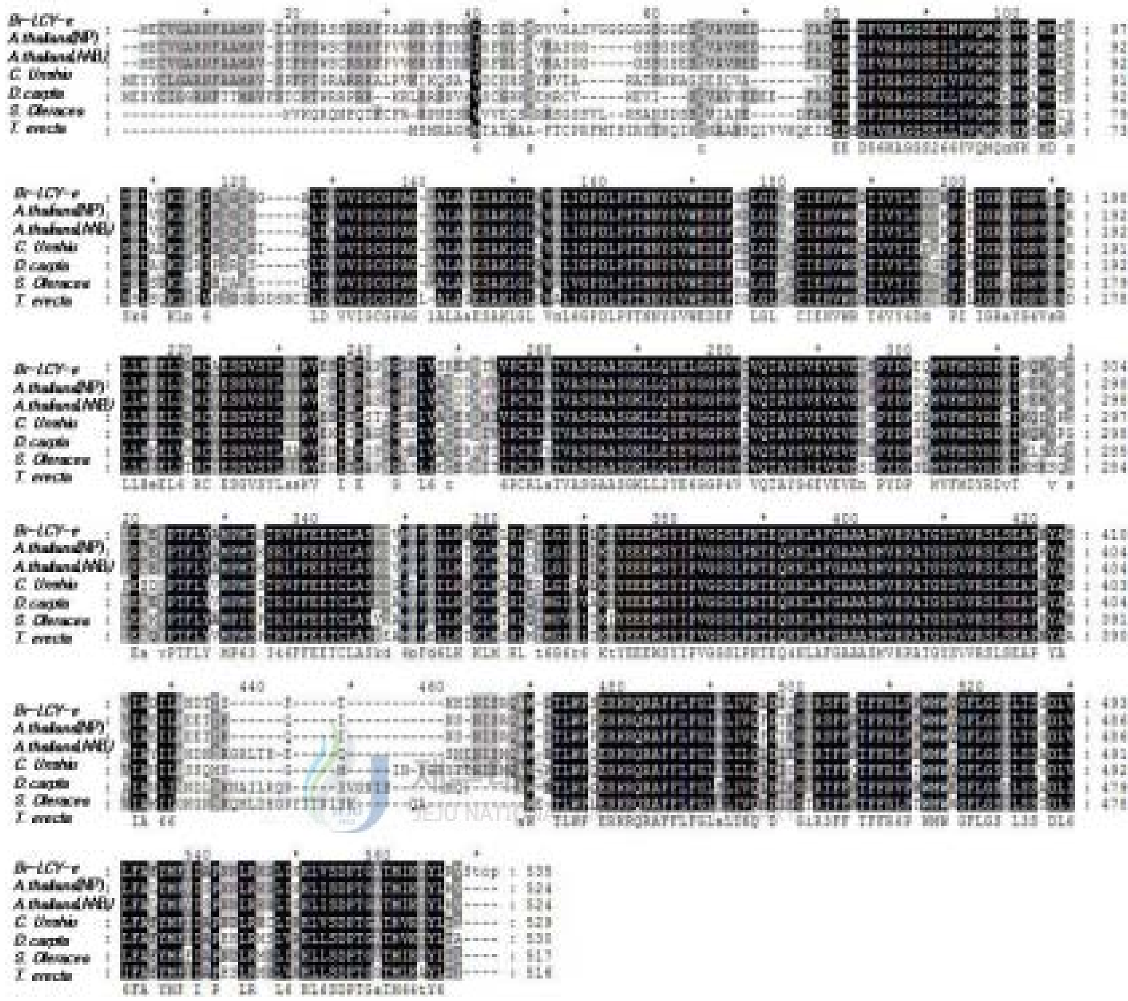

Fig. 14. Multiple alignment of predicted *Br-LCY-e* protein sequence with homologous sequence of *Arabidopsis* and other proteins. The deduced amino acid of *LCY-e* from *Arabidopsis thaliana* (Accession No. NP_200513.1), *Arabidopsis thaliana* (Accession No. AAB53336), *Citrus unshiu* (Accession No. BAE93359.1), *Daucus carota subsp. sativus* (AAB52073), *Spinacia oleracea* (AAL69394) and *Tagetes erecta* (AAG10428) are shown. Numbers indicate the position of amino acid residues. Conserved residues are highlighted (in black when present in all sequences). The alignment was created with GeneDoc program. Gaps in the sequences are indicated by dashes. Numbers of nucleotides are indicated on the right margin.

Isolation of *β-CHX* gene

The cDNAs for *β-CHX* have been previously cloned and characterized in *Arabidopsis*, pepper, tomato and citrus (Sun et al., 1996; Bouvier et al., 1998; Fraser et al., 1994; Kim et al., 2001).

To identify and examine the expression pattern of *β-CHX*, full-length cDNA of β-*CHX* was isolated from leaves and flowers of *B. napus*. The isolated full-length cDNA was sequenced. Nucleotide sequence analysis revealed the coding region of this cDNA was 912bp with a deduced protein of 304 amino acids **(Fig. 15).** A BLAST sequence similarity search (Altschul et al., 1990) revealed that the β-*CHX* gene from *B. napus* has 96% sequence similarity to that of *Brassica rapa* (**Fig. 16**). The deduced amino acid sequence of β-*CHX* was also compared to other plant's β-*CHX* genes and showed high sequence similarity to those of *Arabidopsis thaliana* (85%, NP 194300) and *Citrus unshiu* (77%, AAG10793). β-*CHX* is nonheme di-iron protein that contains 10 conserved His residues required for iron binding and activity. Searching the NCBI for protein domains revealed significant similarity to a carotene hydroxylase. This similarity extends over 139bp, starting at position 132. In addition, the molecular weight of the expressed β-*CHX* protein was estimated to be 33 kDa on SDS-PAGE, which is very close to the value calculated from the gene sequence **(Fig. 17).**

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  1 TGCCTGCAGG TCGACTCTAG AGGGGATCCA GATCTATGGC GGCAGGTCTC
                                           M  A   A  G  L
 51 TCAACCACCG TAACATTCAA CCCGCTCCAC TGCTCTTTCT CATCCTCCTC
     S  T  T   V  T  F  N   P  L  H   C  S  F   S  S  S  S
101 AAGTGTCCGC TTACACCACC CAAGATCCTT AACCGGACTC CCTTCATCTC
      S  V  R   L  H  H   P  R  S  L   T  G  L   P  S  S   L
151 TCCGATTCAG AGGCTTCTCG GTCTGCTACG TGGTTGAGGA GCAGAGGCAG
       R  F  R   G  F  S   V  C  Y  V   V  E  E   Q  R  Q
201 AGCTCTCCCG TCGACAACGA TGAGAGACTC GAGAGCACTA ACGCCATAGA
     S  S  P   V  D  N  D   E  R  L   E  S  T   N  A  I  D
251 TCCCGAGCTC CTAGCGTTGC GTTTGGCTGA GAAGTTGGAG AGGAAGAAGT
      P  E  L   L  A  L   R  L  A  E   K  L  E   R  K  K   S
301 CCGAGAGGTT CACTTATCTA ATAGCCGCAG TGATGTCGAG CTTTGGTATC
       E  R  F   T  Y  L   I  A  A  V   M  S  S   F  G  I
351 ACTTCCATGG CTGTTATGGC TGTTTACTAC AGATTCTCTT GGCAAATGGA
     T  S  M   A  V  M  A   V  Y  Y   R  F  S   W  Q  M  E
401 GGGAGGTGCG ATCCCAATGT CAGAGATGTT TGGTACATTT GCACTCTCTG
      G  G  A   I  P  M   S  E  M  F   G  T  F   A  L  S   V
451 TTGGTGCTGC TGTGGGCATG GAGTTTTGGG CAAGATGGGC CCATAGAGCT
       G  A  A   V  G  M   E  F  W  A   R  W  A   H  R  A
501 CTCTGGCACG CTTCTCTTTG GAATATGCAT GAGTCACATC ACAAACCAAG
     L  W  H   A  S  L  W   N  M  H   E  S  H   H  K  P  R
551 AGAAGGCCCC TTTGAGCTGA ACGATGTGTT TGCAATTATA AACGCTGTTC
      E  G  P   F  E  L   N  D  V  F   A  I  I   N  A  V   P
601 CTGCGATTAG TCTCCTCTCT TATGGTTTCT TCAATAAAGG ACTCGTTCCT
       A  I  S   L  L  S   Y  G  F  F   N  K  G   L  V  P
651 GGTCTCTGCT TTGGCGCCGG ACTAGGAATA ACGGTGTTTG GGATCGCCTA
     G  L  C   F  G  A  G   L  G  I   T  V  F   G  I  A  Y
701 TATGTTTGTC CACGATGGTT TGGTGCACAA GCGTTTCCCC GTAGGTCCCA
      M  F  V   H  D  G   L  V  H  K   R  F  P   V  G  P   I
751 TCGCTGATGT CCCTTATCTC CGGAAGGTCG CTGCCGCTCA CCAGCTACAT
       A  D  V   P  Y  L   R  K  V  A   A  A  H   Q  L  H
801 CACACTGACA AGTTCGATGG TGTGCCATAT GGACTGTTTC TTGGACCAAA
     H  T  D   K  F  D  G   V  P  Y   G  L  F   L  G  P  K
851 GGAATTGGAA GAAGTTGGAG GAGATGAAGA GTTAGACAAG GAGATTACTC
      E  L  E   E  V  G   G  D  E  E   L  D  K   E  I  T   R
901 GGAGAATCAA ATTATACAAA AAGAGTTCGA GCTCTTGAAA GCTTGGAATT
       R  I  K   L  Y  K   K  S  S  S   S  *
951 CGCCCGGGTA CCGAGCTCGC CCTATA
```

Fig. 15. Nucleotide sequence of Br-β-*CHX* and its deduced amino acid sequence. Numbering begins at the first nucleotide of sequence.

