Ph. D. Thesis

Functional Analysis of Carotenoid Biosynthesis Genes in Sweetpotato *Ipomoea batatas*

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ABSTRACT

Sweetpotato *Ipomoea batatas* is known as a hexaploid species. Carotenoids contents in the leaves and tubers of sweetpotato cultivars 'White Star' (WS) and W71 were analyzed chemically. These cultivars were found to contain several carotenoids unique to sweetpotato tubers such as β-carotene-5,6,5′,8′-diepoxide and β-carotene-5,8-epoxide. Two kinds of carotene cyclase genes that encode lycopene β- and ε-cyclases were isolated from the WS and W71 leaves, by RT-PCR and subsequent RACE. Two and three lycopene β-cyclase gene sequences were, respectively, isolated from WS, named *IbLCYb1*, 2, and from W71, *IbLCYb3*, *4*, *5*. Meanwhile, only a single lycopene ε-cyclase gene sequence, designated *IbLCYe*, was isolated from both WS and W71. These genes were separately introduced into a lycopene-synthesizing *Escherichia coli* transformed with the *Pantoea ananatis crtE*, *crtB* and *crtI* genes, followed by HPLC analysis. β-carotene was detected in *E. coli* cells that carried *IbLCYb1-4*, indicating that the *IbLCYb1-4* genes encode lycopene β-cyclase. Meanwhile, the introduction of *IbLCYe* into the lycopene-synthesizing *E. coli* led to efficient production of δ-carotene with a monocyclic ε-ring, providing evidence that the *IbLCYe* gene codes for lycopene ε-(mono) cyclase. Expression of the β- and ε-cyclase genes was analyzed as well.

Two kinds of carotene hydroxylase genes that encode non-heme di-iron carotene hydroxylase (BHY) and heme-containing cytochrome P450-type carotene hydroxylase (CYP97B) were isolated from the W71 and WS leaves, by RT-PCR and subsequent RACE, and found that each gene consists of single copy. These genes were respectively named *IbBHY1*, *IbBHY2* *IbCYP97B1* and *IbCYP97B2*. Whereas, two and four heme-containing cytochrome P450-type carotene hydroxylases (CYP97A) were isolated from W71 and WS, and named *IbCYP97A1*, *2* and *IbCYP97A3*, *4*, *5*, *6*, respectively. When *IbBHY1* and *IbBHY2* were separately introduced into β-carotene-accumulating *E. coli* due to the presence of plasmid pACHP-Beta, and resultant transformed cell cultures were analyzed by HPLC, β-cryptoxanthin and zeaxanthin were detected. This result indicates that both *IbBHY1* and *IbBHY2* hydroxylated the β-ring of β-carotene to synthesize zeaxanthin via β-cryptoxanthin. Contrarily, introduction of *IbCYP97A1-IbCYP97A6* and *IbCYP97B1* and *IbCYP97B2* genes into β-carotene-accumulating *E. coli* did not change the composition of the carotenoids, and β-carotene was detected when analyzed by HPLC. Similarly, introduction of *IbCYPP97A1-IbCYP9A6* and *IbCYP97B1* and *IbCYP97B2* genes into α-carotene accumulating *E. coli* did not change the carotenoids composition. These results suggested that these *IbCYP97A* and *IbCYP97B* gene sequences were not able to function as carotene hydroxylases in *E. coli.*

Introduction of a bacterial ketolase gene *crtW* in cultivar W71 resulted in the accumulation of three novel carotenoids in transgenic W71, identified by high resolution ESI-MS and ^{1}H NMR data analyses.

KEY WORDS

Sweetpotato, W71, WS, Carotenoids accumulation, High performance liquid chromatography, RACE, PCR, *E. coli*, Transformation, Cloning, Gene isolation, Sequence Analysis, Phylogenetic analysis, Functional analysis, Carotenoids extraction, Agrobacterium, Carotenoids ketolase, *crtW*, Metabolic engineering, Plant Transformation, UV-vis, ESI-MS, ^{1}H-NMR, CD Spectral data, COSY and NOESY Experiments, Novel carotenoids.

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Muhammad Zubair Khan
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Dedicated to my beloved life mate Maleeha and my cute little Angel Wardah Zubair

TABLE OF CONTENTS

LIST OF FIGURES

Figure Page

LIST OF TABLES

Table Page

ABBREVIATIONS

AO	aldehyde oxidase
AVRDC	Asian Vegetable Research and Development Center
BHY	carotene β-ring hydroxylase (non-heme di-iron carotene hydroxylase)
BLAST	Basic Local Alignment Search Tool
CAGR	compound annual growth rate
CaMV	cauliflower mosaic virus
CD	circular dichroism
$CDCl_3$	Trichloro (^2H) methane (Deuterated chloroform)
CIP	Centro Internacional de la Papa CIP (International Potato Center)
Cm^r	kanamycin resistance gene
COSY	correlation spectroscopy
CrtB	phytoene synthase (*Pantoea ananatis*)
CrtE	GGPS equivalent in bacteria (*Pantoea ananatis*)
CrtI	phytoene desaturase (Bacterial, *Pantoea ananatis*)
CRTISO	carotenoid isomerase
CrtM	dehydrosqualene synthase (Bacterial PSY)
CrtO	β-carotene ketolase (*Synechocystis*)
CrtW	β,β-carotenoid 4,4′-ketolase (*Brevundimonas* sp.)
CrtX,	zeaxanthin glucosyltransferase (*Pantoea ananatis)*
CrtY	lycopene β-cyclas (bacterial; *Pantoea ananatis*)
CrtZ	β,β-carotenoid 3,3′-hydroxylase (*Brevundimonas* sp.)
CYP97	heme-containing cytochrome P450-type carotene hydroxylase
DMAPP	dimethyl allyl pyrophosphate
DMSO	dimethyl sulfoxide
DNase	deoxyribonucleas
dNTP	deoxy ribonucleotide triphosphate
DW	dry weight
DXP	1-deoxy-D-xylulose-5-phosphate
DXR	DXP reducto-isomerase
DXS	DXP synthase
EDTA	Ethylenediaminetetraacetic acid
ESI	electro-spray ionization
FPP	farnesyl pyrophosphate
FW	formula weight

G3P	glyceraldehyde 3-phosphate
GGPS	geranylgeranyl pyrophosphate synthase
GPP	geranyl diphosphate
^{1}H NMR	proton nuclear magnetic resonance
HIV	human immunodeficiency virus
HPLC	high performance liquid chromatography
HRMS	High Resolution Mass Spectrometry
IDI/id i	isopentenyl diphosphate isomerase (*Paracoccus* sp.)
IPP	isopentenyl diphosphate
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IU	international unit (1.0 IU of β-carotene=0.6μg of β-carotene)
Km^{r}	kanamycin resistance gene
LC/MS	Liquid Chromatography/Mass Spectrometry
LCYb	lycopene β-cyclas
LCYe	lycopene ε-cyclase
LDL	low-density lipoprotein
Mbp	millions of base pairs
MEP	2*C*-methyl-D-erythritol 4-phosphate
MVA	mevalonic acid
NCED	9-cis-epoxycarotenoid dioxygenase
NOESY	Nuclear Overhauser Effect Spectroscopy
NPQ	non-photochemical quenching
NSY	neoxanthin synthase
PDA	photodiode array detector
PDS	phytoene desaturase
PSY	phytoene synthase
PVACs	Pro-vitamin A carotenoids
RACE	Rapid Amplification or cDNA (Complementary DNA) Ends
RNase	ribonuclease
SCF	sequence chromatogram files
SQS	squalene synthase
TAE	Tris-Acetate and EDTA (buffer)
TE	Tris base (tris- amino methane) and EDTA (buffer)
TOF	time-of-flight
2YT	two-time yeast extract and tryptone media
USDA	United States Department of Agriculture
UV	ultra violet
UV-vis	Ultraviolet–visible spectroscopy

v/v	volume per volume
VDE	violaxanthin de-epoxidase
w/v	weight per volume
ZDS	ζ-carotene desaturase
ZEP	zeaxanthin epoxidase
Z-ISO	ζ-carotene-isomerase

1. INTRODUCTION

1.1 Sweetpotato

Sweetpotato [*Ipomoea batatas* (L.) Lam], which is also described as “sweet potato”, belongs to the family Convolvulaceae and occupies the seventh position among the food crops of the world after wheat, rice, maize, potato, barley, and cassava (Ahn et al., 2010; Khan et al., 2016; Rodriguez-Bonilla, et al., 2014).The largest genus in family Convolvulaceae is *Ipomoea* consisting, 600-700 species among which only *Ipomoea batatas* (L.) Lam. is cultivated widely as a food crop around the world (Hirakawa et al., 2015; Austin and Huám, 1996). The genome of sweetpotato is structurally complex and has a size of 4.8-5.3 pg/2C nucleus (Ozias-Akins and Jarret, 1994). Due to the existence of polyploidy, sweetpotato is a hexaploid species (2n=6x=90) that has a basic chromosome number of 15, (Hirakawa et al., 2015) with a huge genome size of 2,200 to 3,000 Mbp (Yan et al., 2014). The genetic studies on this species are exhausting, since it is difficult to generate seeds and to evaluate the effects of polyploidy on the genome (Roullier et al., 2013; Arizio et al., 2014). Such a complex structure of its genome also manifests self and cross-incompatibility causing barrier for genetic studies on several important agronomical characters (Martin, 1965; Hirakawa et al., 2015). Its tubers exhibit various colors such as white, yellow, orange, and purple among different cultivars, and all species include β-carotene as well as other carotenoids, while orange-fleshed lines were shown to contain β-carotene as the predominant carotenoid (Purcell et al., 1968; Teow et al., 2007; Grace, et al., 2014; Holden et al., 1999; Burns et al., 2003). In Japan the high yielding orange-fleshed varieties include *Benihayato* and *Kyushu* No. 114, while, among purple-fleshed, varieties, *Ayamurasaki* is a high anthocyanin rich cultivar developed by the Kyushu National Agricultural Experiment Station (Yamakawa et al., 1997; Nedunchezhiyan et al., 2012).

1.1.1 Economic Importance of Sweetpotato

Sweetpotato is a major staple crop especially in many of the tropical countries (Lebot 2009), subtropical countries and has the potential to provide nutritional benefits to the people of both rural and urban regions (Woolfe, 1992; Fesco and

Boudion, 2002; Alam et al., 2016). It is cultivated in more than 100 countries (Mohan and Nair, 2012) with a world estimated production of 107 million metric tonnes, Asia (74.3%), Africa (21.2%), Americas (3.6%), and Oceania (0.8%). (FAO, 2017). Developing countries highly depend on root and tuber crops as a major source of food, nutrition or income (Scott et al., 2000). Therefore, it is important to critically reassess multipurpose and easily available root and tuber crops with wide ecological adaptability for their usefulness in human nutrition. Among such crops, sweetpotato is a unique one for having a high harvest index supplemented with drought tolerance, and a wide range of adaptability to diverse climatic conditions and farming systems (Diop, 1998; Jiang et al., 2004a). Moreover, the cultivation costs for sweetpotato are low because it requires very few inputs and labour. Such agronomic characteristics make it suitable especially for individuals threatened by migration or lethal diseases such as HIV/AIDS (Jayne et al., 2004; Mwanga et al., 2011). In Asia, approximately half of the sweetpotato total production is used for animal feed and the rest is used for human consumption. Whereas, in Africa, almost all of the production is used as human food (Ahn et al., 2010). Sweetpotato also possesses potential for bioenergy production as it can grow on marginal lands (Kim et al., 2014) and is an important material for industry as a crop for new energy resource (Mohan and Nair, 2012). Reports on the use of sweetpotato tops as a vegetable in many areas of the world (Villareal, et al., 1982; As-Saqui , 1982; Nwinyi, 1992) shows that they are acceptable as edible like other traditional leafy vegetables (Islam, 2016). As compared to other tuber crops, the per unit area production of energy by sweetpotato is desirably high but, it is ranked third after potato and cassawa production around the world (Nedunchezhiyan et al., 2012; Chagonda et al., 2014).

1.1.2 Genetic Origin and Diversity

Sweetpotato cultivation is widespread in tropical, sub-tropical and even in some of the developing countries situated in temperate zones with variable climates (Ahn et al., 2010). Based on the variability in flesh color, white-fleshed to cream-fleshed sweetpotato is widespread in the Pacific whereas in the United States yellow to orange-fleshed sweetpotato is predominantly grown (Mohan et al., 2012). The

main commercial contributors of sweetpotatoes are China, Indonesia, Vietnam, Japan, India, and Uganda (Senanayake et al., 2014). The largest grower is China with 76% of the total world sweetpotato production (Mohan et al., 2012). At CIP the sweetpotato germplasm collection comprises 7,777 accessions, that include 4,615 landraces (native varieties), 1,984 breeding lines (improved varieties), and 1,178 samples of wild sweetpotato. They originate from Asia, Africa, the Americas, and the Pacific Islands (CIP, 2012). The international Potato Centre (CIP) records has reported 6,500 varieties or cultivars of sweetpotato from year 1,895 to 2,000 that include accessions, "wild" varities and selected varities (Ellong et al., 2014; Huaccho and Hijmas, 2000).