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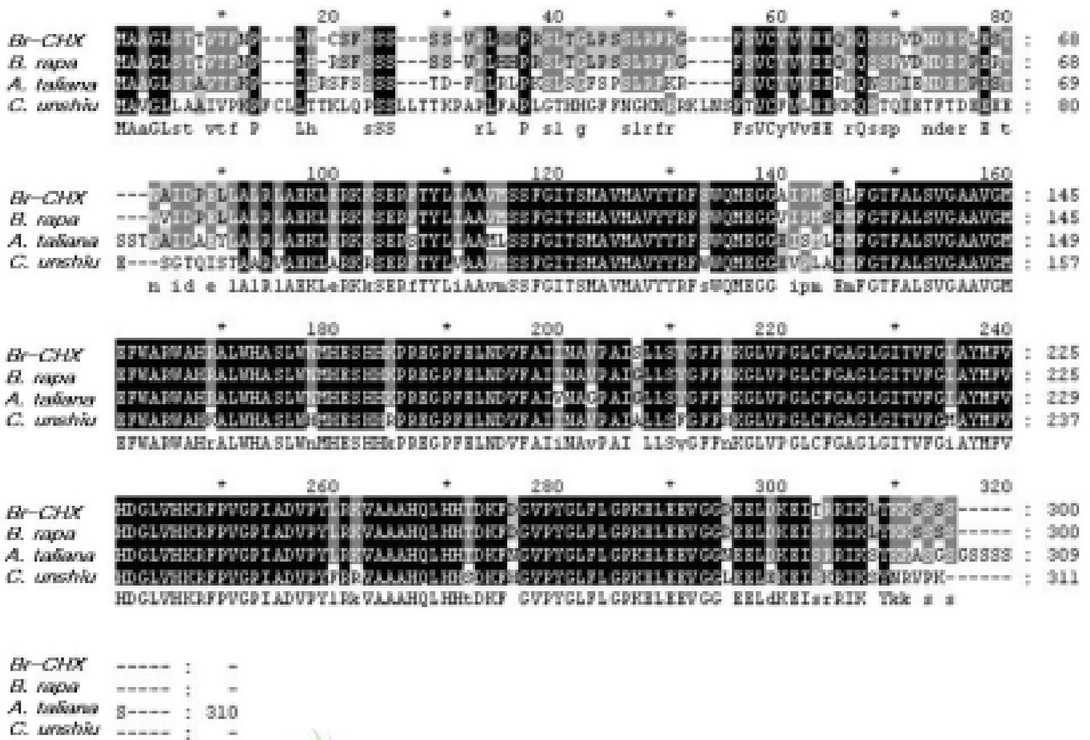

Fig. 16. Alignment of deduced amino acid sequences of β-*CHX* genes. The amino acid sequence of β-*CHX* from *Brasica rapa* (AY545229), *Arabidopsis thaliana* (NP 194300) and *Citrus unshiu* (AAG10793) are shown. Numbers indicate the position of amino acid residues. Conserved residues are highlighted (in black when present in all sequences). The alignment was created with GeneDoc program. Gaps in the sequences are indicated by dashes. Numbers of nucleotides are indicated on the right margin.

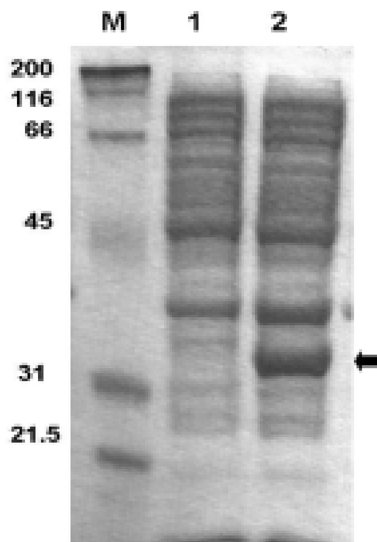

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Fig. 17. Expression of β-CHX in *E. coli*. Proteins were separated by SDS-PAGE and stained with Coomassie blue. Lane M : BRL-broad range protein marker; Lane 1 : control-non induced *E. coli* culture : Lane 2: pellet fraction of induced cells. Thick arrow indicates β-CHX protein of about 33kDa.

Isolation of a cDNA encoding ε-*carotene hydroxylase* (ε-*CHX*)

Degenerate primers were designed by using CODEHOP program (http://bioinformatics.weizmann.ac.il/blocks/codehop.html) comparing three protein sequences from *Arabipdosis* ε-*carotene hydroxylase*. On the basis of the conserved amino acid sequences among *Arabidopsis* in ε-*CHX*, primers were designed.

Reverse transcriptase -PCR were performed in the leave tissues of *B. napus*. We isolated the ε-*CHX* cDNAs with 2,060 bp and 545 amino acids. It has about 73% homology with amino acid sequences from *Arabidopsis* LUT-1, a member of the cytochrome P450 family.

The nucleotide sequence of cDNA isolated *B. napus* showed identity (73% at protein level) to *Arabidopsis* and similar sequence (72% at protein level) to *Daucus carota subsp. sativus*. Searching in the NCBI for protein domains revealed significant similarity with a carotene hydroxylase.

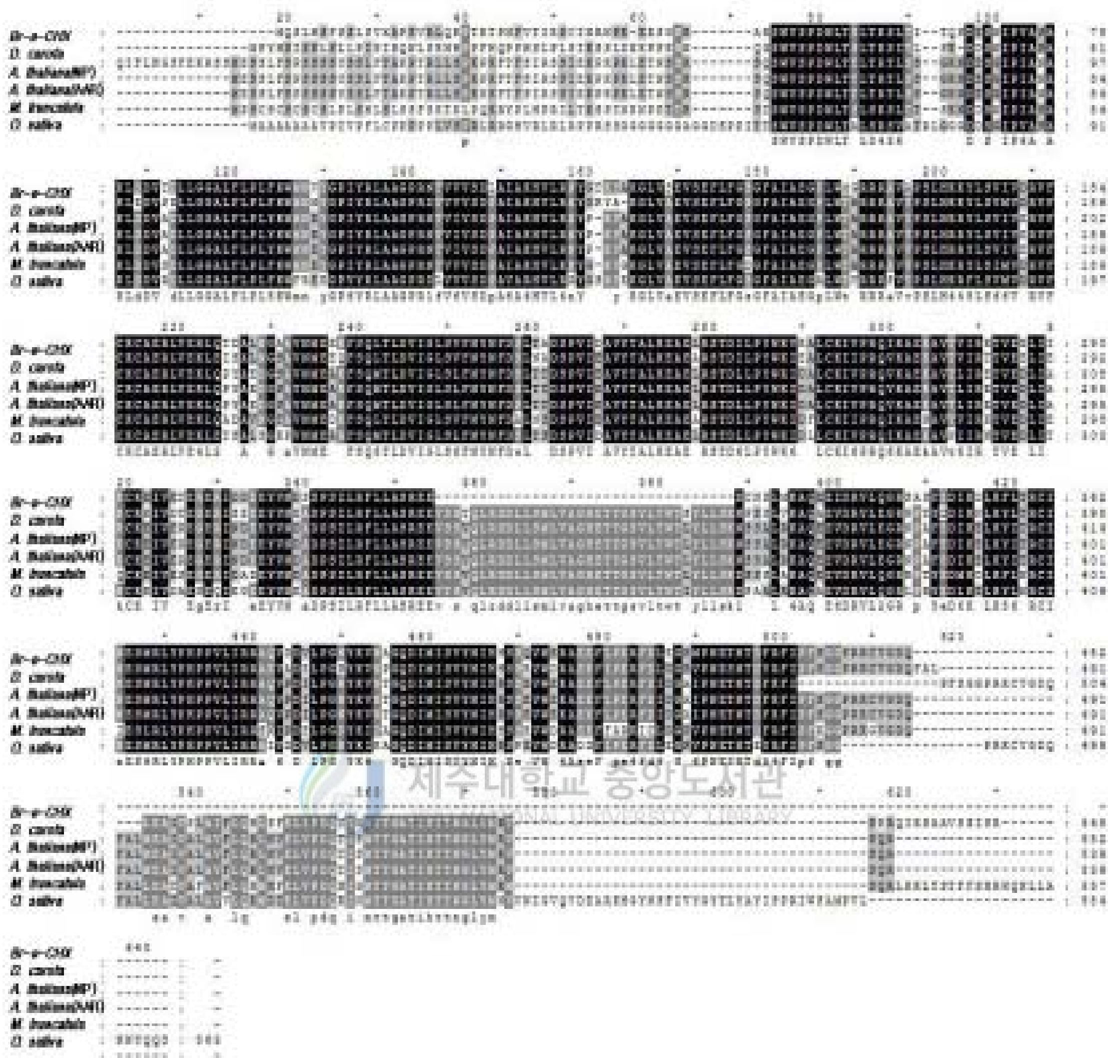

Fig. 18. Alignment of deduced amino acid sequences of ε-*CHX* genes. The deduced amino acid sequences of ε-*CHX* from *Daucus carota subsp. sativus* (ABB52076), *Arabidopsis thaliana* (AM13903), *Arabidopsis thaliana* (NP_190881), *Arabidopsis thaliana* (AAR83120), *Medicago truncatula* (ABC59096), and *Oryza sativa* (NP_922604) are shown. Numbers indicate the position of amino acid residues. Conserved residues are highlighted (in black when present in all sequences). The alignment was created with GeneDoc program. Gaps in the sequences are indicated by dashes. Numbers of nucleotides are indicated on the right margin.

Isolation of *zeaxanthin epoxidase* (*ZEP)* gene.

Zeaxanthin is converted to violaxanthin via antheraxanthin by zeaxanthhin epoxidase (ZEP). The amino acid sequence of the putative partial *ZEP* gene cloned from *B. napus* is 97 % identical to its homologous protein from *Thellungiella halophila*, 94% identical to the *Arabidopsis thaliana*, 85% identical to *Prunus armeniaca* , 85% identical to the *Citrus unshiu*, and 86% identical to the *Lycopersicon esculentum*.

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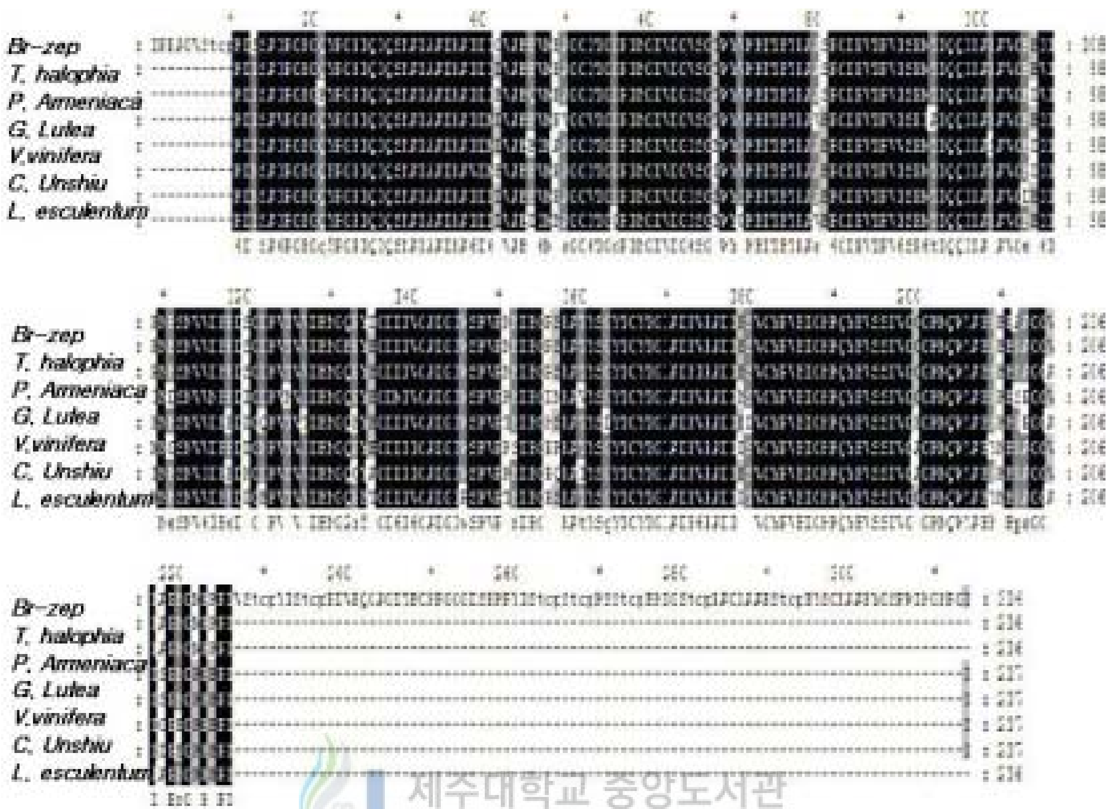

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Fig. 19. Alignment of deduced amino acid sequences of partial *Br-ZEP* genes. The deduced amino acid of ZEP from *Thellungiella halophila* (AAV85824), *Arabidopsis thaliana* (NP_201504.2), *Prunus armeniaca* (AAD42899), *Gentiana lutea* (BAA88842), *Vitis vinifera* (AAR11195), *Citrus unshiu* (BAB78733), and *Lycopersicon esculentum* (CAB06084) are shown. Numbers indicate the position of amino acid residues. Conserved residues are highlighted (in black when present in all sequences). The alignment was created with GeneDoc program. Gaps in the sequences are indicated by dashes. Numbers of nucleotides are indicated on the right margin.

2. Expression analysis of carotenoid biosynthetic genes by Northern blot

Expression pattern of genes involved in carotenoid biosynthesis was investigated by Northern blot analysis. To investigate on the transcriptional level of different genes encoding enzymes of the carotenoid biosynthetic pathway, the expression pattern in leaves, petals and flower buds were investigated. Genes (*LCY-b*, *LCY-e*, and ε-*CHX*) involved in the formation of β-carotene and derived xanthophylls exhibited a low steady-state mRNA transcript level in petals. In flower buds, a strong induction in the amount of the corresponding mRNAs was observed. The expression of the *β-carotene hydroxylase* gene, was increased in the transcript level comparable to leaves and petals.

Expression of carotenoid genes depends on the species investigated (Romer et al., 1993) and the development stage (Bugos et al., 1999). Environmetal factors have been shown to influence their gene expression (Bouvier et al., 1996; Audran et al., 1998; Steinbrenner and Linden, 2001). In addition, the presence of the xanthophyll cycle enhances plant photoprotection (Niyogi et al., 1998). This cycle comprises the three xanthophylls; zeaxanthin, antherxanthin, and violaxanthin and their interconversion (Demmig et al., 1987).

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Northern blot analysis revealed distinct expression patterns for the different carotenoid genes (**Figs. 20, 21**). The expression of *phytoene synthase* gene was observed in leaves, petals and flower buds. All genes involved in the formation of β-carotene and its derivative xanthophylls exhibited a high steady-state mRNA transcript level in leaves and flower buds. The amount of *zeaxanthin epoxidase* mRNA showed no significant difference in different tissues.

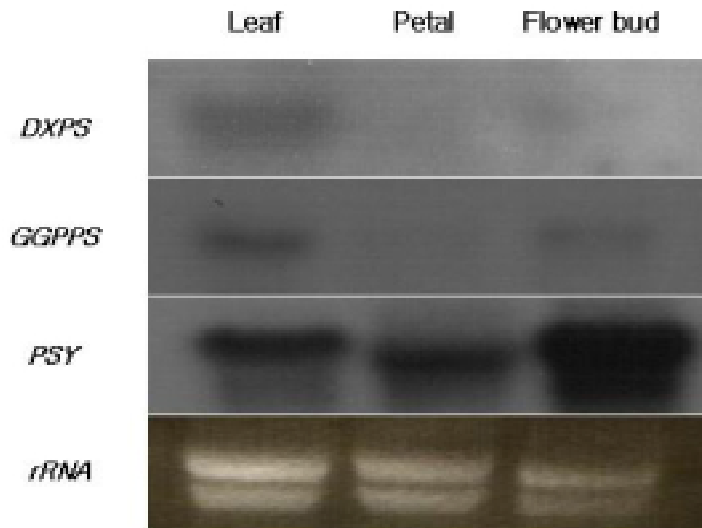

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Fig. 20. Northern blot analysis of carotenoid biosynthetic genes in the different tissues of *B. napus*. *DXPS*, DXP synthase; *GGPPS*, GGDP synthase; *PSY*, Phytoene synthase

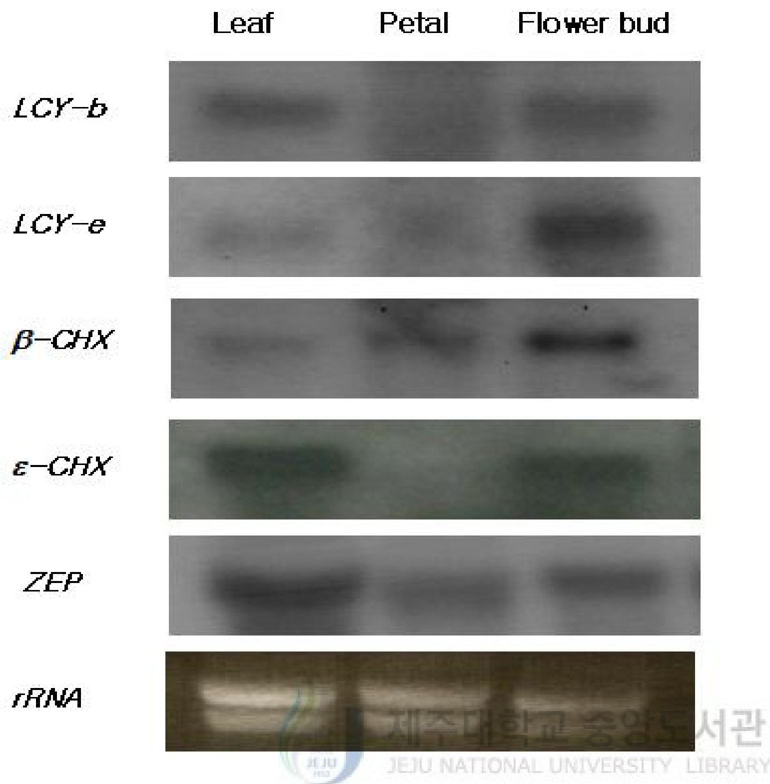

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Fig. 21. Expression of various carotenoid biosynthetic genes in *B. napus.*

LCY-b, lycopene β-cyclase; *LCY-e*, lycopene ε-cyclase; β-*CHX*, β-carotene hydroxylase; ε-*CHX*, ε-carotene hydroxylase; *ZEP*, zeaxanthin epoxidase

3. Analysis of carotenoid levels in different stages of rapeseed

Total carotenoids were extracted from the leaves, petals, flower buds, bud flower and sepals of rapeseed. Carotenoid analysis was performed by HPLC. Analysis condition is shown in **Table 3**. **Fig. 22 and Fig. 23** show chromatograms of *B. napus* using UV absorbance at 450nm. A wavelength of 450nm was chosen to maximize absorbance in the 300 ~ 600nm region of the visible spectrum, which is an optimal area for studying carotenoids of *B. napus*. Carotenoids were identified by their characteristics absorption spectra, distinctive retention times, and in some cases, comparison with standards. The peaks were identified as neoxanthin, violaxanthin, lutein, α-carotene, and β-carotene, respectively, by comparing their absortion spectra and retention times with those of purchased authentic standards (**Table 5**).

Spectral characteristics are campared with those reported in the literature showing that the chosen peaks matched reported (**Table 6**) and confirmed the peaks with molecular mass species of carotenoid. The fraction was examined with LC/MS. The peak 1 elutes and the mass spectrum of the elution showed the molecular ion at mass-to-charge ratio (m/z) 568([M + H^+] . The absorption maximum were close to those of lutein standard and reported previously (Tai and Chen, 2000). The peak 3 elutes and the mass spectrum of the elution showed the molecular ion at mass-to-charge ratio (m/z) 536([M + H^+). The absorption maximum were close to those of β-carotene standard.

Carotenoid composition in the green stages of leaves in rapeseed is similar to that of green stages of fruit in tomato (Ronen et al., 1999) where lutein and β-carotene predominate (**Table 7**). In leaves, lutein is the major carotenoid (approximately 25.7%), with smaller amounts of β-carotene. In petals and flower buds, lutein decreased though the amounts of violaxanthin, neoxanthin, and antheraxanthin increased.

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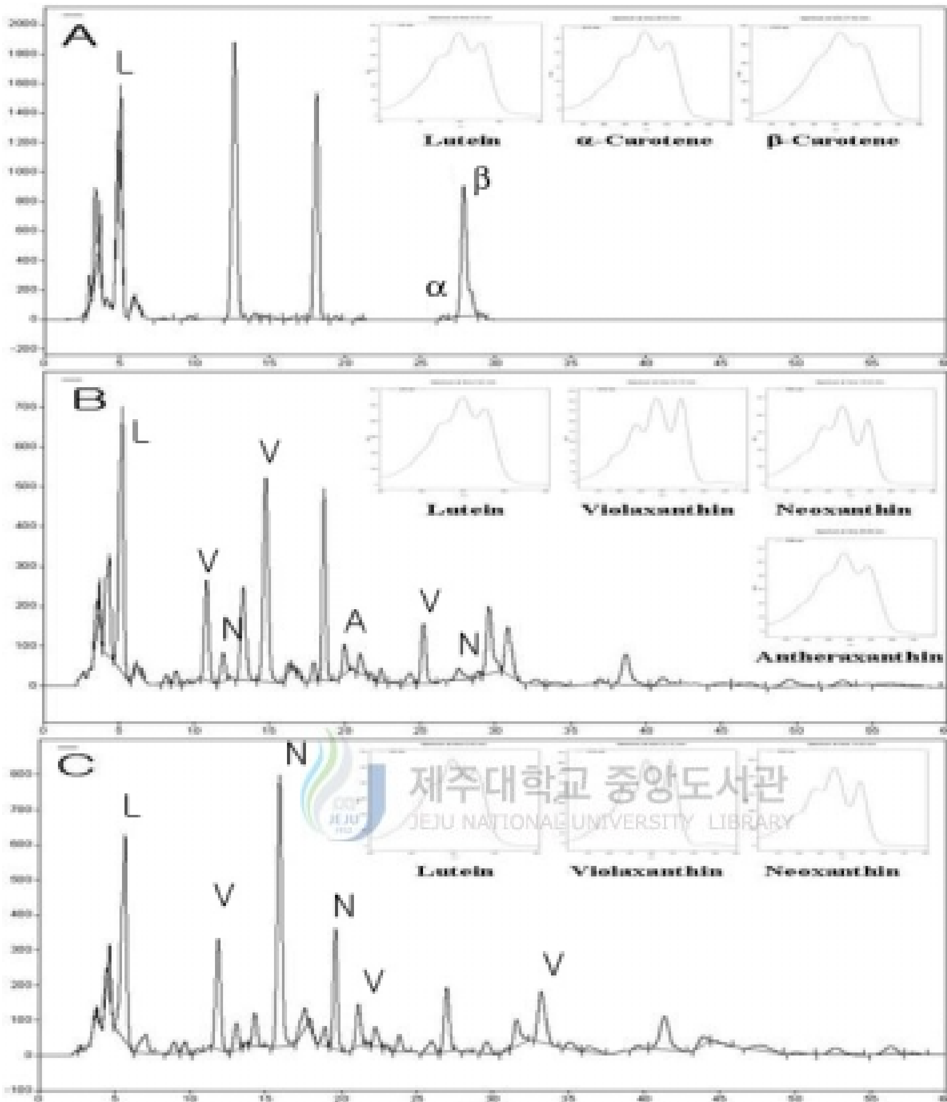

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Fig. 22. HPLC profiles of crude carotenoids extracted from *B. napus*.