Archaeological records show that sweetpotato, native to Americas is one of the oldest domesticated and clonally propagated crops in the Americas (Roullier et al., 2013). Sweetpotato genetic diversity explored by use of molecular markers provided evidence that Central America is the primary centre of diversity and most likely its centre of origin (Haung and Sun, 2000). Based on analysis of morphological characters of sweetpotato and the wild *Ipomoea* species, the centre of origin of *I. batatas* was supposed to be somewhere between the Yucatan Peninsula of Mexico and the mouth of the Orinoco River in Venezuela more than 10,000 years ago (O'-Brien, 1972; Zhang et al., 2004; Austin, 1987; Rodriguez-Bonilla, et al., 2014).

Sweetpotato was introduced to Western Europe from West Indies after the first voyage of Columbus in 1492. Then, it was transferred to Africa, India, South East Asia and the East Indies in the 16th century, by Portuguese explorers, while, direct transfer of the plant was done by Spanish trading galleons from Mexico to the Philippines (Srisuwan et al., 2006; Rossel et al., 2001). Some of the genetic and cytological studies on its genome indicate that the diploid *Ipomoea trifida* is its closest wild relative. (Roullier et al., 2013; Buteler et al., 1999; Srisuwan et al., 2006). Because both *Ipomoea trifida* and *Ipomoea batatas* are cross-compatible with each other (Shiotani and Kawase, 1989; Katayama et al., 2006). The complete mechanism of the polyploidization in sweetpotato is yet not fully discussed (Roullier et al., 2013). Studies based on the cytogenetic analyses of a

series of interspecific hybrids between sweetpotato and *Ipomoea trifida* proposed that sweetpotato is an autohexaploid derived from diploid *Ipomoea trifida* (Shiotani and Kawase, 1989). New Guinea is considered the most important secondary centre of diversity for sweetpotato particularly the highlands region where the total number of cultivars grown has been roughly estimated to be about 5,000 (Bourke, 2009; Roullier et al., 2013).

1.1.3 Nutritional Composition and Health benefits of Seetpotato

In comparison with other tuber crops sweetpotato comprises higher contents of carbohydrates, many minerals, and more protein estimates than other vegetables (Shih et al., 2007; Ji et al., 2015). It also contains much higher levels of provitamin A, vitamin C, and minerals than rice or wheat (Wang et al., 1997). 100 g of raw sweetpotato contains: 1.57 g protein; 20.12 g carbohydrates; 3.0 g total dietary fiber; 41.8 g total sugars, 30.0 mg calcium; 0.61 mg iron; 25 mg magnesium; 47.0 mg phosphorous; 337.0 mg potassium; 55.0 mg sodium; 0.3 mg zinc; 2.4 mg vitamin C; 0.5 mg niacin; 0.2 mg; vitamin B6; 14187 IU vitamin A; 0.2 mg vitamin E; 11 µg vitamin B-9; and 8509 µg β-carotene (USDA National Nutrient Database, 2016). Sweetpotato starch is easy to digest therefore, it is a valuable constituent in the preparation of excellent weaning meals (Antonio et al., 2011). It is a source of food supply to combat malnutrition in the developing nations, since the tuberous roots (tubers) are enriched with starch and dietary fiber, along with carotenoids, anthocyanin, ascorbic acid, potassium, calcium, iron, and other bioactive ingredients (Khan et al., 2016; Purcell and Wallter, 1968; Yoshinaga et al., 1999; Teow et al., 2007; Grace, et al., 2014). For people of South-east Asia and Africa, this crop is the main source of β-carotene (Khan et al., 2016; Liao et al., 2008). The tubers of the Japanese cultivar “Benimasari” were found to accumulate unique carotenoids such as β-carotene-5,8,5′,8′-diepoxide (40.5% of the total carotenoids), β-carotene-5,8-epoxide (6.5%) and ipomoeaxanthin A (3.2%), in addition to β-carotene (10.5%) (Maoka et al., 2007). Sweetpotato may have many health positive effects since it contains high amounts of numerous phytochemicals in roots or leaves (Ji et al., 2015; Tsuda et al., 1998). Sweetpotato cultivars with color-fleshed tubers have been reported for their excellent bioactivities such as antimutagenic (Mazza et al., 2002; Garzon et al., 2009) free radical scavenging

(Tsoyi et al., 2008), hepatoprotactive, reduction of liver injury (Wang and Maza 2002; Pisha and Pezzuto, 1994), anticancer (Hagiwara et al., 2002; Kurata et al., 2007; Islam et al., 2006), anti-oxidative activities (Islam et al., 2006; Kano et al., 2005; Cho et al., 2003), antimicrobial activity, anti-hypertension, anti-inflammatory, anti-diabetic anticaries effect, ultraviolet protection (Islam et al., 2006), and Chemo-preventive activities (Tsuda et al., 2003; Kamei et al., 1995). Previous reports suggest that tubers of *Ipomoea batatas* may be useful for treating peptic ulcers (Panda and Sonkamble, 2012).

Starch composition is 50% on average of the total carbohydrates. The composition of sugars vary among varieties and even between the same cultivars depending upon the maturity stage. Sucrose is dominant with fructose and glucose but, sweetpotato shows a low glycemic index of 50 (Ellong et al., 2014; Joseph 2006) which makes it suitable for diabetic or overweight individuals (Ellong et al., 2014). Total carotenoids in sweetpotato range from trace to amounts above 9 mg/100 g (Hagenimana et al., 1998). Among minerals, potassium is predominantly present in sweetpotato (Laurie et al., 2012). The leaves of sweetpotato are rich in protein, vitamin B, iron, calcium, and zinc, and are resistant to diseases and pests as compared to numerous other vegetables (Yoshimoto, 2001; Islam, 2016; Pace, et al., 1985; AVRDC 1985; Woolfe, 1992). The carotenoids present in the sweetpotato leaves can scavenge free radical agents as singlet oxygen quencher (Foote, 1976; Hue et al., 2011; Hue et al., 2012; Islam, 2016).

In a recent analytical report by Islam (2016), total phenol, carotenoid, anthocyanin and flavonoids contents of the sweetpotato leaves ranged from 2.0 to 22.5 (g /100 g DW), 0.9 to 23.4 (β-carotene equivalents/100 g; BET/100 g), 2.2 to 24.5 (color value/ g DW) and 62.8 to 272.2 (catechin equivalents; µg/g), respectively (Islam, 2016). Consumption of sweetpotato in Asia, range from its use as additional food of minute status to a very vital supplementary food to rice and/or other root and tuber crops (Sosinski et al., 2002). It is cooked or used to make cakes, chapatis, mandazia, bread, buns and cookies (Aguoru, et al., 2015). In United States and some other developed countries sweetpotato is strictly used as a luxury food. In Japan, it is used in novel plant products and/or nutraceuticals (Wanda, 1987). By using absorption spectroscopy Ishiguro et al., (2010) analyzed carotenoids in some eight cultivars of yellow-fleshed sweetpotato and

compared them with carotenoids in yellow-fleshed cultivars. By HPLC analyses they revealed some 17 different carotenoids from yellow and orange fleshed-sweetpotato. In yellow fleshed-sweetpotato the major carotenoids included β-carotene, 5,8; 5',8'-diepoxide (32%-51%) and β-cryptoxanthin 5,8-epxide (11%-30%), whereas, β-carotene with amounts ranging from 80%-92% were dominant in in orange fleshed cultivars.
(Kammona et al., 2014) analysed and compared the carotenoid composition in some Malaysian orange, yellow, purple and white sweetpotato flesh tubers. They reported the highest total carotenoid content from orange sweet potato followed by yellow, purple and white sweetpotato. Among the individual carotenoids analyzed, β-carotene existed in all types ranging from 91.95±2.05 µg/g DW in white sweetpotato to 376.03±11.05 µg/g DW in orange sweet potato. Traces of zeaxanthin were reported with values 5.44±3.23 µg/g DW and 20.47±2.03 µg/g DW in yellow and white sweetpotato, respectively. Lutein was available only in orange sweetpotato at trace amount of 0.91±1.03 µg/g DW. Purple sweetpotato contained only β-carotene (113.86±14.17 µg/g DW) with absence of other carotenoids

Islam et al., (2016) performed HPLC analyzes of *trans*- and *cis*- β-carotene from raw and boiled sweetpotato which included three orange-fleshed, three yellowish-cream-fleshed and one white-fleshed varieties of sweetpotato. The deep-orange-fleshed variety Kamalasundari (BARI SP-2) showed the highest amounts of β-carotene amomg all the varieties followed by yellow varieties. On the other hand, from one of the two white-fleshed varieties, only trace amounts of β-carotene were obtained with no amounts at all from the other one. Their results proposed that the orange-fleshed variteis of sweetpotato contain the highest amounts of β-carotene in raw as compared to those which were boiled.

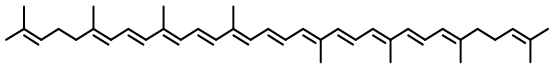
The development of an efficient and reproducible transformation system is needed for genetic manipulation of sweet potato to either improve the crop or establish it as a novel ‘transgenic plant bioreactor’ (Song et al., 2004).

Despite of huge economic value sweetpotato has not received due importance as compared with common staple crops such as wheat, maize and rice. World

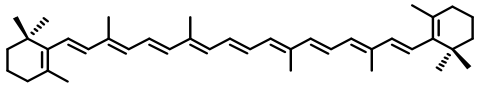
increasing hidden hunger especially in developing countries needs new foods and nutrition sources on sustainable bases. In this regard, sweetpotato not only offers immense nutritional, medicinal and industrial, potential but is also a new horizon in modern industrial biotechnological uses.

1.2 Carotenoids and their Distribution

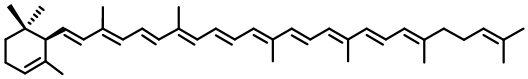
Carotenoids, the visible colors of life are the 40-carbon isoprenoids synthesized naturally by fungi, bacteria, algae, cyanobacteria (Walter and Strack, 2011; Okada *et al*., 2008; Misawa, 2010), and conspicuously by green plants including, bryophytes (Takemura et al*.,* 2014) and higher plants (Sugawara et al*.,* 2009; Misawa, 2010; Fraser and Bramley, 2004). Being intracellular, carotenoids are commonly located in the membranes of chloroplasts, mitochondria or endoplasmic reticulum (Margalith, 1999). Approximately 750 carotenoids have been reported so far (Farré, et al., 2013; Britton et al., 2004).



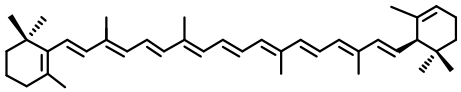
Lycopene



β-carotene



δ-Carotene



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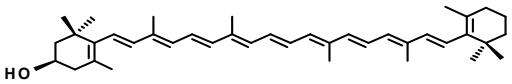
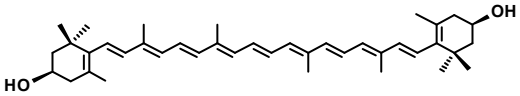
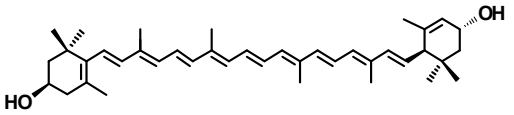


β-Cryptoxanthin



Zeaxanthin



lutein

Figure 1. Structures of some major dietary carotenoids.

1.2.1 Role of Carotenoids in Plants

Carotenoids have received increasing attraction during last decades due to their unique physiological role in plants light absorbance process during photosynthesis and protecting photosynthetic apparatus from photo-oxidization by quenching the chlorophyll triplets and singlet oxygen (Yuan et al*.,* 2015; Giuliano, 2014; Arizio et al*.,* 2014; Niyogi, 1999; Niyogi, 2000; Joseph Hirshberg, 2001). Carotenoids are the agents which aid colors and fragrance to the flowers and fruits attractive to animals consequently aiding to pollination and seed dispersal (Yamamizo et al*.,* 2010; Tanaka et al*.,* 2008; Howit and Pogson, 2006). Carotenoids also act as precursors for isoprenoid volatiles and signaling biomolecules such as abscisic acid and strigolactones which regulate plant development and function as plant protectants against environmental stress (Nisar et al., 2015; Walter and Strack, 2011; Cazzonelli, 2011).

1.2.2 Role of Carotenoids in Animals

Animals, with very few exceptions (Moran and Jarvik, 2010; Tonhosolo et al., 2009) are unable to synthesize them (Ruiz-Sola and Rodríguez Concepcióna 2012; Cazzonelli, 2011; however, carotenoids are accumulated by crustaceans, crabs, fish, crayfish, prawns and mammals and in insects such as butterflies. The animal and human diet must include carotenoids as essential nutrients (Misawa, 2010).

In marine animals, astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione) has been reported as the most commonly stored carotenoid pigment (Misawa et al., 1995). It is responsible for the red/pink coloration of crustaceans (Matsuno, 1989; Renstorm et al., 1981), and the flesh of salmonoids (Fraser et al., 1997). Astaxanthin has received much attraction for its likely role in preventing cardiovascular diseases and aging caused by UV-light in human body (Misawa, 2011).