A : Profile of leaf pigments. ; B: flower buds. C: petals

L, lutein; N, neoxanthin; V, violaxanthin; A, antheraxanthin;

α, α-carotene; β, β-carotene;

Numbers above the arrows indicate carotenoids peak number.

Absorption spectra of peaks are presented in the inserts.

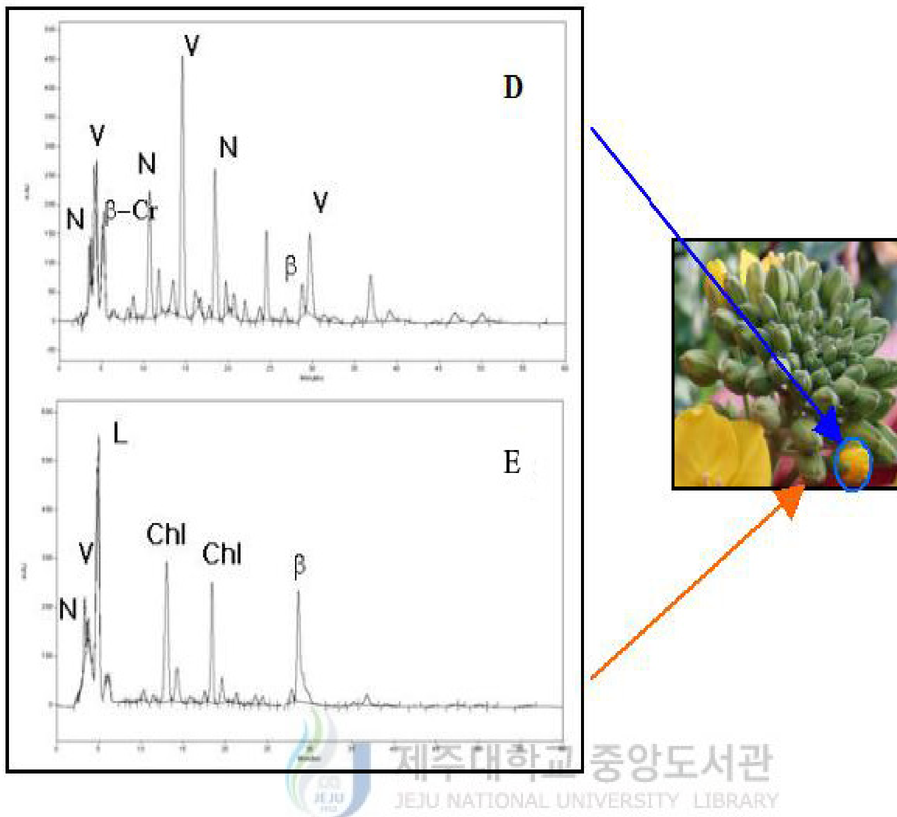

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Fig. 23. The HPLC profiles of crude carotenoids extracted from *B. napus*.

D : Profile of Bud flowers; E: Sepals

L, lutein; N, neoxanthin; V, violaxanthin; A, antheraxanthin;

α, α-carotene; β, β-carotene

Numbers above the arrows indicate carotenoids peak number.

Absorption spectra of peaks are presented in the inserts.

Table 6. Identification of carotenoids in the different tissues of rapeseed

Abbreviation	Carotenoid compound	Max ABS wavelength	% III/II[a]	M. W	Max ABS wavelength	%III/II
					Britton, 1995 & 2004	
L	Lutein	424, 448, 476	54	568	421, 445, 474	60
α	α-Carotene	424, 445, 472	-	536	423, 444, 473	-
β	β-Carotene	424, 456, 480	-	536	(425), 450, 478	25
A	Antheraxanthin	419, 442, 472	35	584	422, 444, 472	55
V	Violaxanthin	420, 444, 472	93	600	419, 440, 470	95
N	Neoxanthin	418, 442, 472	73	600	415, 439, 467	80

Source : Britton (1995) and Britton et al., 2004.

[a] Ratio of the height of the longest-wavelength absortion peak, designated III, and that of the middle absorption peak, designated II, taking the minimum between the two peaks as baseline, multiplied by 100.

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Table 7. Carotenoid composition in mature leaves, petals, and flower buds of rapeseed

Carotenoid	Leaf	Petals	Flower buds
Lutein	25.7	23	17.95
α-Carotene	1.28	n.d	1.19
β-Carotene	62.55	n.d	n.d
Violaxanthin	n.d	28.96	35.4
Neoxanthin	n.d	45	7.9
Antherxanthin	n.d	n.d	3.4
Unknown	12	2.14	16.22
Total carotenoids	100 (%)	100 (%)	100 (%)

Values are percentage of total carotenoids (peak area).
Total carotenoid concentration is per fresh weight.

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4. Expression analysis of β-*CHX* gene by real-time RT-PCR in rapeseed

The gene expression was increased higher in bud flower than sepals (**Fig. 24**). This result is closely similar to HPLC results (**Fig. 23**). Concentration of violaxanthin and neoxanthin were higher in bud floweres rather than sepals by HPLC analysis. This means that β-*CHX* gene is active in bud flower than sepal tissues.

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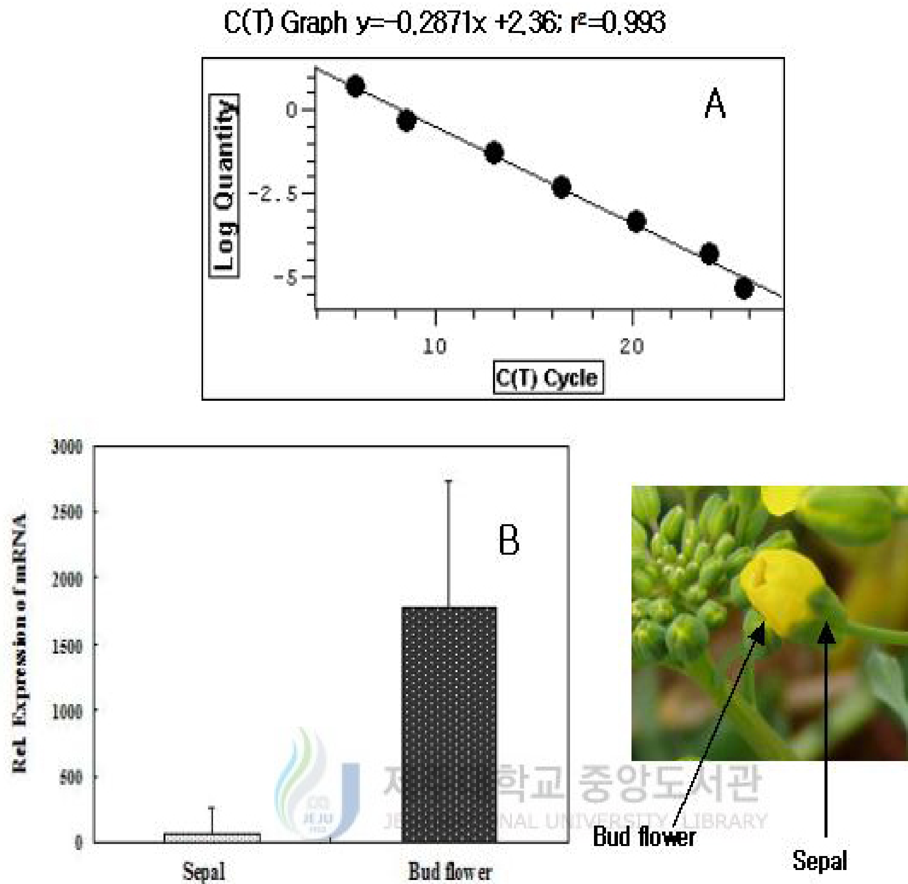

Fig. 24. Real-time RT-PCR analysis for β-CHX gene expression in bud flower and sepals. (A) Standard curve for β-CHX gene expression. The curve derives from plotting Ct against log quantity of fluorescence. This result shows R^2 >0.99 with 97% efficiency of the PCR, with a slope of -0.2871. (B) Real-time RT-PCR of the β-carotene hydroxylase gene expression in bud flowers and sepals. The level of gene expression was calculated afrter normalization against *Br-GAPDH* in each sample and is presented as relative mRNA expression units (n=3).

IV. Discussion

1. Carotenoid biosynthetic pathway genes

Carotenoid formation is a highly regulated process. Concentration and composition of leaf xanthophylls are affected by light intensity (Ruban et al., 1994) and the accumulation of specific carotenoids in chromoplasts of fruit and flowers is developmentally regulated (Giuliano et al., 1993).

The initial steps of the pathway involve condensation of three molecules of acetyl-CoA to produce the C_6 compound 3-hydroxy-3-methylglutaryl-Coenzyme A (HMG-CoA), which after reduction yields MVA. In the next two steps, mevalonate kinase and mevalonate 5-diphosphate kinase catalyze MVA to form mevalonate 5-diphosphate, which is subsequently decarboxylated to yield isopentenyl diphosphate (IPP). The mevalonate pathway provide IPP for the synthesis of some sesquiterpenes, sterols, and triterpenes and is localized in the cytosol.

After the discovery of the MEP pathway, previous studies suggest that the rate-limiting nature of PSY activity was dependent on the availability of non-limiting levels of MEP-derived precursors (Lois et al., 2000). The supply of plastidial precursors regulates the production of other isoprenoids such as chlorophylls, tocopherol, and gibberellins (Esteves et al., 2001; Fray et al., 1995). A major unsolved problem for the biotechnological production of high levels of carotenoids in plants is how to increase the flow of metabolic precursors to carotenoid synthesis without affecting other interacting metabolic pathways (Sandmann, 2001). The production of IPP and dimethylallyl diphosphate (DMAPP) catalyzed by the HMG-CoA erductase (HDR) enzyme represent a key step in controlling the production of plastidial isoprenoid precursors and carotenoid biosynthesis in plant cells (Botella-Pavia et al., 2004).

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Carotenoid accumulation during fruit ripening in tomato serves as a model system to investigate the regulation of the process. In tomato, accumulation of lycopene begins at the breaker stage of fruit ripening after the fruit has reached the mature green stage. Cloning of the genes for *PSY, PDS,* and *GGDPS*, it was possible to demonstrate that the mRNA levels of these genes increase significantly during the breaker stage (Fraser et al., 1994; Giuliano et al., 1993; Pecker et al., 1992).

In *Rhodococcus erythropolis*, expression vectors containing *dxs*, which encodes 1-deoxy-D-xylulose-5-phosphate (*DXP*) synthase, or *CrtE*, which encodes geranylgeranyl pyrophosphate synthase, increased the carotenoid accumulation approximately two fold when compared to the controls (Bremman, 2003).

In this study, mRNA levels of *PSY, PDS,* and *GGPPS* increased higher in flower buds rather than petals in rapeseed. In contrast, the mRNA of *LCY-b and LCY-e* decreased in petals. In tomato, the mRNA of lycopene β-cyclase decrease at the breaker stage. Evidenece for transcriptional up-regulation of carotenoid genes in flowers has been described for *Psy, Pds* and CrtL-b (Corona et al., 1996; Giuliano et al., 1993; Pecker et al., 1996).

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These data indicate that the relative abundance of mRNA of genes for the carotenoid biosynthesis enzymes changes between the green tissues and non-green tissues. While mRNA of *LCY-ε* is undetectable in petals of old flowers. Chromoplasts in petals develop without going through green stage (Ronen et al., 1999).

Carotenoid β- and ε-hydroxylases add hydroxyl group to the β- and ε- rings of carotenes, respectively. In this study, we have isolated and characterized a full-length cDNA clone encoding β-*CHX* from *Brassica napus* cv. Halla. The cDNA clone showed high sequence homology with β-*CHX* genes from various plant species and encoded 304 amino-acid residue polypeptides with a predicted molecular weight of **33** kDa. Also, we isolated the ε-*CHX* cDNAs with 2,060 bp and 545 amino acids from *B. napus*. It has about

73% homology with amino acid sequences from *Arabidopsis* LUT-1 a member of the cytochrome P450 family. The cytochrome P450 ε-hydroxylase carries out a type of hydroxylation reaction that is mechanically distinct from the non heme diiron β-hydroxylases and has evolved from cytochrome P450- type fatty acid hydroxylases (Tian et al., 2004), independent of the β-hydroxylases. This would be analogous to the nonheme diiron carotenoid β-hydroxylases that has consensus iron-binding histidine motifs shared with the membrane fatty acid desaturase (Shanklin & Cahoon, 1998). ε-Hydroxylase homologs have been identified in monocot (e. g. rice) and dicot (e. g. soybean) databases. The ε-hydroxylase product, lutein, has only been identified in land plants.

2. Gene expression in rapeseed

The levels of mRNA of *LCY-e* and *LCY-b* in leaves, petals, and flower buds of *B. napus* were measured by northern blot analysis. The results indicate that the mRNA level for *LCY-e* and *LCY-b* increases in flower buds relative to petals, while these genes are not expressed at all in capsules. This result corresponds to the accumulation of violaxanthin and neoxanthin in flower buds. In flower buds of *B. napus*, the major carotenoid accumulated is violaxanthin. In this tissue, the mRNA levels of *Psy, Pds* (Giuliano et al., 1993; Pecker et al., 1992) and *CrtL-e* (Pecker et al., 1996) increase while the mRNA levels of *LCY-e* are undectable in petals of old flowers. This phenomenon demonstrates that chromoplasts in petals develop without going through green stages.

To determine the expression pattern of β-*CHX*, we compared its mRNA level in leaves, petals and flower buds at different developmental stages. The highest transcript level of β-*CHX* was found in flower buds. β-*CHX* mRNA was more abundant in the flower buds and petals than leaves (**Fig. 21).**

The increasement of the steady-state transcript levels of carotenoid biosynthetic

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genes (β-*CHX*, *LCY-b*, and *LCY-e*) is likely to be related to the large increase of total carotenoids. Expression profiles of β-*CHX* and *ZEP* were similar, this implies a coordination in gene expression of these two xanthophyll biosynthetic genes (β-*CHX*, *ZEP*), which are necessary to increase the content of β-carotene derivatives with light-harvesting functions (violaxanthin and neoxanthin). The regulation of the β-*CHX* steady-state transcript level may represent a way to meet the requirement of additional β-carotene derivatives for the assembly of the photosynthetic apparatus (Young, 1991).

3. Analysis of carotenoid composition

Carotenoids were extracted from leaves, flower buds and petals, and were subjected to HPLC analysis **(Fig. 22).** In leaf tissues, both lutein and β-carotene were the predominant carotenoids (**Fig. 23**). In flower buds, β-carotene was not detected, and both lutein and α-carotene peaks were slightly low, while other xanthophylls such as antheraxanthin, violaxanthin and neoxanthin were predominantly detected (**Fig. 22**). Petals showed carotenoid profiles different from those of leaves and flower buds. Violaxanthin and neoxanthin accumulated in the petals (**Fig. 22**), which might have resulted from active hydroxylation of carotene in flower buds, and then subsequent accumulation of more neoxanthin than violaxanthin using β-catotene as a substrate in the petals (**Fig. 22**).

The major pigment identified in *Brassica napus*' leaves, was lutein and β-carotene, which are the most abundant carotenoid in the photosynthetic tissues and accumulates in the chloroplast. An HPLC profile of leaves is similar to that observed in most chloroplasts of higher plants (Ryberg et al., 1993), where lutein is the most abundant carotenoid, followed by β-carotene in abundance. During the development of flowers, plastids differentiate into chromoplasts that are specialized organelles for the sequestration of lipophilic molecules such as carotenoids. This process in chromoplasts has been reported in a number of non-photosynthetic plant tissues such as tomato and pepper fruits, daffodil flowers, and

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marigold flowers (Camara et al., 1995). The carotenoid composition of chromoplasts varies widely among different plants. For example, tomato fruits accumulate lycopene, capsicum fruits accumulate violaxanthin and β-carotene (Ben-Amotz and Fishler, 1998) and marigold flowers accumulate lutein (Moehs et al., 2001). In flower petals, the chromoplasts originate from the conversion of a fully developed chloroplast, and during this transition the chlorophyll concentration decreases and the carotenoids content increases. Sometimes the carotenoid composition of chromoplasts also changes (Bartley and Scolnik, 1995). The transition from chloroplast to chromoplast is concomitant with the degradation of chlorophylls, the upregulation of carotenoid biosynthetic genes and the increased production of floral or fruit specific carotenoids (Nielsen et al., 2003). Our results showed that lutein and β-carotene were the major carotenoids in the leaves. In contrast, modified forms of β-carotene, violaxanthin, and neoxanthin were the major compounds in the floral tissues **(Table 7).**

Carotenoids in the chromoplast accumulate to high levels in certain fruits and flowers. These compounds are thought to serve as visual attractants for insects and animals to aid pollination and seed dispersal (Goodwin, 1986).

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Here we reported the isolation of gene encoding carotenoid biosynthetic pathway and investigated its expression patterns in different tissues from *B. napus*. Other xanthophyll peaks were confirmed, and their contents in petals and flower buds were higher than in leaves. The carotenoid and xanthophyll compounds are color determinants, and the intensity of the petal's bright- yellow color is determined by the amount of other xanthophyll, violaxanthin and neoxanthin in the flowers of *B. napus.*

The results showed that all the carotenoids from the floral tissues contain the same types of pigments except for β-carotene, even though the individual pigment' ratios vary. This may show a direct relationship between the color of the inflorescence and the nature of carotenoids. The color differences in marigold flowers are due not to the accumulation of different carotenoids or pathway intermediates, but rather to the accumulation of different

amounts of the same carotenoid, lutein esters (Moehs et al., 2001). Therefore the high level of the β-hydroxylase gene expression appeared to be closely related to flower development and pigment accumulation in the petals.

4. Regulation of carotenoid biosynthesis

Carotenoids are the main group of plastidial isoprenoids synthesized in plants and enhancing their production is an important biotechnological purpose due to their industrial and nutritional value (Bartley and Scolinik, 1995; Fraser and Bramley, 2004; Hirschberg, 2001; Sandmann, 2001). There is still much to be discovered about how plants regulate the carotenoid biosynthetic pathway. Because the pathway contains many steps, and the precursors of carotenoid biosynthesis are shared with a large range of isoprenoids with important functions, regulation is likely to be complex and to occur at several levels. Transcriptional activation of gene expression seems to be the major form of regulation in many fruits and flowers (Fraser and Bramley, 2004; Hirschberg, 2001).

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Overexpression studies in *Arabidopsis* led to the conclusion that *DXS* is a limiting enzyme for the biosynthesis of plastidial isoprenoids in plant cells (Esteves et al., 2001), but the relative contributions of other MEP pathway enzymes to the supply of precursors remain to be investigated. Here we show that increased levels not only *DXS* but also of *PSY* lead to higher carotenoid levels in leaves, suggesting that the assignment of a single rate-limiting step in the isoprenoid pathway mignt not be appropriate in a metabolic context. Several enzymes may share control over the flux of a pathway, with different enzymes exhibiting different degrees of control (Fell, 1992; Thomas and Fell, 1998).

The regulation of the pathway may benefit from a degree of redundancy at the gene level and the segregation of different gene products to different tissues (Taylor and Ramsay, 2005). It appears that at least three of the enzyme in the pathway, including the key rate-limiting enzyme phytoene synthase, are coded for by different forms expressed in a tissue-specific

manner in green tissue or in floral and fruit tissue, and hence targeted primarily to either chloroplasts or chromoplasts.

In the case of carotenoids, *PSY* exhibits the highest flux control coefficient among the enzymes such as LCY-b also results in increased levels of total carotenoids (Ronen et al., 2000; Rosati et al., 2000)

In recent years, the PSY-catalyzed production of phytoene from GGPP was considered to be the first limiting step in the production of carotenoids. The regulation patterns for *DXS, PSY, PDS, LCY-b, LCY-e* at the different tissues were investigated in this study. The results suggest that *DXS* and *PSY* are at rate-limiting steps during carotenoid synthesis in leaves and also *PSY* is at rate-limiting step during carotenoid synthesis in the flower buds and petals. In the previous reports, the *PSY* transcript was observed to increase 10-fold during development of tomato flowers (Giliano et al., 1993). Zhu et al. (2002) found that the steady-state levels of *PSY* transcript increased 6- to 7-fold in *Gentiana lutea* petals from the young bud stage to the fully mature stage. The induction of *PSY* transcript was related to pigment accumulation. In tomato, Fraser et al., (1999) found that despite the absence of a mutated *PSY-1* in a r, r mutant and with down regulation conferred by an antisense gene, enzymatic formation of phytoene still exists, because another gene (*PSY2*) is expressed and the tissue has the enzymatic capability to synthesize carotenoids.

Moechs et al. (2001) reported that the expression of *LCY-b* was not induced during marigold petal development , wherease Del Villar-Martinez et al (2005) reported that expression of this gene was induced and might play an important role in lutein synthesis. Thus, we suggest that *LCY-b* and *LCY-e* were not induced during brassica petal development. During fruit ripening the genes mediating lycopene synthesis are up-regulated and those mediating its cyclization are down-regulated , resulting in the accumulation of this pigment in ripe fruits (Ronen et al., 1999). In conclusion, future attempts to efficiently increase metabolic flux through the MEP pathway to carotenoids and other plastidial isoprenoids should take into account the requirement to target multiple steps simultaneously.

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Part II

Overexpression of Its β-Carotene Hydroxylase Gene from Rapeseed in Transgenic Tobacco (*Nicotiana Tobacum* cv. nc).