Both α-carotene and β-carotene have provitamin A activity and are converted to retinol in the human body (Zeb and Mehmood, 2004; Jaswir et al., 2011; Thane

and Reddy, 1997; Park et al., 2009; Carillo-Lopez et al., 2010). Carotenes such as the lycopene and β-carotene play a potential role in human nutrition and act as a protectants against diseases such as lycopene protects against cardiovascular (Böhm, 2012), aging related diseases, macular degradation of eye (Gupta et al., 2003; Chichili et al., 2006), certain types of cancers including gastrointestinal, cervix, breast and prostate cancer (Peto et al., 1981; Fiedor and Burda, 2014; Fraser and Bramley, 2004; Stahl et al.,1998; Woodall et al., 1997; Erdman et al., 2009; Jaswir et al., 2011; Giovannucci et al., 2002). Beneficial effects of dietary carotenes α-carotene β-carotene on human health related to enhancement of immune system and minimizing the risk of cancer are due to their anti-oxidant potential (Fiedor and Burda, 2014; Das et al*.,* 2007). β-carotene, α-carotene and β-cryptoxanthin are Pro-vitamin A carotenoids (PVACs) and hence they are the main precursors of vitamin A (VA) in the human body (Arscott and Tanumihardjo, 2010).

1.2.3 Carotenoids use in Disease Prevention

Carotenoids especially astaxanthin, have been reported to enhance both the non-specific and specific immune system and protect cell membranes and cellular DNA from mutation (Misawa, 2010; Bendich A. 1989). Intake off fruits and vegetables rich in carotenoids mainly lycopene, α-carotene β-carotene, β-cryptoxanthin, zeaxanthin and lutein lowers the risk of morbidity and mortality by cardiovascular diseases and atherosclerosis (Fiedor and Burda, 2014). Epidemiological studies have reported that lycopene can lower the risk of prostate cancer (Nakazawa, et al., 2009) and in its ability to quench singlet oxygen, it is two to tenfold stronger than β-carotene and α-tocopherol, respectively (Rao, Guns and Rao, 2003). Clinical studies have reported that the lycopene enriched foods are protective against oxidative DNA damage in leukocytes in vitro (Pool-Zobel et al., 1997) and prostate tissue in vivo (Bowen et al.,2002). β-carotene is useful in reducing the risk ischemic heart disease and myocardial infarction risk (Hennekens et al., 1996). In the macular region of human eye including eye lense, two xanthophylls, lutein and zeaxanthin exist in high concentrations and are regarded very important carotenoids for eye health. Reports suggest that these two carotenoids protect eye from high energy UV light and are excellent reactive

oxygen species scavengers (Edge et al., 1997). The role of lutein and zeaxanthin as macular pigments and their function in eye health has been reported in previous studies (Abdel-Aal et al., 2013). It has been anticipated that phytoene and phytofluene which are colorless precursors of other carotenoids, possess light absorption in UV-A and UV-B range, protect skin by their photo-protective characteristics (Stahl and Sies, 2012; Engelmann et al., 2011). Astaxanthin, is also known as the super anti-oxidant. Since it contains particular molecular configuration, making it extremely powerful antioxidant consequently protecting cells against oxidation by quenching singlet oxygen and dissipating the energy as heat. It has the strong potential for scavenging free radicals and effectively breaks peroxide chain reactions (Kurashige et al., 1990; Jorgensen, 1993). Studies have showed that the LDL high cholesterol levels in mice decreased when supplemented with astaxanthin. Neither beta-carotene nor canthaxanthin produced the same effect. Astaxanthin or other carotenoids can decrease the oxidation of the lipid-carriers and thereby reduce the risk of atherosclerosis (Murillo, 1992). It also has positive effects in case of antitumor activity (Jyonouchi et al., 2000).

1.2.4 Industrial Uses of Carotenoids

All carotenoids show antioxidants activities appearing in a variety of colors in red, yellow and orange. They are used as natural pigments in food, food supplements and as nutraceuticals. Moreover, they are used in pharmaceutical and cosmetic industry and various biotechnological purposes (Martin et al., 2008; Misawa, 2010; Kirti et al., 2014). In a previous report the global market for carotenoid was $766 million in 2007. The expected projection for the year 2015 was $919 million with a compound annual growth rate (CAGR) of 2.3%. In 2007, β-carotene alone shared the market value at $247 million; this segment was predicted to be worth $285 million by 2015 with CAGR of 1.8% (FOD025C, 2008; Kirti et al., 2014). In horticultural crops they appear as a trait of attractiveness adding value to the marketing potential of fruits and vegetables (Azadi et al., 2010; Clotault et al., 2008). Green algae *Haematococcus pluvialis* which is the natural source of astaxanthin has been reported for huge amounts ranging from 10,000-40,000 ppm (mg/kg) astaxanthin in addition to other important carotenoids such as

beta-carotene, lutein and canthaxanthin (Fraser et al., 1997; Turujman, 1997). Industrially, astaxanthin has been utilized as a feed supplement for cultured fish and shellfish (Fujita et al., 1983; Matsuno, 1991). Other diverse biological functions of astaxanthin include an involvement in cancer prevention (Tanaka et al., 1994), enhancer of immune responses (Jyonouchi et al., 1993), and a free radical quencher (Miki et al., 1994 Fraser et al., 1997). It is evident, therefore, that astaxanthin is a molecule with potential both to the pharmaceutical and food industries (Fraser et al., 1997).

1.2.5 Classification and Nomenclature of Carotenoids

A systemic nomenclature for carotenoids has been reported (Fraser and Bramely, 2004) however, carotenids are broadly classified into two major groups, carotenes and xanthophylls. (Botella-pavía and Rodríguez Concepción, 2006; Mezzomo and Ferreira, 2016). Carotenes, the hydrocarbon carotenoids include α-carotene,β-carotene, and lycopene whereas, xanthophylls which are oxygenated hydrocarbon derivatives having at least one oxygen as keto, hydroxyl, methoxy, epoxy,or carboxylic acid groups, include β-cryptoxanthin, lutein, and zeaxanthin,astaxanthin, fucoxanthin, violaxanthin and neoxanthin (Basu et al., 2001 ;Jaswir et al*.,* 2011; de Quiros and Costa, 2006). With 40 number of carbon atoms in a single molecule, the number of hydrogen atoms varies with no other elements. Tail to tail linkage of two C-20 geranylgeranyl diphosphate molecules leads to parental C-40 carbon skeleton and all of the individual variations are derived from it. Hydrocarbon ring either on one end or on the both ends is observed. Carotenes being hydrocarbons lack oxygen. Cyclisation at one or both ends of the parental structure results into seven end groups that include β, ε, Ψ, κ, Φ ,χ ,and γ. Change in the hydrogenation level and the addition of oxygen containing functional groups also modify the basic parental structure (Kirti et al*.,* 2014). Carotenes are abundantly found in photosystem reaction centers, while xanthophylls mostly enriched in the light harvesting complexes (Ruiz-Sola and Rodríguez Concepción, 2012; Niyogi et al*.,* 1997).

1.3 Biosynthesis of Carotenoids

In higher plants the genes which encode the enzymes that catalyze carotenoids biosynthesis for nearly every step of carotenoids biosynthetic pathway have been identified, sequenced and characterized (Cunningham, 2002).

1.3.1 Biosynthesis of IPP and DMAPP

Two pathways: the cytoplasmic mevalonate pathway (MVA) from metazoa and fungi and the plastid localized methylerythritol 4-phosphate (MEP) pathway for IPP production in plants and eubacteria have been reported (Eisenreich et al., 2004; Rodríguez Concepción and Boronat, 2002; Moise et al., 2014)

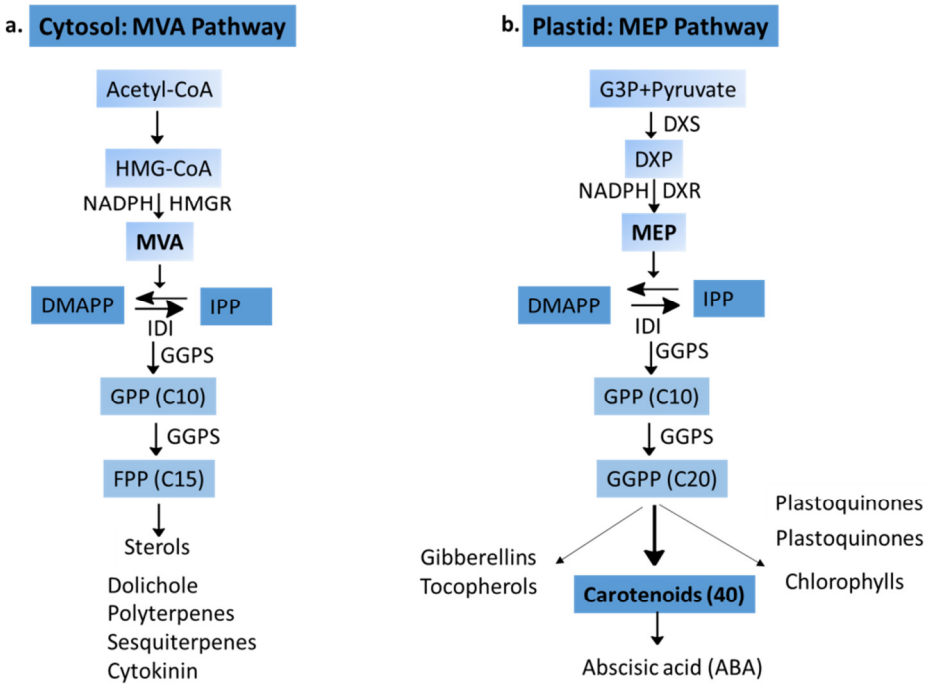


Figure 2. Cytoplasmic mevalonate pathway **a**, mevalonic acid pathway (MVA) and **b**, the plastid localized methylerythritol 4-phosphate (MEP) pathway for isoprenoid precursors IPP and **DMAPP** biosynthesis. **HMG-CoA,** 3-hydroxy-3-methylglutaryl-coenzyme A; **DXP**, 1-deoxy-D-xylulose-5-phosphate; **MVA,** mevalonic acid; **G3P**, glyceraldehyde 3-phosphate; **DMAPP**, dimethylallyl pyrophosphate; **IPP**, isopentenyl diphosphate; **IDI**, Isopentenyl diphosphate isomerase; **GPP**, geranyl diphosphate **FPP**, farnesyl pyrophosphate; **GGPS**, eranylgeranyl pyrophosphate synthase.

Since carotenoids are basically isoprenoids all are derived from the basic C-5 isoprene units, isopentenyl Carotenoid biosynthesis in plants uses initial molecules of IPP and DMAPP. Both IPP and DMAPP are derived from either, mevalonic acid

pathway (MEV) which evolved in archaea, existing in cytosol or the methylerythritol 4-phosphate (MEP) pathway in plastids (Moise et al., 2014; Lichtenthaler, 1999; Phillips et al., 2008; Cazzonelli, 2011; Eisenreich et al*.,* 2004).

Pyruvate and glyceraldehyde 3-phosphate (G3P) are the initial substrates used in the MEP pathway to from deoxy-D-xylulose 5-phospahte (DXP) which is then catalyzed by DXP synthase (DXS) leading to the formation of MEP via reductive action of DXP reductoisomerase (DXR) on DXP and its intramolecular rearrangement (Carretero-paulet et al., 2006; Ghirardo et al., 2014). Depending on the availability, carotenoids are derived from two isoprene isomers, isopentenyl diphosphate (idi) and its allylic isomer dimethylallyl diphosphate (DMAPP).

After a number of subsequent reactions, IPP and DMAPP are formed .Isopentenyl diphosphate isomerase (idi) has the ability to reversibly interconvert IPP and DMAPP (Lichtenthaler, 1999). Agranoff et al., (1960) discovered IDI gene (Moise et al., 2014) and then it was cloned by Hahn et al., (1999). A series of condensation reactions leads to the formation of geranylgeranyl diphosphate (GGPP), the carotenoids biosynthesis precursor catalysed by geranylgeranyl diphosphate synthase, GGPS (Cazzonelli, 2011). GGPS was first identified in carotenoid biosynthesizing bacteria *Pantoea agglomerans* (Math et al., 1992) and then in plants (Kuntz et al., 1992; Moise et al., 2014).

1.3.2 Biosynthesis of Phytoene

Phytoene (a 15-cis isomer) which is the C40 isoprenoid is formed by head-to-head condensation of the two molecules of GGPP catalyzed by enzyme phytoene synthase (PSY) which was firstly isolated and cloned from plants (Dogbo et al., 1988; Armstrong et al., 1990) and from bacteria (Misawa et al., 1990; Armstrong et al., 1990). Formation of phytoene is the first devoted and rate limiting step in the carotenoid biosynthesis pathway in plants. (Moise et al., 2014; Welsch et al*.,* 2008; Welsch et al*.,* 2010).There are several paralogues of PSY in some plant species and are regulated differentially and show various tissue and intracellular patterns of expression (Shumskaya et al., 2012). Additionally, PSY expression is upregulated (Kachanovsky et al., 2012; Moise et al., 2014).Much about the activity of PSY can be deduced based on its similarity to crtM (dehydrosqualene synthase and squalene synthase (SQase). crtM is a bacterial type PSY that catalysis

formation of 15-cis-dehydrosqualene (C30 carotenoid). But PSY shows preference for catalyzing the biosynthesis of C40 carotenoids (Umeno et al., 2002).

1.3.3 Biosynthesis of Lycopene

In plants, with the introduction of four double bonds by the activity of two enzymes; phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS), phytoene is first converted to tetra-cis lycopene. Carotenoid isomerase (CRTISO) then isomerizes cis bonds at 7, 9 and 7′,9′position of tetra-cis lycopene to all -trans –lycopene named as Ψ,Ψ-carotene (Park et al., 2002; Isaacson et al., 2002; Nisar et al., 2015).

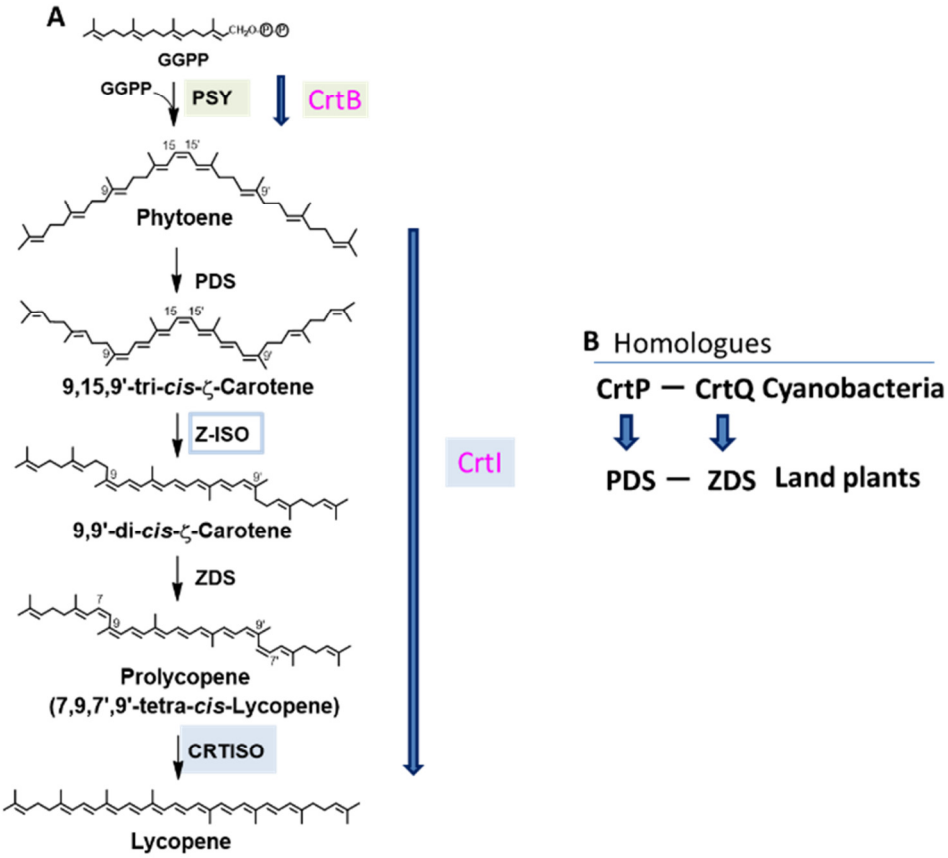


Figure 3. Comparison of the lycopene synthesis pathway among plans, bacteria and cyanobacteria. **A**, lycopene synthesis in plants, and the CrtI based phytoene synthesis pathway from carotenoid biosynthesizing bacteria compared with **B**, cyanobacteria. **GGPP**, geranylgeranyl pyrophosphate synthase (plants); **PSY**, phytoene synthase (plants); **CrtB**, phytoene synthase (bacterial); **PDS**, phytoene desaturase (plants); **CrtI**, phytoene desaturase (bacterial) **Z-ISO**, ζ-carotene-isomerase (plants); **ZDS**, ζ-carotene desaturase(plants); **CRTISO**, carotenoid isomerase (plants); **CrtP**, phytoene desaturase (cyanobacterial); **CrtQ**, ζ-carotene desaturase (cyanobacterial).

Bacterial lycopene biosynthesis however, is catalyzed by bacterial type phytoene desaturase **(CrtI)** which was identified from *Rhodobacter capsulatus***.** (Giuliano et al., 1986; Armstrong et al., 1989; Moise et al., 2014). In contrast, lycopene biosynthesis in plants, cyanobacteria, and green algae requires four enzymes with sequence of desaturation and isomerization reactions commonly proceeds in the order PDS →Z-ISO→ZDS→ CRTISO. It was found that fungal CrI is similar to that of bacterial one (Fraser et al., 1992; Bartley et al., 1990; Schaub et al., 2012; Moise et al., 2014). PDS was first identified in cyanobacteria (Chamovitz et al., 1990) followed by cloning of related cDNA from soybean, pepper, tomato and maize (Bartely et al., 1991). ZDS was first functionally identified by heterologous expression of a candidate PDS in *E. coli* strains that were specific to accumulate ζ-carotene (Linden et al., 1994). The gene for Z-ISO was initially identified by mutant screening of maize followed by functional complementation assys in *E. coli* (Chen et al., 2010). By using biochemical complementation supplemented with screening of carotenoids deficit strains and map based cloning, CRTISO gene was identified independently from cyanobacteria, tomato and *Arabidopsis thaliana* (Isaacson et al., 2002; Moise et al., 2013).

1.3.4 Biosynthesis of Carotenes from Lycopene Cyclisation

In plants the crucial stage begins when the cyclases: lycopene β-cyclase (LCYb) and lycopene epsilon cyclase (LCYe) attack on all-trans-lycopene and initiate cyclisation reactions that result into the formation of cyclic end group carotenoids with the addition of either β-ring and/or ε-ring . This leads to the formation of two branches in the carotenoid biosynthesis pathway in higher plants (Harjes et al., 2008; Cunningham et al., 1996; Takemura et al*.,* 2014; Cazzonelli *2011;* Nisar et al., 2015) Since lycopene cyclisation is accelerated by different cyclase families so, the rise in the activity of β contrasted with ε- lycopene cyclases can turn lycopene towards synthesis of provitamin A carotenoids with enhanced nutritional value (Harjes et al., 2008) CRTY is a representative β- Cyclase from most of the proteobacteria but absent in cyanobacetria. However, cyanobacteria and green plants have a related LCY. On these bases, LCY enzymes can be classified into three sub-types based on the production of ionone ring So, there exist cyclase

(LCY) enzymes which catalyse the formation of β-ionone ring for example LCYB, or ε- ionone ring for example LCYe, and the bifunctional lycopene cyclases that generatecarotenoids with either β or ε- ionone ring i.e., LCYB/LCYE (Cunningham et al., 1994; Moise et al., 2014) It was *Rhodobacter sphaeroids* a photosynthetic bacteria in which the first Bacterial type CRTY was identified by sequencing the carotenogenic gene cluster (Coomber et al., 1990). Plant type LCYB was first identified by utilizing map-based cloning and introgression lines (Ronen et al., 1999; Moise et al., 2014). Lycopene β-cyclase (LCYb) is singly capable to introduce two β-rings at both ends of lycopene changing it to β-carotene which belongs to β, β branch of carotenoids and named β-β-carotene. On the other hand, the combined catalytic action of lycopene β-cyclase (LCYb) and lycopene epsilon cyclase (LCYe) introduces one β and one ε-ring into lycopene consequently changing it to α-carotene［(6′R)- β, ε-carotene］which belongs to β- branch of carotenoids. However, LCYe alone typically adds one ε-ring to the lycopene to make monocyclic δ-carotene ［(6′R)- ε,Ψ-carotene］which is converted to α-carotene by LCYb.

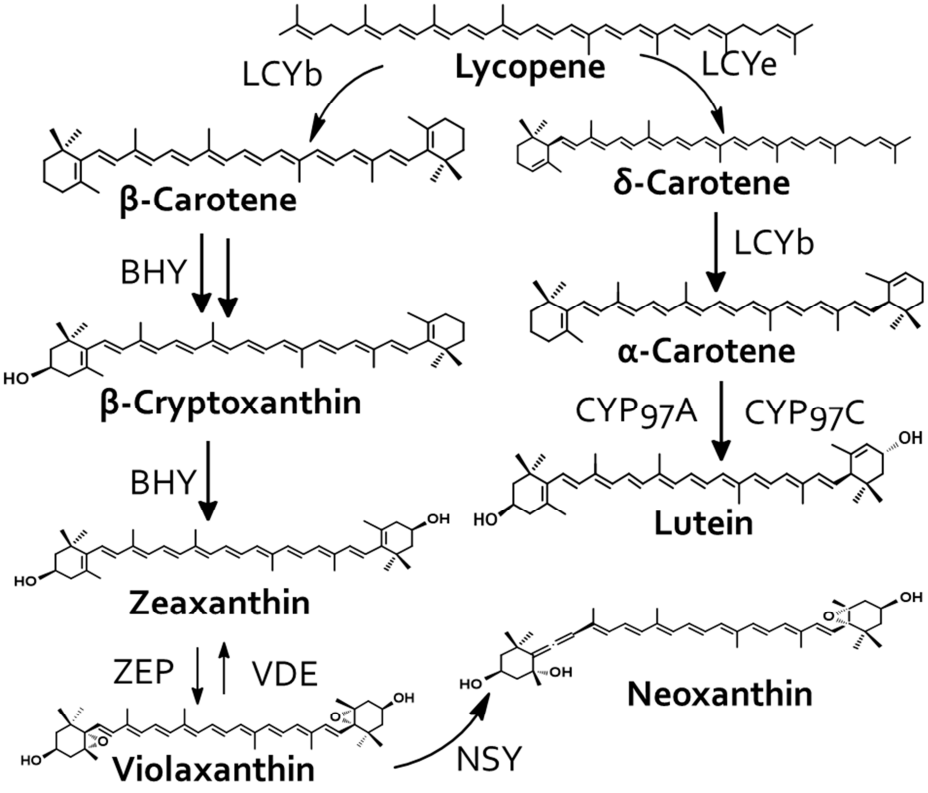


Figure 4. Biosynthetic pathway of carotenes and xanthophylls in higher plants.

Subsequent isomerization, hydroxylation or epoxidation of α-carotene and β-carotene modifies them to various structures (Yamamizo et al., 2010) thus, β-carotene which is one of the major carotenoids found in leaves of higher plants is changed to zeaxanthin, anthraxanthin, violaxanthin and neoxanthin (Takemura et al., 2014).

In most of the plants and algae, carotenoids with two ε- rings are commonly not found (Goodwin, 1980) however, in lettuce *LCYe* has been reported for generating bicyclic ε-ε-carotene along with its hydroxylated derivative lactucaxanthin by cyclsing both Ψ-ends of lycopene (Phillip and Young, 1995; Takemura et al., 2014; Cunnigham and Gantt, 2001). Various specific domains and residues have been identified which determine whetehr *LCYe* introduces one or two ε- ionone ring (Cunnigham and Gantt, 2001)

High level of structural similarity (30%) between the amino acid sequences of *LCYb* and *LCYe* exists (Hirschberg, 2001) suggests that both *LCYb* and *LCYe* share a common ancestor for their origin through gene duplication (Takemura et al., 2014). In tomato, two *LCYb* genes which separately exist in chloroplast and chromoplast have been reported that have 53% of identical amino acid sequences (Hirshberg, 2001; Bouvier et al., 1994). In case of Arabidopsis, only one *LCYb* gene was reported (Cunnigham et al., 1996; Ronen et al., 2000, Alquezar et al., 2009). Contrarily, only one copy of *LCYe* gene found in all higher plants by now. Highly regulated processes such as the control of gene expression levels are the factors determining the concentration and composition of carotenoids inside plant tissues (Ronen et al., 1999; Takemura et al., 2014). The allelic variation in the genes controlling carotenoids biosynthesis pathway is the important factor that determines the accumulation of carotenoids in a plant (Clotault et al*.,* 2010; Arizio et al., 2014). The amino acid sequences of all LCY β-type enzymes have a conserved motif called FLEET motif which is essential for establishing activity (Cunnigham et al., 1996; Moise et al., 2014).

1.3.5 Biosynthesis of Xanthophylls

The hydroxylation of α-carotene and β-carotene leads to the formation of the xanthophylls such as lutein and zeaxanthin which are the main carotenoid

pigments in the photosynthetic apparatus of plants. Both zeaxanthin and lutein are synthesized by hydroxylation at the 3 and 3' carbon atom of β,β-carotene or β,ε-carotene by different hydroxylases which are specific to β or ε-rings for their hydroxylation activity (Deli et al., 2001). Hence, β-xanthophylls such as β-cryptoxanthin and ε-xanthophylls such as lutein are catalysed by two different classes of carotene hydroxylases. Among these one is a heme-containing cytochrome P450 i.e., CYP97 and the other is non-heme, di-iron BHY (Quinlan et al., 2012). Spesific memebers of β-ionone ring hydroxylase family have been realized as a critical target in breeding plants with a high provitamin A carotenoid quantity (Vallabhaneni et al., 2009). Carotene hydroxylases were firstly identified from carotenoid biosynthetic clusters in *Erwinia urdoyora/herbicola* i.e., *Pantoea* species (Perry et al., 1986). Followed by their identification in plants (Sun et al., 1996). Among bacterial and plant carotene hydroxylases there exists a sequence homology based on 40 % identity and 60% conserved substitutions. Marine bacteria, in contrast to higher plants and cyanobacteria have another family of carotenoid hydroxylases which catalyze the hydroxylation of β-ionone ring at C-2 position (Nishida et al., 2005; Moise et al., 2005).