I. Introduction

Carotenoids are naturally occurring colored pigments common to all photosynthetic organisms with antioxidative properties (Krinsky, 1989) which are involved in photoprotection and light harvesting. The unsaturated C_{40} hydrocarbons produce distinct red, orange, and yellow colors in some parts of the plant, including the flowers and fruits in higher plants. Genes and cDNAs that encode some of the carotenogenic enzymes have been isolated and characterized in bacteria, algae, fungi, and more recently, in higher plants such as tobacco, tomatoes, and peppers (Hirschberg, 2001; Rahman, 2001). Because of the importance of carotenoids, the gene-encoding enzymes of carotenoid biosynthesis in higher plants have been potential targets for metabolic engineering. The higher plants possess a special dynamic protection mechanism, the xanthophyll cycle (Hager, 1975; Demmig et al., 1987). This cycle involves the β-carotene derived xanthophylls- zeaxanthin, antherxanthin, violaxanthin, and their interconversion.

Xanthophyll lutein is the most abundant carotenoid in all plant photosynthetic tissues, where it plays an important role in light harvesting complex assembly and photoprotection

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(Demmig-Adams et al., 1996; Tian and Dellapenna, 2001). Other xanthophylls such as β-crytoxanthin, zeaxanthin, and violaxanthin are abundant in citrus fruits, crop potato tubers and hook (*Sandersonia aurantiaca*) flowers, functioning as color determinants (Goodner et al., 2001; Brown et al., 1993; Nielsen et al., 2003). Xanthophylls are also precursors for biosynthesis of the plant hormone abscisic acid (ABA). *Arabidopsis* mutants defective in carotenoid hydroxylase exhibited decreased production of violaxanthin, neoxanthin and ABA, and increased water loss, under drought-stress conditions (Tian et al., 2004). The important enzymatic steps in xanthophyll biosynthesis are hydroxylation reactions catalyzed by β-carotene hydroxylase (β- CHX). The β-carotene hydroxylase (β-CHX) catalyze the β-ring hydroxylation for the formation of zeaxanthin, violaxanthin and neoxanthin. Further modification by zeaxanthin epoxidase produces violaxathin that is finally converted to neoxanthin by neoxanthin synthase (**Fig. 2**).

Genes for β-*CHX* have been isolated from a variety of photosynthetic and non-photosynthetic bacteria, green algae and higher plants such as *Arabidopsis*, tomato, pepper and citrus (Sun et al., 1996; Bouvier et al., 1998; Cunningham et al., 1998; Kim et al., 2001). β-CHX synthesis is closely related to the pigmentation of flowers and fruits and the production of ABA. A strong increase in β-*CHX* synthesis was observed in the yellow and orange varieties of the marigold (*Targetes erecta* L.) flower (Moechs et al. 2001). During the coloration of citrus fruits β-*CHX* synthesis has been found to increase in their flavedo and juice sacs (Kato et al., 2004). Overproduction of β-*CHX* enhanced stress tolerance to UV-light, intense light, and high temperature. Inhibition of β-*CHX* synthesis by antisense polynucleotides has been shown to alter the accumulation of β-carotene-derived xanthophylls in *Arabidopsis* (Davison et al. , 2002; Gotz et al. 2002 ; Rissler and Pogson, 2001).

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Rapeseed (*Brassica napus cv*. Halla) is an indigenous plant in Jeju Island, Korea and traditionally has been cultivated as an ornamental plant and an oil source. Its bright-yellow flower is rich in xanthophylls. In this study, we engineered transgenic tobacco plants overexpressing the β-*CHX* gene from *B. napus* constitutively. Carotenoid composition and

expression levels from putative transformants leaves were analyzed using high performance liquid chromatography (HPLC). Also, this study aimed to show the effects of the constitutive expression of *Br* β-*CHX* gene in transgenic tobacco plants. Here, the consequences of an enhanced biosynthetic capacity were examined in transgenic tobacco.

II. Materials and Methods

1. Plant materials

The tobacco (*Nicotiana tobacum* cv. NC) was used in this study. The seeds were sterilized by immersion in 25% (v/v) YUHANROX solution (commercial bleach containing 4% sodium hypochloride) for 10 min and were rinsed three times with sterile distilled water. The surface sterilized seeds were blot-dried on sterile filter paper for 5 min. The seeds were then placed on petri dishes containing hormone-free MS medium (Murashige and Skoog, 1962) supplemented with 30 g/ℓ sucrose and solidified with 8 g/ℓ plant agar. Each culture plate was incubated at 25℃ under a 16/8 hr (day/night) photoperiod.

2. *Agrobacterium* strain and construction of plant vector

The *Agrobacterium tumefaciens* strain LBA4404 carrying the binary vector pBI121 was used. *Agrobacterium* was cultured on YEP medium (An, 1987) supplemented with 50㎎/ℓ kanamycin sulfate (Sigma-Aldrich) using standard procedures (Curtis et al., 1994).

The full-length β-*CHX* cDNA was introduced into the pBI121 vector at the BamHI and SstI site to replace the GUS gene (Jefferson et al., 1987). The resulting construct was named pBIBr-CHX(+) (**Fig. 25**). This vector was introduced into *A. tumefaciens* strain *LBA4404* (CLONTECH).

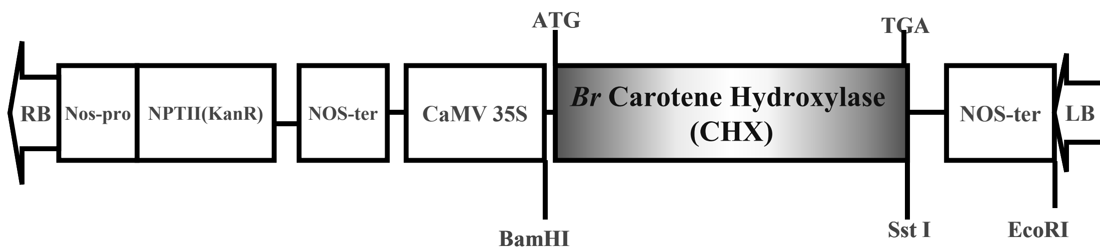

Fig. 25. Structure of the T-DNA region of pBI121 binary vector including *Br* β-*CHX*. RB, right border; Nos-pro, NOS promoter; NPTII (Kan R), neomycin phosphotransferase gene (kanamycin resistance); NOS ter, NOS terminator; LB, left border.

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3. Transformation

Agrobacterium-mediated transfromation of *N. tobaccum* cv. nc was conducted by the method of Horsch et al., (1995). Leaves were cut into around 0.5cm discs. The explants were inoculated with *A. tumefaciens* suspensions for 5 to 10 min. Then transferred to Murashige and Skoog (MS) medium supplimented with 1 ㎎/ℓ benzyladenine (BA) and 0.01 ㎎/ℓ naphthylacetic acid (NAA). After 2 days of coculture, explants were transferred to selective regeneration medium consisting of MS medium with 1 ㎎/ℓ BA, 0.01 ㎎/ℓ NAA, 100 ㎎/ℓ kanamycin, and 500 ㎎/ℓ carbenicillin. The developing shoots were transferred to MS medium with 100 ㎎/ℓ kanamycin and 500 ㎎/ℓ carbenicillin for rooting. The rooted plants (T_0) were transferred to soil and grown in a growth chamber.

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4. DNA isolation and confirmation of transgenic plants by PCR

All DNA manipulations were carried out according to standard methods (Sambrook et al., 2001). For PCR analysis, DNA was isolated from the young leaves of putative transgenic plants according to Gawel and Jarret (1991). Genomic DNA was amplified by PCR using β-*CHX* specific primers and full gene primers to represent 900 bp. PCR amplification of a 900bp β-*CHX* fragment was performed using primers; the forward primer (5'-ATG GCG GCA GGT CTC TCA ACC AC-3') and the reverse primer (5'-AAC TTC TTC CAA TTC CTT TGG TCC-3'). These primers were designed on the basis of the conserved regions of the previously reported β-*CHX* sequence (Accession No. AY545229). To amplify the presence of a full sequence of β-*CHX* genes, the 35S forward primer, 5'-CACTATCCTTCGCAAGAC-3' and the Nos -T reverse primer, 5'-GCAAC AGGATTCAATCTTAAG-3' were designed from regions between 35S promoter and Nos terminator. Plant genomic DNA (1 ㎕, approximately 200ng) was mixed with a pair of primers, 10 pmoles each, 5 ㎕ of 10×buffer, 5 ㎕ of 2.5 mM

dNTP mixtures, and 0.5 ㎕ of Taq DNA Polymerase (Takara Taq 0.25units), and adjusted with double distilled water to a final volume of 50 ㎕. The PCR was performed for 30 cycles of 1 min at 94℃, 1 min at 52℃, and 1 min at 72℃ with a thermal cycler (MJ Research, USA).

5. RNA extraction and reverse transcription (RT)-PCR analysis

Transgenic plants were analyzed for the presence of PSY transcripts. Total RNA was prepared from leaves of both the wild type and transgenic plants using Trizol reagent according to the manufacturer's instructions (MRC). The integrity of the total RNA was examined by running 2 ㎍ of each sample in a 1.2% agarose gel.

A 3 ㎍ of total RNA was treated with reverse transcriptase (MBI) at 42℃ for 60 min. First-strand cDNA was amplified for 30 cycles by a specific primer β-*CHX* of gene. The initial denaturation step was at 94℃ for 5 min. PCR was performed under the following conditions: a denaturation step at 94℃ for 1 min, annealing step at 55℃ for 1 min, extension step at 72℃ for 30 cycles of 1min and a final extension step at 72℃ for 5 min.

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6. Northern blot analysis

Northern blot analysis was performed against total RNA preparations. Ten ㎍ of total RNA was separated on a 1.2% denaturing agarose gel, and then transferred to positively charged nylon membrane (Schleicher & Schull, USA). According to the instruction manual (BD Bioscience, USA), hybridization was carried out using α-^{32}P labeled DNA probe that was made by PCR (Sambrook et al., 2001) as previously mentioned in 'DNA isolation PCR amplification' section. The membrane was washed using standard saline solution and exposed to X-ray film at -70℃.

7. HPLC analysis

Carotenoids were extracted from leaves with different stages described above. For the isolation of crude carotenoids, approroximately 1 g of leaves was extracted three times with 50 ㎖ extraction solvent (MeOH : Ethyl acetate= 1: 1) containing 0.1% butylated hydroxy toluene. The extracts evaporated for dryness. Carotenoid extracts were seperated on a C_{18} reverse phase column at 1 ㎖/ min (waters chromatography). Carotenoids were separated by HPLC with a linear gradient beginning with a 100% eluate A (80% of acetonitrile, 15% of methanol, and 5% of ethylacetate to 100%), and eluate B at 15 min (60% of acetonitrile, 25% of methanol, and 15% of ethylacetate). Spectra were recorded using photodiode array detector (UV 6000LP) and the absorbance of the carotenoid extracts was determined at 450 nm. The absorption spectra of individual peaks were taken and compared with published data (Britton, 1995). The standard β-carotene and lutein were purchased from Sigma Chemicals. The liquid chromatograph apparatus consists of a Thermo seperation products.

III. Results

1. Production of the *35S* ::*BrCHX* plant and expression of *BrCHX* transgenic tobacco

The previously identified Br β-*CHX* gene from *B. napus* was placed under control of the cauliflower mosaic virus (CaMV) 35S promoter (**Fig. 26**) and introduced into tobacco by *Agrobacterium tumefaciens*-mediated transformation. Callus formation was observed on both cut surface segments 3 weeks after culture and adventitious shoots were developed from callus 4 weeks after culture (**Fig. 26**). Fifty putative transgenic plants (T_0) were obtained (**Fig. 26**). Rooted putative transgenic tobacco plants were acclimatized and grown in a plastic house.