Hydroxylation of the two β-ionone rings in β-carotene gives zeaxanthin, whereas hydroxylation of the one β-ring and one ε-ring in α-carotene leads to lutein formation. β-rings in the carotenes is potentially hydroxylated by either the P450-type CYP97A or di-iron BHY enzymes, while ε-ring of α-carotene is hydroxylated by CYP97C, which is an another P450 carotene- hydroxylase. From genetic evidences, it can be in-sighted that the enzyme which catalyzes the hydroxylation of ε-ionone ring in *Arabidopsis* was linked with *lut1* locus. This finding directed to the identification of the gene coding for ε-ring hydroxylase. Such a novel ε-ring hydroxylase was found to be a cytochrome P450 enzyme member of the CYP97C family (Tian et al., 2004; Moise et al., 2014).

CYP97C hydroxlases preferentialy the ε-ring however, in vivo studies showed that it can also show activity to catalyse β-ring hydroxylation (Fiore et al., 2006). Another P450hydroxylase from CYP97A family have been reported for activity pertaining to the hydroxylation of β-rings (Kim and DellaPenna,, 2006). CYP97B carotene hydroxylase is also present in higher plants however, their biosynthetic role is not clear (Kim et al., 2009a; Yang et al., 2014). CYP97A has been found to

preferably catalyse hydroxylation of β-rings (Moise et al., 2014). Studies show that CYP97C together with the CYP97A β-hydroxylases synthesize lutein by preferentially hydroxylating the ε-ring of substrates of whose β-ring have already been hydroxylated (Quinlan et al., 2007; Kim and DellaPenna,, 2006). It has also been identified that different carotenoid hydroxylases function in coordination to synthesize xanthphylls (Quinlan et al., 2012; Takemura et al., 2014; Moise et al., 2014)

Zeaxanthin is easily converted to violaxanthin, via antheraxanthin, by introduction of a 5,6-epoxy group to the 3-hydroxy β-rings, catalyzed by a zeaxanthin epoxidase (ZEP).

On the contrary, in leaves growing under high light intensity, violaxanthin de-epoxidase (VDE) catalyzes the de-epoxidation reaction in two steps, leading to the transformation of violaxanthin into zeaxanthin. However, when light conditions return to normal values, zeaxanthin is transformed into violaxanthin. This reversible inter-conversion is known as the xanthophyll cycle and is of key importance for NPQ (non-photochemical quenching) and for plant adaptation to changes in environmental conditions (Gómez-García. Ochoa-Alejo, 2013). In the last step of carotenoid biosynthesis, by the action of a neoxanthin synthase (NSY), violaxanthin is converted into neoxanthin (Botella-pavía and Rodríguez Concepción, 2006). Violaxanthin can also be converted into xanthoxin and subsequently into abscisic acid by reaction catalyzed by 9-cis-epoxycarotenoid dioxygenase (NCED) and an aldehyde oxidase (AO).

It has been proposed that neoxanthin can also be converted into xanthoxin by the NCED (Seo and Koshiba, 2002).

1.3.6 Biosynthesis of Ketocarotenoids

Ketocarotenoids comprise numerous types that have huge commercial value and medicinal utilization.Two different families of ketocarotenoids have been numerated. One of which belongs to *Synechocystis* β- Carotene ketolase (crtO) which introduces a 4-keto group in the β-ionone ring at the one end of β-Carotene to synthesize firstly the echinone and then ketolates the second end to generate cathaxanthin (Misawa, 2010; Fernandez-Gonzalez et al., 1997; Moise et al., 2014).

The second group of carotenoid ketolases include crtW which is found in marine bacteria and green algae. It catalyses introduction of keto groups at C-4 to make canthaxanthin (Misawa, 2010; Kajiwara et al., 1995; Moise et al., 2014). Experiments which included complementation assays in *E. coli* expressing carotenoid biosynthesizing gene indicated that in contrast to crtO, crtW could also introduce 4-keto groups into the substituted β-ring of zeaxanthin leading to the formation of ketocarotenoids for example astaxanthin (Makino et al., 2008; Choi et al., 2007; Moise et al., 2014).

1.4 Isolation and Functional Identification of Carotenoids Biosynthesis genes

The heterologous complementation expression system in *E. coli* offer unique tool for functional analysis of isolated new carotenoids biosynthesis genes from different organisms (Wurtzel et al., 1997). Carotenoid biosynthetic pathway in microorganisms, such as *Erwinia uredovora* (now, *Pantoea ananatis*) or *Erwinia herbicola* is specified by a gene cluster encoding biosynthetic enzymes that function in a pathway starting with the synthesis of geranylgeranyl pyrophosphate (GGPP) and ending in the synthesis of zeaxanthin glucosides (Sandmann, 1990; Misawa et al., 1990; Hundle et al., 1993). Complete carotenoid gene clusters or part of it from *E. uredovora* and *E. herbicola* have been introduced into *Escherichia coli,* which is otherwise a non-pigmented bacterium and such transformed *E. coli* engineered in a way that they accumulate a range of colorful carotenoids (Misawa et al., 1990; Misawa et al., 1993). Since carotenoids are derived from isoprenoid precursors *E. coli* can accumulate carotenoids by coupling an endogenous isoprenoid biosynthetic pathway with enzymes encoded by transformed genes of carotenogenic organisms such as *E. uredovora*. Hence, the biosynthetic pathway can be reconstructed in vivo even if the enzymes are of such diverse origin as those encoded by bacteria and plants (Misawa et al., 1994; Li et al., 1996; Chamovitz et al., 1992). The expression of carotenoid genes in *E. coli* has been useful for identifying function of gene products (Chamovitz et al., 1992; Cunningham et al., 1993;Sandmann, 1993), manipulation of the pathway (Hundle

et al., 1993; Sandmann and Misawa, 1992); investigating transcriptional regulators of carotenoids biosynthesis genes (Penfold Pemberton,1994), and isolation of new genes encoding enzymes of the carotenoid biosynthetic pathway (Kajiwara et al., 1995) or enzymes catalyzing the synthesis of carotenoid precursors (Ohnuma et al., 1994).

Misawa et al., (1995) isolated and functionally identified the carotenoids biosynthesis genes cluster that included *CrtB* (phytoene synthase), *CrtI* (phytoene desaturase), crtW (β-Carotene ketolase) and *crtZ* (β-Carotene hydroxylase) from *A. aurantiacum* (now, *Paracoccus* sp. Strain N81106). The functional identification of the isolated gene cluster led them to propose astaxanthin biosynthetic pathway for the first time.

Misawa et al (1990) isolated and functionally identified the carotenoids *crtE* (GGPS equivalent), *crtX*, **(**Zeaxanthin glucosyltransferase) *crtY* (lycopene β-cyclase), *crtI* (phytoene desaturase), *crtB* (phytoene synthase), and *crtZ* **(**β-Carotene hydroxylase) gene cluster form *Erwinia uredovora* (now *Pantoea ananatis*) by analyzing carotenoids accumulated in *E. coli* transformants in which these genes were expressed. By analysis of accumulated carotenoids in the transformed *E. coli* by these individual genes they found that carotenoids in that pathway appear to be close to those in higher plants rather than to those in bacteria.

1.5 Sweetpotato Carotenoids Biosynthesis Genes, Cloning and Genetic Engineering

Although sweetpotato is highly important as a valuable source of carotenoids especially β-carotene, very little research has been done on molecular biological aspects of its carotenoid biosynthesis (Khan et al., 2016; Kim et al., 2014; Liao et al., 2007; Arizio et al., 2014).

Otani, (1993) developed and reported the first successful transformation protocol for the production of transformed (transgenic) sweetpotato plants that was based on the formation of hairy roots using leaf discs as explants for *Agrobacterium rhizogenes*. However, the regenerated transgenic plants showed some morphological abnormalities such as short storage-root and internodes. Later on, to overcome such anomalies, a modified and successful *Agrobacterium*

rhizogenes-mediated transformation protocol was developed via somatic cell embryogenesis (Otani et al., 1996; Otani et al., 2001; Luo et al., 2006).

Liao et al., (2008) isolated and functionally characterized an isopentenyl diphosphate isomerase (idi) gene from sweetpotato cultivar YUSU 303 from South-east China. They isolated a full lenghth cDNA of idi gene by SMART^{TM} RACE cDNA Amplification Kit (Clontech, USA). Isolated idi was 1155bp with an open reading frame of 892bp encoding a polypeptide of 296 amino-acids (GenBank accession No. DQ150100). Isolated idi gene was cloned in pTrc expression vector and was fed to *E. coli* which contained pAC-BETA plasmid for β-carotene accumulation. *E. coli* were cultured and carotenoids were analyzed by color complementation. Cultures of *E. coli* which were transformed with idi gene turned orange indicative for β-carotene and suggested its potential activity in promoting β-carotene biosynthesis.

Kim et al., (2012) isolated a partial sequence with of phytoene synthase (PSY) which contained 354bp from a cultivar Shinhwangmi (accession No.HQ828092). It showed 94% sequence identity with a PSY isolated from Ipomoea species Kenyan (GenBank accession No. AB499050.1. However, no gene function of isolated PSY from sweetpotato could be reported.

Ling et al., (2013) isolated a lycopene ε- cyclase (LCYe) gene from sweetpotato cultivar Nongdafu 14 from China. However, they did not functionally characterize it. They isolated a full lenghth cDNA of *idi* gene by GeneRacer TM Kit (Invitrogen Carlsbad, CA, USA). Isolated LCYe was 1805bp with an open reading frame of 1236bp encoding a polypeptide of 411 amino-acids. Quantitative Real time PCR analysis showed that *IbLCYe* expression levels were desirably higher in roots as compared to those in leaves. Isolated LCYe gene was expressed in tobacco cultivar Winconsin 38. Carotenoids from transgenic tobacco plants were extracted and analysed by HPLC which revealed transgenes accumulating more β-carotene as compared to control plants.

Kim et al., (2014) isolated a partial lycopene β-cyclase (IbLCY-β) from a cultivar Yulmi of sweetpotato. They synthesized primers by using a partial sequence of IbLCY-β from data base with accession number JX393306. And amplified a partial cDNA of IbLCY-β by RT-PCR. By using isolated IbLCY-β an IbLCYb-β-RNAi

vector was constructed and then used to transform white fleshed sweetpotato. Transformed sweetpotatos were cultured and analysed for accumulated carotenoids. Their results showed a total increase in the carotenoids contents along with increase in resistance against salt stress in transgenic sweetpotato as compared to the control. Significant levels of carotenoids genes expression were observed in all palnt parts with highest expression in leaves to lowest in the fibrous roots. But in case of transgenic calli expressions of IbLCY-β were dramatically reduced and found high in non-transgenic calli. Lycopene was not produced both by transgenic and non-transgenic sweetpotato.

In an another experiment Kim et al., (2012) cloned a partial cDNA encoding β-carotene hydroxylase (CHY-β) from storage roots of sweetpotato cultivar Shinhwangmi and constructed a RNA-i-IbCHY-β vector for transformation of white-fleshed cultivar Yulmi and evaluation of inhibition effects of β-carotene hydroxylase (CHY-β) in transgenic lines. Down- regulation of IbCHY-β gene expression altered the content and degree of carotenoids between transgenic and non-transgenic cells with an increase in the β-carotene and total carotenoids in transgenic sweetpotato cells along with an increase in their ant-oxidation potential.

1.6 Metabolic Engineering of the Carotenoid Biosynthetic Pathway to Enhance Carotenoid Content in Higher Plants

The pathway engineering approach using a variety of carotenoid biosynthesis genes is becoming a potential approach in one of the most effective methods to generate large quantities of structurally diverse carotenoids (Misawa, 2011; Lee and Dannert-Schmidt, 2002; Giuliano et al., 2008). Astaxanthin (3,30-dihydroxy-4,40-diketo-b-carotene) is a high-value ketocarotenoid that is biosynthesized only by a few organisms typically at low levels. This red pigment (produced through chemical synthesis) has been used in large amounts in aquaculture. Currently, natural astaxanthin is employed as a health food and is investigated for the treatment of a number of human diseases including cancers (Guerin et al., 2003). The limited renewable sources and growing demand for natural astaxanthin have attracted tremendous interest in engineering heterologous hosts, especially plants with the ability of sequestering 10 to 50-fold

higher carotenoids than microorgansims, to produce the high-value pigment during the past decade (Misawa 2009; Zhu et al., 2009). The most promising approach reaching high astaxanthin yields was by chloroplast transformation using a bacterial ketolase gene (Hasunuma et al., 2008). Plastid genome transformation of lattuce (*Lactuca sativa*) has similarly been site-specifically modified with the addition of three transgenes, which encoded β,β-carotenoid 3,3′-hydroxylase (*crtZ*) and β,β-carotenoid 4,4′-ketolase (4,4′-oxygenase; *crtW*) from a marine bacterium *Brevundimonas* sp. strain SD212, and isopentenyl diphosphate isomerase (*idi*) from a marine bacterium *Paracoccus* sp. strain N81106. The resultant transplastomic lettuce leaves generated 49.2 % astaxanthin fatty acid diester, 18.2 % astaxanthin monoester and 10.0 % astaxanthin in its free forms along with the 17.5 % of other ketocarotenoids. The ketocarotenoids produced in transplastomic lettuce were 94.9% of total carotenoids. The wild type native carotenoids analysed were 3.8% lactucaxanthin, and 1.3% lutein in the transplastomic lattuce (Harada et al., 2013). Likewise, by the introduction and heterologous expression of *crtW* gene, astaxanthin and other intermediates have been produced and reported in carrot (*Daucus carota*) roots (Jayaraj et al., 2008) canola (*Brassica napus*) seeds (Fujisawa et al., 2009) and maize (*Zea mays*) endosperms (Zhu et al., 2008).

Through pathway engineering that utilizes the marine-bacterial carotenoid 4,4'-ketolase (4,4'-oxygenase) gene named *crtW,* unique keto-carotenoids such as astaxanthin diglucoside, 2,2'-dihydroxyastaxanthin, and 2,2'-dihydroxycanthaxanthin have been produced in *Escherichia coli* (Yokoyama et al., 1998; Nishida et al., 2005) and 4-ketoantheraxanthin in tobacco (*Nicotiana tabacum*).plants (Shindo et al., 2008). Breitenbach et al. (2014) also synthesized α-echinenone (4-keto-α-carotene) in rice callus using *crtW*. Recently, 4-keto-zeinoxanthin was produced in *E. coli* cells by introducing the bacterial *crtW* gene and carotenogenic genes from liverwort (Maoka et al., 2014).

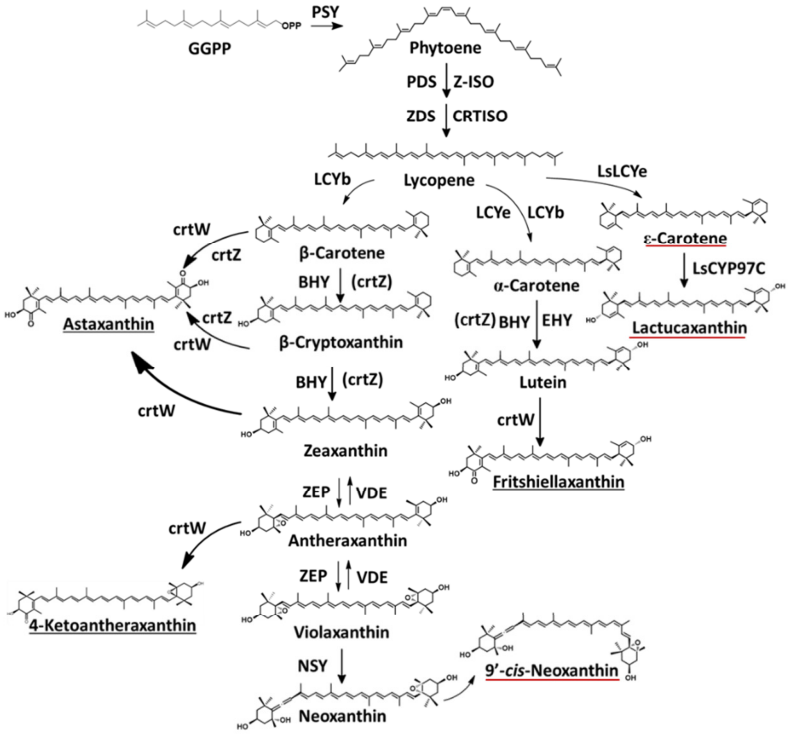


Figure 5. Carotenoid biosynthetic pathway in higher plants along with g the summarized illustration for the introduction and function of heterologous *crtW* and *crtZ* genes. Expressed in tobacco (Hasunuma et al., 2008) and lettuce (Harada et al., 2014) leaves, the, carotenoids shown black represent native carotenoids accumulated by both tobacco and lattuce, black and underlined are those reported from both of the transgenic tobacco and lattuce. Carotenoids underlined red are reported from lettuce only. **GGPS**, Geranylgeranyl pyrophosphate synthase; **PSY**, phytoene synthase; **PDS**, phytoene desaturase; **ZDS**, ζ-carotene desaturase; **CRTISO**, Carotenoid isomerase; **LCYb**, lycopene β-cyclase; **LCYe**, lycopene ε-cyclase; **LsLCYe**, lattuce **LCYe**; **LsCYP97C**, lettuce heme-containing cytochrome P450-type carotene ε- ring hydroxylase;**BHY**, Non-heme di-iron type carotene β-ring hydroxylase; **EHY**, carotene ε- ring hydroxylase; **ZEP**, zeaxanthin epoxidase; **VDE**, violaxanthin de-epoxidase; **NSY**, neoxanthin synthase

1.6.1 Metabolic Engineering of the Carotenoids Biosynthetic Pathway in Sweetpotato

Metabolic engineering of carotenoid biosynthetic pathway using a combinatorial approach has led to the efficient production of interesting carotenoids of high commercial value and pharmaceutical potential (Umeno and Arnold, 2004; Misawa, 2010; Misawa, 2011). It was reported that sweetpotato contained not only β-carotene but also several epoxy carotenoids unique to the sweetpotato tubers *e.g.*, β-carotene-5, 8-epoxside and β-carotene-5, 8, 5'8'-diepoxside (Maoka et al.,

2007). Therefore, it was assumed that new structural carotenoids with epoxy and keto groups can be produced by expressing the ketolase *crtW* gene in sweetpotato tubers. Recently, marine-bacterial genes that include the *crtW* gene encoding carotenoid 4,4'-ketolase (Shindo et al., 2008) was introduced into sweetpotato cultivar W71 under the control of the CaMV promoter consequently, novel carotenoids with epoxy and keto groups 1, 2, and 3 were obtained along with a series of ketocarotenoids. The structural elucidation of these novel epoxy-keto carotenoids along with biosynthetic pathway in sweetpotato was also proposed (Maoka et al., 2016)

'White Star' (WS) and W71, which produce white- and orange-fleshed tubers, respectively, are important sweetpotato cultivars, since they are amenable to *Agrobacterium*-mediated transformation (Khan et al., 2016; Maoka et al., 2016; Gama et al., 1996; Takahata et al., 2010). Chemical analysis of the carotenoids, isolation and functional characterization of carotenoids biosynthesis genes and of these two cultivars have not been performed so far (Khan et al., 2016).

Although HPLC is a routine analytical tool to analyses various gene products from plants, highly developed and comprehensive metabolome analytical techniques with respect to particular tissues now offer precise analytical approaches such as nuclear magnetic resonance (NMR), and accurate mass spectrometry (MS) techniques (Keeler and James, 2010; Fraser and Bramley, 2006, Moing et al., 2011). Novel carotenoids produced by foreign carotenoids genes expressed has been successfully nalysed and identified by UV-vis, ESI-MS, ^{1}H-NMR, CD Spectral data and COSY and NOESY experiments (Maoka et al., 2016).

1.7 OBJECTIVES

The present study was planned to:

1) Elucidate carotenoid biosynthesis genes in sweetpotato cultivars WS and W71 through chemical analysis of their carotenoids as well as structural and functional identification of their carotene cyclases and carotene hydroxylase genes.

2) Transform sweetpotato cultivar W71 tubers with β-carotene ketolase (crtW) genes and elucidate the accumulation of novel carotenoids.

2. MATERIALS AND METHODS

2.1 Plant Material

The present study, comprised sweetpotato [*I. batatas* (L.) Lam] carotenoid analysis from leaves and tubers, isolation and functional identification of carotenoids biosynthesis genes along with their expression analysis, in two cultivars of sweetpotato. For these purposes, WS and W71, were used which produce white- and orange-fleshed tuberous roots (tubers), respectively. Both cultivars were bred in the USA (Takahata et al., 2010; Mullen et al., 1981). The vines (ca. 25 cm in length) of each cultivar were planted into Wagner pots (159 mm in diameter and 190 mm in height) containing culture soil [Engeibaido 1 (Nihon Hiryo, Tokyo, Japan)] in a greenhouse. After 5 months of growth, tubers were harvested from each cultivar. Cultivar W71 was used for metabolic engineering of carotenoids biosynthetic pathway in sweetpotato, using ketolase (*crtW*)

2.1.1 Bacterial Strains

Competent cells of *Escherichia coli* DH5α (Toyobo) were used as host strain for all type of general plasmids DNA manipulation aimed for cloning and sequence analysis of carotenoids biosynthesis genes with appropriate cloning vectors at each cloning stage. For gene expression analysis, *Escherichia coli* strain BL21 (DE3) was used with appropriate expression vector for each gene. BL21 (DE3) is a chemically competent *E. coli* cell suitable for transformation and high level protein expression using a T7 RNA polymerase-IPTG induction system. Whereas for the transgenic expression of bacterial ketolase gene, *crtW* (β-Carotene 4,4'-oxygenase), in Sweetpotato cultivar W71, *Agrobacterium* strain EHA101 was used with appropriate binary expression vector and promoter. Originally, this gene was reported from *Brevundimonas aurantiaca* accession number AY166610 by Misawa et al., (1995).

2.2 Carotenoid Analysis from Leaves and Tubers of Sweetpotato

2.2.1 Carotenoids Extraction from Leaves and Tubers of Sweetpotato Cultivars

Extraction of carotenoids from the leaves and tubers of the two sweetpotato cultivars and subsequent chromatographic and spectroscopic analyses were carried out as follows: Total carotenoids were extracted from leaves and tubers with acetone at room temperature. The extract was concentrated to a small volume in vacuo and partitioned with *n*-hexane-diethyl ether (1:1, v/v) and water. The organic layer was evaporated to dryness and submitted to LC/MS analysis. The total carotenoid contents were calculated employing an extinction coefficient of $E_{cm}^{1\%} = 2400$ at λ max. The ether-hexane solution was evaporated. In the case of leaf carotenoid analysis, chlorophylls were removed by treatment with 5% (w/v) KOH/MeOH. The residue was subjected to analysis in a LC/photodiode-array detection (PDA)/MS system.

2.2.2 HPLC Analysis of Carotenoids Extracted from Leaves and Tubers

The LC/PDA/MS analysis of carotenoids was carried out using a Waters Xevo G2S QTof mass spectrometer equipped with an Acquity UPLC system. UV-Visible (UV-VIS) absorption spectra were recorded from 200 to 600 nm by PDA. The electro-spray ionization (ESI) time-of-flight (TOF) MS spectra were acquired by scanning from *m/z*100 to 1500 with a capillary voltage of 3.2 kV, cone voltage of 40 eV, and source temperature of 120 °C. Nitrogen was used as a nebulizing gas at a flow rate of 30 L/h. An Acquity 1.7 μm BEH UPLC C18 column (Waters) was used as a stationary phase with the mobile phase of: AcCN-water (85:15, v/v)→AcCN-MeOH (65:35), with a column temperature of 40 °C, at the flow rate of 0.4 mL/min.

2.2.3 Identification and Quantification of Carotenoids in Sweetpotato Leaves and Tubers

Carotenoids from leaves and tubers of sweetpotato W71 and WS cultivars were identified by analyzing the spectroscopic data of individual carotenoids. Carotenoid composition in the leaves of both sweetpotato cultivars was calculated based on the HPLC chromatograms.

2.3 Isolation and Analysis of Nucleic Acids

2.3.1 Isolation and Purification of RNA from Sweetpotato

Following the protocol reported by Takahata et al., (2010), total RNA, from cultivars WS and W71 was isolated and further purified by using an RNeasy Mini Kit (QIAGEN, Hilden, Germany) according to the protocol provided by the manufacturer. Total Polyadenylated mRNA then treated with DNase I to remove any contaminating genomic or foreign DNA.

2.3.2 Synthesis of the First-Strand Complementary DNA (cDNA Synthesis)

Using 1µg of total RNA, first strand cDNAs were synthesized with an oligo-dT primer in a reaction catalyzed by reverse transcriptase (RT), ReverTra Ace (Toyobo, Osaka, Japan). Incubations were first at 42 °C for 20 min, swiftly shifted to 99 °C for 5 min, and at the end cooled down at 4 °C and stored at –30 °C until the next use. Total reaction consisted of 20µl constituting 1µg RNA, 4µl of 5×RT buffer, 2µl dNTP mix, 1µl oligo dT primer, 1µl RNase inhibitor, 2µl Reverse TraAce and 8.8µl of sH2O. After mixing in ice the reaction tubes were first incubated at 42°C for 20min then swiftly shifted to 99°C for 5 min and then cooled to 4°C and stored at -30°C for use in PCR amplifications that utilized whole cDNA.