The presence of the *Br*-β-*CHX* gene in putative transformants were confirmed by PCR analysis to reveal the expected DNA fragment bands of 900 bp (**Fig. 27**). Some transgenic tobacco lines represented chlorisis of leaves (**Fig. 28**). So, these lines were analyzed by RT-PCR and northern blot analysis (**Fig. 29**). Transformants were analyzed by RT-PCR for the expression of β-*CHX*. The reverse transcripts were amplified by specific primers. RT-PCR products with the predicted size, 900 bp for β-*CHX* gene were obtained from all transgenic plants (**Fig. 29**). Northern blot analysis revealed that the expression level of β-*CHX* was much higher than the non-transformant. These results showed that the β-*CHX* gene cloned from *B. napus* could be expressed in transgenic tobacco using plant expression vector.

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Fig. 26. Transgenic tobacco harboring β-*carotene hydroxylase* isolated from rapeseed (*B. napus* cv. Halla).

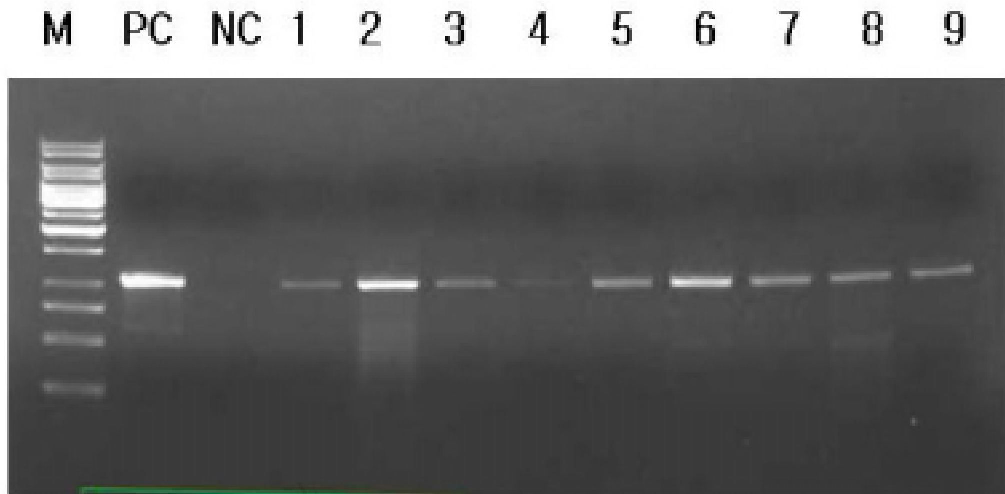

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Fig. 27. PCR analysis of putative transgenic plants. PCR bands were detected for *Br* β-*CHX* full gene by 35S primer and Nos terminator specific primers.

M, 1kb plus ladder marker; PC, Plasmid (positive control); NC, Non-transformant (negative control); Lane 1-9, putative transformants.

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Fig. 28. Putative transgenic tobacco showing chlorisis of leaves.

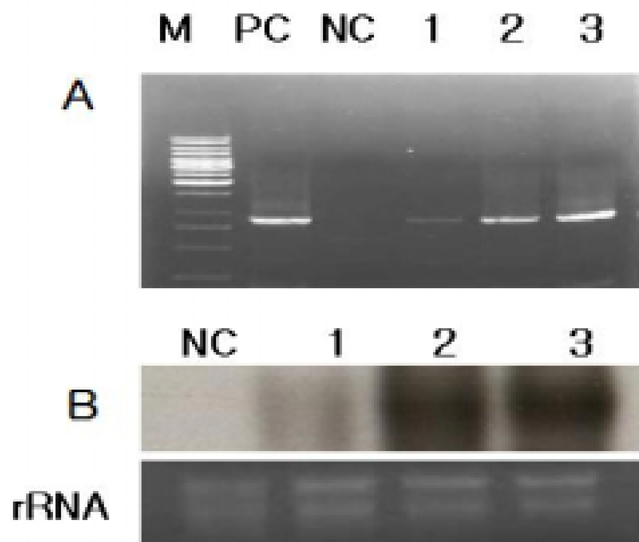

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Fig. 29. RT-PCR (A) and Northern blot analysis (B) of putative transformants.

A: RT-PCR showing chlorisis lines; B: Northern blot analysis showing chlorisis lines
M, 1kb ladder marker; PC: Positive control (plasmid); NC: negative control (non-transformant plant), 1, transformant #27; 2, transformant #35; 3, transformant #56.

2. Carotenoid composition of *BrCHX* transgenic tobacco

Carotenoid pigments in putative transformants were analysed by HPLC, which indicated that violaxanthin, neoxanthin, and antheraxanthin were the increasing predominant carotenoids contrast to non-transgenic plant (**Figs. 30, 31**). As expected, the amount of xanthophylls was roughly 2 ~ 4 fold greater in sense β-*CHX* transgenic lines than in wild-type. Putative transformants had already a lutein content of 118.8 ㎍ /g fresh weight, and a β-carotene content of 202 ㎍ /g fresh weight which decreased 2-fold rather than non-trangenic control (**Fig. 32**)

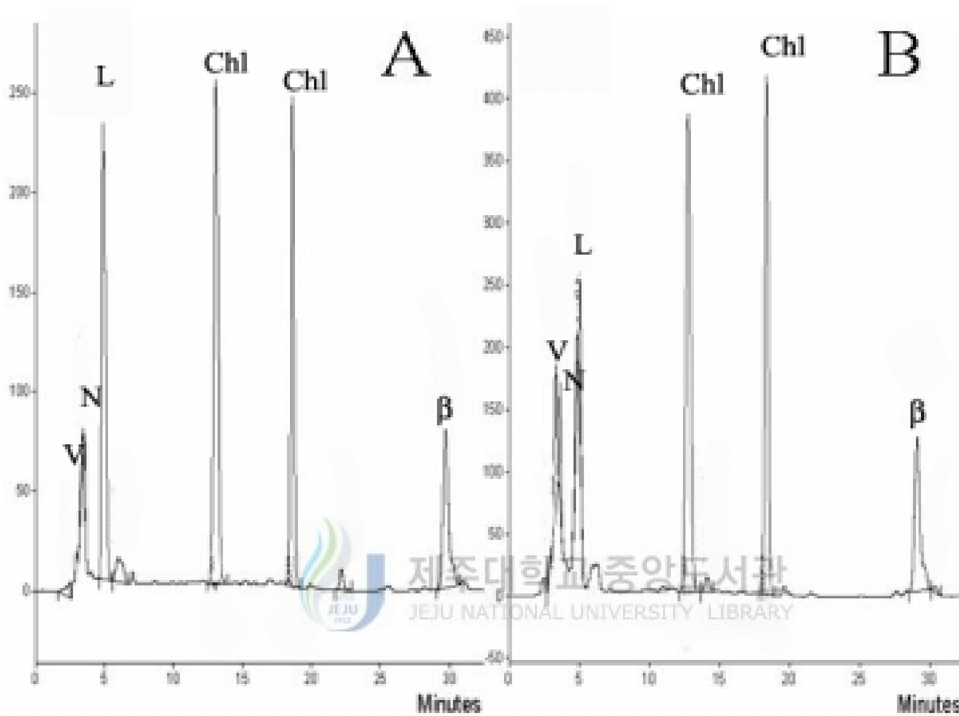

Fig. 30 . The HPLC profiles of crude carotenoids from non transgenic plant (A) and transgenic plant of tobacco (B) .

L, lutein; N, neoxanthin; V, violaxanthin, β, β-carotene; Chl, chlorophylls

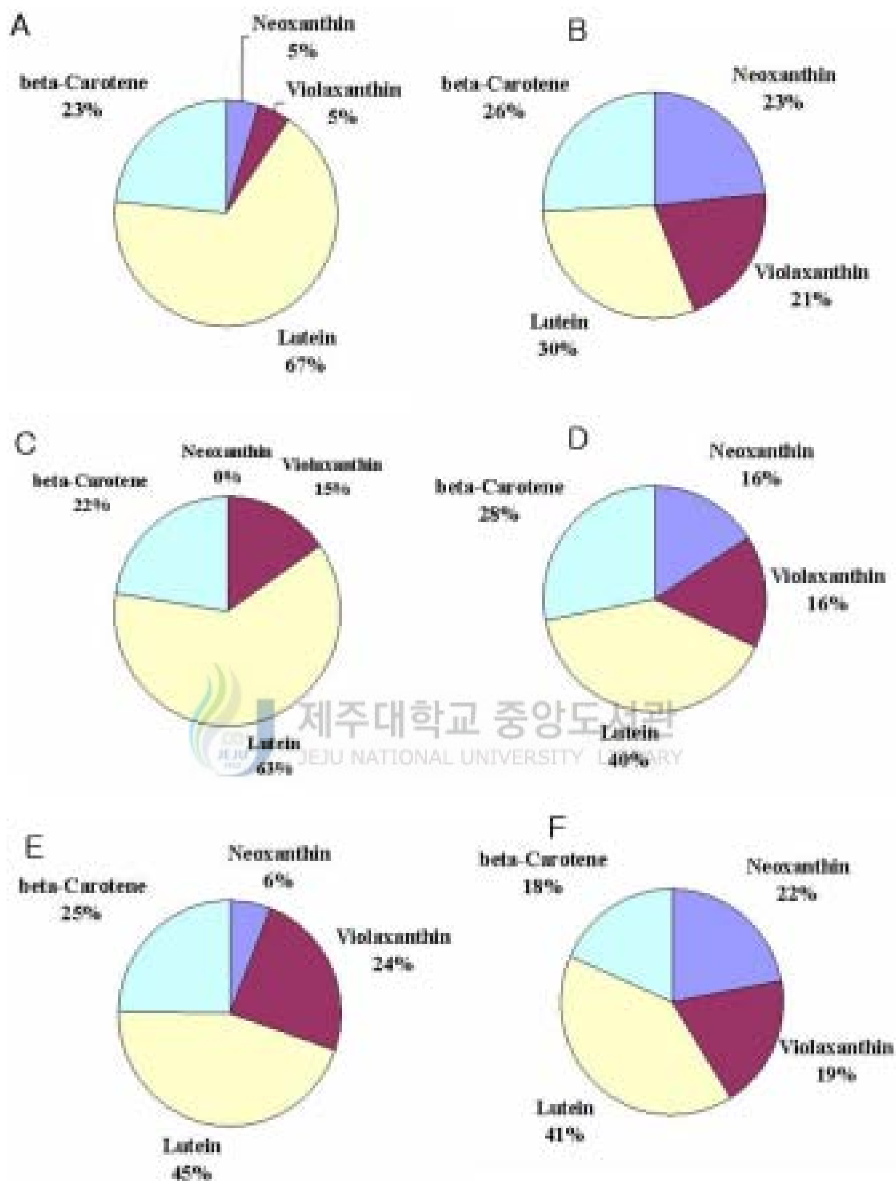

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Fig. 31. Comparisons of carotenoid compositions between transgenic and non-transgenic lines. A: Control; B~F: Putative transformants.