2.3.3 Agarose Gel Electrophoresis

For plasmid DNA or gel-extracted DNA analyses, agarose gels were prepared as 1% agarose gels in 1x Tris-Acetate-EDTA (1 x TAE buffer). For RNA electrophoresis, 1 x TAE buffer was prepared in sterilized water. After run completion, gels were viewed on AE-6905H Image Saver HR with a blue-light transilluminator installed gel documentation system (BIOINSTRUMENT ATTO) and documented using a computer installed with it.

2.3.4 Extraction and Purification of DNA Fragments from Agarose Gels

DNA fragments of the target sizes were excised from agarose gels using sterile scalpel blades and purified using QIAquick Gel Extraction Kit (Qiagen) by following the protocol provided by the manufacturer.

2.3.5 Isolation and Purification of Plasmid DNA

E. coli colonies, transformed with either any of the PCR-product amplified by appropriate primer and ligated in a suitable cloning vector or, those which were fed with a suitable expression vector for a gene isolate were used to inoculate 3 ml of autoclaved and cooled down 2YT media supplemented with appropriate antibiotic (Sigma) and incubated at 37 °C with shaking bath (rpm) overnight or ~ 16 hr. Plasmids were extracted by using the QIAprep Spin Miniprep Kit (QIAGEN) according to the protocol provided by the manufacturer.

2.3.6 Restriction Digestion Reactions

Restriction digestion reactions were performed in a total volume of 50-100 µl consisting of 1X reaction buffer, 2 – 10 ng of plasmid DNA and 1 unit of appropriate restriction enzyme. For example during cloning of the RACE-PCR products in pGEMT easy vectors, (Promega), restriction digestion with *EcoRI* restriction enzyme was used to excise gene fragments. Reactions were incubated for at least 1.0-1.5 hr in incubator at 37 °C. After incubations the products of restriction were analyzed by gel electrophoresis.

2.3.7 DNA Sequencing

After appropriate size confirmation by restriction with a suitable restriction enzyme (as per each experiment requirement), the identity of insert from PCR, RACE) reactions were confirmed by DNA sequencing using either gene-specific primers or M13 universal primers. The sequencing reaction (5µl) consisted of 1µM primer, 150 – 300 ng of plasmid DNA, 5x sequencing buffer and 0.5 µl of BigDye Terminator v3.1 enzyme mix (Applied Biosystems). The thermocycling conditions included an initial denaturation at 96 °C for 1 min followed by 30 cycles at 96 °C for 10 sec, 50 °C for 5 sec and 60 °C for 4 min. The reaction was held at 4°C and DNA

sequences were analyzed by capillary electrophoresis on 3130 Genetic Analyzer Applied Biosystems (Thermo Fisher Scientific).

2.3.8 DNA Sequence Analysis

DNA sequences were analyzed using DNASIS DNA analysis software (Hitachi Solutions, Tokyo, Japan). The ABI files were converted into DNA sequence chromatogram files (SCF) using DNASIS pro. Homology search was performed by BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). By using algorithm program for aligning and DNA sequences (Zhang et al., 2000) and MegaBLAST module of BLAST that does the initial phase of finding short seeds for matches by searching a database index (Morgulis, et al., 2008) Amino acid sequences were aligned by CLUSTAL W (http://www.clustal.org/), and a neighbor-joining tree was constructed with a 500 bootstrap replication support using MEGA7 software (Takmura et al., 2013).Transit peptides of the gene products were predicted by ChloroP (http://www.cbs.dtu.dk/services/ChloroP/). Fake sequences and poorly aligned ends were manually trimmed using BioEdit software (https://www.**bioedit**.com) to increase the quality of the analysis, (Hall, 1999).

2.4 Amplification and Quantification of Nucleic Acids

2.4.1 Designing of Primers

Primers were designed for RACE-PCR based on the partial sequences of an *I. batatas* lycopene β-cyclase gene (*IbLCYb*; accession no. GQ283003) and an *I. batatas* lycopene ε-cyclase gene (*IbLCYe*; accession no. HQ828090) in the Genbank database. The primers for *IbBHY*, *IbCYP97A* and *IbCYP97B* were synthesized based on the homology search and the partial sequence data of sweetpotato β-Carotene hydroxylase gene (*IbBHY*: Genbank accession number HQ828095), and cytochrome P450-type monooxygenase *ItCYP97A* cytochrome P450, *ItCY97B* from *Ipomoea trifida* data bases respectively. The length of all primers ranged from 18 – 26 bp with a GC content of 35 – 36 %. The melting temperature (Tm) target was 55 – 68 °C. Internal secondary structures for example hairpins and dimers resulting from primer self- or cross-annealing, as well as sequence segments with repetitive bases and known single nucleotide polymorphisms (SNPs), were avoided.

2.4.2 Polymerase Chain Reaction (PCR)

PCR-amplification was carried out using a Takara PCR thermocycler (Takara, Japan). Generally, all PCR reactions constituted Emerald PCR mix (Takara, Shiga, Japan) 10 µM of each primer (F and R), 10 ng of template and sH_2O to a final volume of 14 µl. All PCRs were initially denatured at 95°C for 2 min followed by 30 cycles of 95°C for 30 sec, 52°C for 30s and 72°C for 1.5 min per bp or kb of expected PCR-product. A final extension step of 72°C for 4 min was included and samples were held at 4 °C until electrophoresis, gel extraction and purification or use in the subsequent procedure of ligation and plasmid or construct preparation etc.

2.4.3 Rapid Amplification of cDNA Ends (RACE)

Rapid amplification of cDNA ends (RACE) was performed in order to amplify the unknown flanking sequences of carotenoids biosynthesis genes in sweetpotato. Both 5′- and 3′-RACE were carried out using a SMART^{TM} RACE cDNA Amplification Kit (Clontech, USA), following manufacturer's instructions.

2.4.4 Quantitative Real-Time PCR Analysis

Expression analysis of the two genes was performed by real-time PCR. Total RNA was extracted from sweetpotato leaves and tubers using an RNeasy Plant mini kit (Qiagen, Hilden, Germany), and treated with DNase I. One microgram each of total RNA was reverse-transcribed with oligo-dT primer using PrimeScript RT Master Mix (Takara, Shiga, Japan). Amplification in real-time PCR was performed using SYBR Premix DimerEraser (Takara), and data analysis was carried out using the ABI 7300 Real Time PCR System (Life Technologies). The expression levels of various genes were normalized by the actin gene IbACT7 as reference gene for the internal control.

The primers used in real-time PCR are shown in Table 4 (Results Chapter).

2.5 Cloning and Functional Identification of Carotenoids Genes

2.5.1 Ligation Reactions for RACE-PCR/PCR-Products with pGEMT Easy Vector, pBluescriptII K^+ Vector, pETDuet Vectors and pRSFDuet Vectors

DNA fragments generated by PCR, 3´-RACE-PCR and/or5´-RACE-PCR including nested RACE-PCR, or plasmids fragments of restriction digestion were purified from agarose gels using QIAquick Gel Extraction Kit (Qiagen) and ligated with pGEM®-T Easy vector (Promega). Gel-Purified DNA fragments were ligated in a final reaction volume of 3 µl with an insert: vector ratio of approximately 1:1. All ligations contained 1 U of Ligation High Ver.2 Ligase (Takara). Reactions were incubated at 16 °C for overnight or up to 16 hr. For purpose of sequencing and complete cDNA isolation, target fragments were ligated with pBluescriptII K^+ Vector (Cm^r), cloning vector (Toyobo, Tokyo, Japan) for *E. coli* with an insert: vector ratio of 1.4:0.6. pBluescriptII K^+ Vector (Cm^r), is a useful cloning vector supplied with a *lacZ* gene which makes it spesific for X-gal (Appedix) blue/white colony screening. This method of screening is convenient for distinguishing a successful recombinant cloning products from unsuccessful ones. For gene expression vectors such as pETDuet, Amp^r and pRSFDuet, Km^r Vectors, (Merck Millipore, Darmstadt, Germany) insert: vector ratios of 1.4:0.6 and/or 1:1 were used. For ligations, Ligation high Ver.2 (Toyobo) was applied.

2.5.2 Preparation of Heat-Shock Competent *E. coli*

Based on Inoue et al., (1990), DH5 α cells were prepared and stored in 50-500µl aliquots, in liquid N_2 and stored at -80°C.

2.5.3 Transformation of Competent *E. coli* DH5α and BL21 (DE3) Cells

For transformation, 200 µl of frozen competent cells were thawed on ice. Approximately, 1.5µl of ligation reactions were added to the competent cells and the mixture was then incubated on ice for 30 min. Cells were heat-shocked at 42 °C for 45-60sec and swiftly transferred again to ice for 2 min. Approximately 1mL of liquid SOC medium was added and the tubes were incubated at 37 °C for 1 hr. After incubation, the tubes then containing the transformed *E. coli* were

centrifuged @10000rpm for 2min and supernatant was discarded leaving approximately 200 µl of the bacterial culture transformed cells which were then vortexed and inoculated in petri dishes on 2YT supplemented with 100 µg/ml of appropriate antibiotic depending upon the experimental requirement. Plates were incubated at 37 °C overnight. After overnight incubation, desired colonies (e.g., white colonies in case of pBluescriptII K^+ vector) which represented the transformed *E. coli* were selected to inoculate into 2YT liquid culture tubes with appropriate antibiotics overnight for subsequent plasmid DNA extraction and analysis.

2.5.4 Culture Media for *E. coli*

E. coli culture tubes were made by selecting and inoculating single colonies from paltes containing transformed *E. coli* by inoculation into 2YT media, supplemented with appropriate selection antibiotics. *E. coli* liquid cultures were incubated at 37°C for up to 16 hrs on shaking bath.

2.5.5 Plasmids Used for the Heterologous Complementation Expression System in *E. coli* for Functional Identification of Isolated Carotenoids Biosynthesis Genes from Sweetpotato

pACCRT-EIB plasmid comprising gene cluster for lycopene synthesis (Misawa et al., 1990; Cunningham et al., 1993) was used to elucidate *IbLCYb* and *IbLCYe* genes from sweetpotato cultivars WS and W71. pACHP-Beta plasmid which has genes for β-carotene synthesis was used to explore the activity of IbBHY, IbCYp97A and IbCYP97B genes . Whereas, *E. coli* competent cells having pACCRT-EIB and pETDuet-MpLCYb/MpLCYe (Takemura et al., 2014) that accumulate lycopene and α-carotene were used for functional analysis of IbCYp97A and IbCYP97B from WS and W71 cultivars. pACHP-Beta, has been constructed by manipulating *Haematococcus pluvialis* pACCAR16DcrtX (Misawa et al. 1995) plasmid. First, the idi gene was isolated from pHP11 (Kajiwara et al. 1997; accession no. AB019034) with XhoI and NotI, and inserted into the AvaI-SalI site of pACCAR16DcrtX (Misawa et al., 1995) with an amplified NotI SalI fragment including the *Pantoea ananatis crtE* gene (Takemura et al., 2015).

2.6 Analysis of Carotenoids from Transformed *E. coli*

2.6.1 Extraction of Carotenoids

Extraction of carotenoids from recombinant *E. coli* was carried out by the method described (Fraser et al., 2000). First of all vortexed the tubes that contained the transformed *E. coli* which had accumulated carotenoids in their bodies after 2 days of culturing in 10mL of 2YT with appropraite selective antibiotic and IPTG at 21°C on a shaker in an incuabtor @200rpm. Also took equal number of new clean conical tubes, tagged and poured the vortexed culture to clean conical tubes one by one corresponding to the tag on original culture tubes.

Now took new sterilized microtubes (three for each culture containing conical tube), tagged correspondingly and weighed. Now centrifuged the conical tubes @8000rpm at room temperature and discarded the supernatant by pipette gently and completely and noted down the color of pallet. Added 1mL of STE buffer into pallet of each conical tube, mixed, and dissolved the pallet completely in it by pipette. Shifted the dissolved pallet in equal volumes to three new microtubes from each conical tube and centrifuged all microtubes @15000rpm for 1minute. Discarded the supernatant completely and dried pallets were weighed again to calculate the weight of pallet.

2.6.2 Chloroform Based Carotenoids Extractions

Added 200µl of methanol to all microtubes containing dried pallet and mixed on vortex at maximum speed for 5-10 min and finger-tapped during vortex to dissolve pallet in methanol well. Then, added 50µl of Tris-HCl and 150µl of 1M NaCl and mixed by vortexed for 2-5 min. At the end added 200µl of chloroform vortexed, mixed, and centrifuged the microtubes @8000 for 5 min at 4°C. After centrifugation, took new clean tubes tagged and poured lower phase correspondingly. All the tubes were shifted to concentrator (Tomy) and put the tubes into holes with lids opened. Set and turned the concentrator on for 1:30 hr. After the samples were completely dried out shifted them to -21°C until analyzed by HPLC.