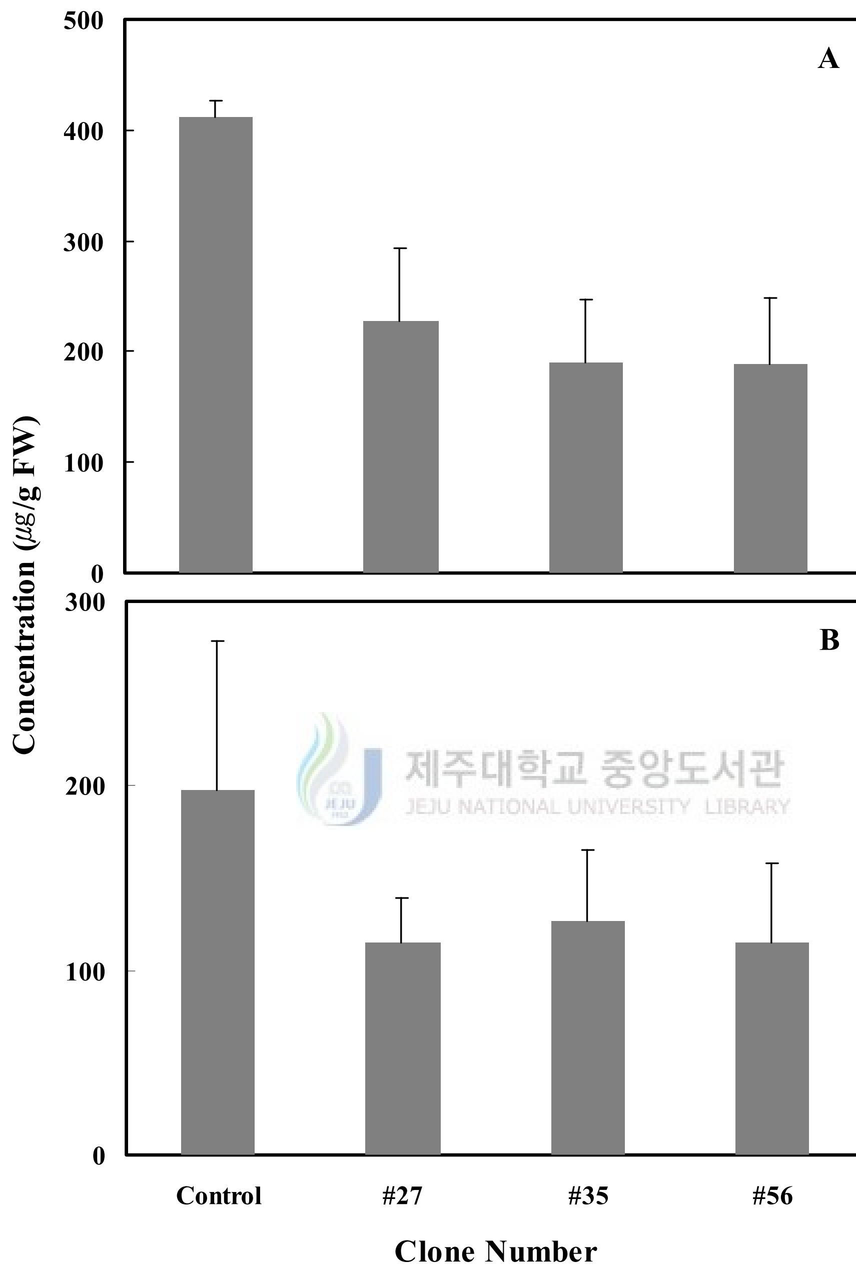

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Fig. 32. Changes in carotenoids between transgenic and non-transgenic lines. A: β-carotene; B: Lutein

IV. Discussion

In *Arabidopsis*, where *β-carotene hydroxylase* was overexpressed, there was a marked (at least two fold) increase in the amounts of violaxanthin in the sense *chyB* lines (Davison et al., 2002). Though overexpression of *β-carotene hydroxylas*e which catalyzes the conversion of β -carotene to zeaxanthin did not unduly perturb the rest of the carotenoid biosynthetic pathway, there was a slight fall in the amount of β-carotene, presumably owing to increased conversion to the xanthophylls (Davison et al., 2002).

The antisense gene constructs can be utilized for increasing β-carotene in plants. β -CHX enzyme catalyzes the hydroxylation of the β-rings of β-carotene to form xanthophylls. Antisense construct for β-*CHX* gene can be introduced into plants to block the conversion of β-carotene to xanthophylls, therby increasing accumulation of β-carotene. Expression of the antisense β-*CHX* in *Arabidopsis* resulted in a 22% increase in β-carotene (Rissler et al., 2001).

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In order to show the effect of the biosynthetic capacity for xanthophyll formation, *N. tobacum* cv. nc was transformed with a β-*CHX* gene encoding β-carotene hydroxylase from rapeseed. The presence of the *Br* β-*CHX* gene in putative transgenic tobaccos were confirmed by PCR analysis to reveal the expected DNA fragment bands of 900bp (**Fig. 27**). Characterization of the tobacco transformants by RT-PCR and Northern blot analysis proved that the rapeseeds's β-*CHX* transgene is expressed (**Fig. 29**). These results showed that the *CHX* gene cloned from *B. napus* could be expressed in transgenic tobacco using plant expression vectors. As shown by **Fig. 28**, transformants showed leaf chlorisis. Ruban et al (1994) reported the concentration and composition of leaf xanthophylls are affected by light intensity. Therefore, these phenomenon would be relate to light intensity in transformants lines by expression of β-*CHX*.

As shown by **Fig. 30,** expression of β-*CHX* showed increasement of violaxanthin and neoxanthin and decrement of lutein in transgenic tobacco. The identities of violaxanthin and neoxanthin were confirmed by UV spectrum reported previously (Britton et al., 2004), and mass spectrometry. We concluded that the tobacco transformants encode the enzyme β-carotene hydroxylase. Our results showed that increasing expression of the β-carotene hydroxylase gene brings about an increase in the content of the xanthophyll compounds. But, lutein and β-carotene were decreased predominantly (**Fig. 32**).

Overexpression of *β-carotene hydroxylase* did not unduly perturb the rest of the carotenoid biosynthetic pathway in the sense *chyB* lines, there was only a slight fall in the amount of β-carotene, presumably owing to increased conversion to the xanthophylls (Davison et al., 2002) **.**

The effect of the genetic manipulation on carotenoid content and composition showed that some alterations in pigment distribution indicating either a stringent control of carotenogenesis, feedback regulation or a limited substrate supply. It is suggested that genetic manipulation of a single enzyme in carotenoid metabolism can bring about a pronounced increase in xanthophylls.

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요 약

식물유래 카로티노이드는 엽록체와 잡색체 막에 있는 적색, 황색, 노란색을 띄는 지용성 색소로서 식물의 꽃과 과실에 색깔을 부여하는 색소이다. 카로티노이드는 식물의 광합성기구의 구성분이며 빛으로부터 방어, abscisic acid 합성용 기질, 그리고 활성산소 제거능을 가지고 있다.

유채는 제주에서 많이 재배되어 온 농작물로서 유채 꽃은 관광자원으로 많이 활용되고 있다. 유채꽃은 isoprenoid 유래 카로티노이드 색소를 함유하고, 카로티노이드 연구의 중요한 재료로서 가치가 높다.

과거에 비해 최근 10년 사이에 빠른 속도로 카로티노이드 생합성에 관련된 효소 유전자들이 클로닝됨에 따라, 카로티노이드 생합성 경로가 많이 밝혀져 왔다. 다음과 같은 11개의 유전자 cDNA들이 클로닝 되었다; HMG Co A reductase (HMGR), 1-deoxy-D-xylulose-5-phosphate synthase (DXPS), geranylgeranyl pyrophosphate synthase (GGDPS), phytoene synthase (PSY), phytoene desaturase (PDS), lycopene β-cyclase (LCY-b), lycopene ε-cyclase (LCY-e), β-carotene hydroxylase (β-CHX*)*, ε-carotene hydroxylase (ε-CHX*)*, zeaxanthin epoxidase (ZEP), and glyceraldehyde 3 phosphate dehydrogenase (GAPDH). 본 연구에서는 유채에서 카로티노이드 생합성의 조절단계를 밝히고자 하였고, β-carotene과 크산토필 축적에 관여하는 9개의 유전자 (D*XPS, GGDPS, PSY, PDS, LCY-b, LCY-ε, β-CHX, ε-CHX*, 그리고 *ZEP*)에 대한 연구가 진행되었다.

카로티노이드 생합성 조절에 중요한 효소들을 합성하는 유전자들인 *DXPS, PSY, LCY-b, LCY-ε, β-CHX,* 그리고 *ε-CHX* 는 RACE법에 준하여 클로닝되었으며, 그들의 염기 및 아미노산 서열을 분석한 결과 *Arabidopsis*와 73-94%의 유사성을 보였다.

또한 카로티노이드의 농도와 조성, 생합성 유전자 발현간의 상관관계를 파악하고자 하였다. 유채의 잎, 꽃잎, 꽃봉오리에서 HPLC로 카로티노이드 화합물을 분석, LC/MS로

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동정하였다. Northern 분석 결과, 카로티노이드 생합성 유전자의 발현 패턴이 조직에서 서로 다름을 보였다. *PSY*유전자 는 유채의 잎, 꽃잎, 꽃봉오리 모든 조직에서 발현이 되었고, β-carotene과 유래 크산토필에 관여하는 유전자들은 잎과, 꽃봉오리에서 mRNA의 발현이 높았다. *ZEP* 유전자는 유채 조직에서 거의 일정하게 발현하였다.

크산토필 생합성의 중요한 단계는 두가지 형태의 hydroxylase에 의한 히드록실 반응이다. 하나는 β-ring의 히드록실 반응을 촉매하여 zeaxanthin, violaxanthin, neoxanthin을 형성하는 β-carotene hydroxylase 이고, 다른 하나는 ε-ring의 히드록실 반응을 촉매하여 루테인을 형성하는 ε-carotene hydroxylase이다. Zeaxanthin은 zeaxanthin epoxidase에 의해 violaxanthin이 형성되고, 이는 다시 neoxanthin synthase에 의해 neoxanthin이 형성된다.

카로티노이드 생합성 유전자 중 중요한 조절 단계에 해당하는 β-carotene hydroxylase의 기능 및 발현을 파악하고자 식물발현 벡터에 유채에서 분리된 β-*CHX* 를 결합시켜 재조합체를 만들고, *Agrobacterium*법에 준하여 담배를 형질전환시켰다. 유채에서 분리된 β-*CHX* 유전자는 912개의 nucleotide로 구성되었으며 304개의 amino acid의 33.4 kDa polypeptide에 상당하였다. 50개의 담배 형질전환체들이 재분화, 선택되었고, genomic DNA PCR, RT-PCR , Northern blot 분석으로 확인되었다. 그리고 β-*CHX* 유전자의 발현과 카로티노이드 조성을 분석한 결과 대조구에 비해 형질전환체에서 violaxanthin과 neoxanthin의 함량이 증가함을 볼 수 있었다. 이는 β-carotene hydroxylase 가 카로티노이드 생합성 경로에서 합성조절에 중요한 효소임을 제시한다.

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