2.6.3 HPLC Analysis and Identification of Extracted Carotenoids

Dried residues were re-suspended in ethyl acetate and applied to HPLC in a Waters Alliance 2695–2996 (PDA) system (Milford, CT, USA). HPLC was

performed according to the method described (Yokohama, 1995) using a TSKgel ODS-80Ts column (4.6×150 mm, 5 µm; Tosoh, Tokyo, Japan) at a flow rate of 1.0 mL/min at 25 °C with solvent A (water-MeOH, 5:95, v/v) for 5 min, followed by a linear gradient from solvent A to solvent B (tetrahydrofuran: MeOH, 3:7, v/v) for 5 min, and solvent B alone for 8 min.

Individual carotenoids were identified by comparing retention times and absorption spectra with those of the authentic standards (Takemura et al., 2014). To separate α- and β-carotene, an isocratic method was applied. HPLC was performed on a Nova-pak HR 6 µ C18 column (3.9×300 mm; Waters) at a flow rate of 1.0 mL/min at 25 °C with the solvent [acetonitrile (AcCN)-MeOH-2-propanol, 90: 6:4, v/v/v] for 80 min.

2.7 Maintenance of Desirable *E. coli* Clones by Cryopreservation

The successful colons from culture plates were maintained by cryopreservation. For this purpose, colony of choice was inoculated in 3mL of liquid 2YT culture tubes with appropriate antibiotic based on the cloning or expression vector used and incubated overnight. Next day, the culture tubes containing cultures of choice were shifted to clean bench and 1mL of culture was poured into cryovials containing 70µl dimethyl sulfoxide (DMSO), tagged properly and stored at −80 °C.

2.8 Transformation of Sweetpotato W71 Tubers with Bacterial Ketolase *crtW* Genes

2.8.1 Preparation of CrtW Gene constructs for Expression in W71 Tuber

The *crtW* gene constructs were prepared by the following the method reported earlier by Hasunuma et al., (2008).

2.8.2 Transformation of *Agrobacterium* Strain EHA101 Cells with crtW Gene Construct

After performing all construction with the auxiliary plasmid (modified pUC19), the gene cassette part were excised with 8 base recognition enzymes PacI and SalI and ligated into the pZH2B binary vector as described earlier (Kuroda et al., 2010; Hajdukiewicz et al., 1994).

2.8.3 *Agrobacterium* Mediated Transformation and Culturing of W71 Sweetpotato Tuber.

The pZH2B binary vector harboring the crtW construct was used to transform the *Agrobacterium* strain EH101 (Cm^r) by electroporation as described earlier (Fujisawa et al., 2009). Culturing of transformed sweetpotato W71 tuber cells was performed by following the protocol reported by Otani et al., (2003) with modifications that will be reported later.

2.9 Analysis of Carotenoids from transgenic Sweetpotato

2.9.1 Extraction of Carotenoids

Carotenoids were extracted from 10 g of the tubers of the *crtW* gene-expressed sweetpotato with 5% KOH methanol at room temperature. The extract was partitioned with *n*-hexane-diethyl ether (1:1) and water. Then, the organic layer was evaporated to dryness and submitted for silica gel column chromatography and HPLC-PDAanalysis described in detail in the results chapter (Maoka et al., 2016).

2.9.2 HPLC-PDA Analysis and Identification of Extracted Carotenoids

Carotenoid was extracted from 100 g of *crtW* gene-induced sweetpotato tubers with acetone at room temperature. The acetone extract was concentrated to a small volume in *vacuo* and partitioned with *n*-hexane-diethyl ether (1:1) and water. The organic layer was evaporated to dryness and submitted for silica gel column chromatography.

The fraction(s) eluted with n-hexane and/or acetone contained different carotenoids which were identified separately by by UV-vis, ESI-MS, ^{1}H-NMR, CD spectral data and COSY and NOESY experiments (Maoka et al., 2016).

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3. RESULTD AND DISCUSSIONS

3.1 CHEMICAL ANALYSIS OF CAROTENOIDS FROM SWEETPOTATO

3.1.1 HPLC Analysis of Carotenoids from the leaves and tubers of Sweetpotato

Two sweetpotato (*I. batatas*) cultivars, WS and W71, were selected as representative genotypes, exhibiting white and orange tuberous roots (tubers), respectively, since they have also been used in *Agrobacterium* mediated transformation (Gama et al., 1996; Takahata et al., 2010; Maoka et al., 2016). Carotenoid pigments were extracted from the two cultivars and subjected to HPLC-PDA-HRMS analysis. Figure 6 shows HPLC chromatograms of carotenoids extracted from the leaves (A) and tubers (B) of cultivar WS. Table 1 contains the spectroscopic data of the individual carotenoids appearing in Figure 6. While, Table 2 shows the carotenoid composition in the leaves and tubers of sweetpotato cultivars WS and W71, which was calculated based on the HPLC chromatograms in figure 6 for WS whereas, data for W71 is not shown here. Carotenoid composition in the leaves of both sweetpotato cultivars was substantially the same as that of other higher plant leaves mediating photosynthesis, i.e. their carotenoids contain approximately 45–50% lutein and approximately 30% β-carotene as the two predominant carotenoids, along with neoxanthin and violaxanthin, and often with small amounts of β-cryptoxanthin, antheraxanthin, zeaxanthin and α-carotene (Goodwin, 1988). Meanwhile, the tubers of cultivars WS and W71 were found to contain unique carotenoids that were composed of β-carotene-5,8-epoxide (9.4% and 4.6% of the total carotenoids, respectively), β-carotene-5,6,5′,8′-diepoxide (35.7% and 9.2%), and β-carotene-5,8,5′,8′-diepoxide (25.1% and 13.8%). Moreover, the WS and W71 tubers were found to contain ipomoeaxanthin A (4.5%) and β-cryptoxanthin-5′,6′-epoxide (2.2%), respectively. Their chemical structures are shown in Figure 7. Such rare carotenoids had previously been identified as unique constituents of the tubers of the Japanese sweetpotato cultivar “Benimasari” (Maoka et al., 2007). It is likely that sweetpotato tubers generally

contain rare carotenoids that include a 5,6-epoxy-β-ring and/or a 5,8-epoxy-β-ring in their structures, since WS and W71 were bred in USA, they should be genetically distant from the Japanese cultivar “Benimasari”.

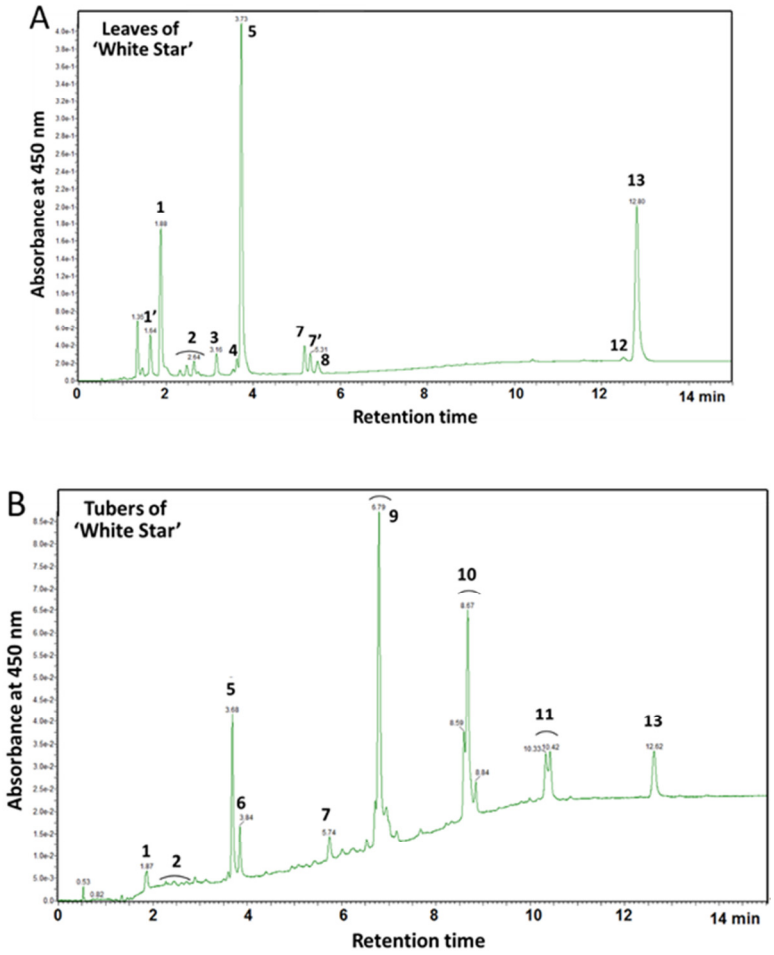


Figure 6. HPLC chromatograms of carotenoids extracted from the leaves (A) and tubers (B) of sweetpotato (*Ipomoeoa batatas*) cultivar WS. An Acquity 1.7 μm BEH UPLC C18 column (Waters) was used, as described in the Materials and Methods Chapter. Identification of peaks: **1′**, neochrome; **1**, neoxanthin; **2**, violaxanthin (including auroxanthin); **3**, antheraxanthin; **4**, zeaxanthin; **5**, lutein; **6**, ipomoeaxanthin A; **7**, 9-*cis*-lutein, **7′**, 13-*cis*-lutein; 8, β-cryptoxanthin; 9, β-carotene-5,6,5′,8′-diepoxide (stereoisomers); **10**, β-carotene-5,8,5′,8′-diepoxide (stereoisomers); **11**, β-carotene-5,8-epoxide (stereoisomers); **12**, α-carotene; **13**, β-carotene. Neochrome (peak 1′) and auroxanthin (some in peak 2) are considered to be formed from neoxanthin and violaxanthin, respectively, during the extraction process. Peaks 9, 10 and 11 are individually composed of

several peaks, which are thought to contain stereoisomers, and sometimes geometrical isomers generated during the extraction process.

Number[a]	Carotenoid	UV-VIS nm	ESI TOF MS *m/z*
1′	Neochrome	405, 428, 450	601.4232 (M^+, $C_{40}H_{57}O_4$)
1	Neoxanthin	414, 439, 469	601.4232 (M^+, $C_{40}H_{57}O_4$)
2	Violaxanthin	414, 439, 469	601.4232 (M^+, $C_{40}H_{57}O_4$)
2	Auroxanthin	376, 399, 423	601.4232 (M^+, $C_{40}H_{57}O_4$)
3	Antheraxanthin	425, 446, 473	568.4299 (M^+ $C_{40}H_{56}O_2$), 551.4246 (M^+-H_2O)
4	Zeaxanthin	(425), 451, 477	568.4299 (M^+ $C_{40}H_{56}O_2$)
5	Lutein	425, 446, 473	568.4299 (M^+ $C_{40}H_{56}O_2$), 551.4246 (M^+-H_2O)
6	Ipomoeaxanthin A	425, 446, 473	586.4410 (M^+ $C_{40}H_{58}O_3$)
7	9-cis-Lutein	422, 441, 468	568.4299 (M^+ $C_{40}H_{56}O_2$), 551.4246 (M^+-H_2O)
7′	13-cis-Lutein	420, 439, 465	568.4299 (M^+ $C_{40}H_{56}O_2$), 551.4246 (M^+-H_2O)
8	β-Cryptoxanthin	(425), 451, 477	552.4327 (M^+ $C_{40}H_{56}O$)
9	β-Carotene-5,6, 5′,8′-diepoxide	405, 426, 451	569.4348 (MH^+ $C_{40}H_{57}O_2$)
10	β-Carotene-5,8, 5′,8′-diepoxide	379, 399, 424	569.4348 (MH^+ $C_{40}H_{57}O_2$)
11	β-Carotene-5,8-epoxide	400, 426, 451	552.4327 (M^+ $C_{40}H_{56}O$)
12	α-Carotene	425, 446, 473	536.4383 (M^+ $C_{40}H_{56}$)
13	β-Carotene	(425), 451, 477	536.4383 (M^+ $C_{40}H_{56}$)
	β-Cryptoxanthin-5′,6′-epoxide[b]	425, 446, 473	568.4299 (M^+ $C_{40}H_{56}O_2$)

Table 1. Spectroscopic data of the individual carotenoids analyzed from sweetpotato cultivars W7 and WS. [a]Numbers correspond to those used in Figure 6. β-Cryptoxanthin-5′, 6′-epoxide was detected only in cultivar W71.

	WS leaf	W71 leaf	'White Star' tuber	W71 tuber
Total carotenoid (μg/g FW)	34	170	0.5	4.0
Carotenoid composition	%	%	%	%
β-Carotene	30.7	31.1	7.3	59.3
α-Carotene	0.6		nd	nd
β-Cryptoxanthin	2.4			3.2
Zeaxanthin	0.7			trace
Lutein	43.2	46.8	13.7	2.0
cis-Lutein	3.3	3.3		
Antheraxanthin	1.8			
Violaxanthin (+ Auroxanthin)	12.3	3.5	0.9	
Neoxanthin (+ Neochrome)	3.6	6.4	1.5	
β-Carotene-5,8-epoxide	nd	nd	9.4	4.6
β-Carotene-5,6,5′,8′-diepoxide	nd	nd	35.7	9.2
β-Carotene-5,8,5′,8′-diepoxide	nd	nd	25.1	13.8
β-Cryptoxanthin-5′,6′-epoxide	nd	nd	nd	2.2
Ipomoeaxanthin A	nd	nd	4.5	nd
Others	1.4	8.9	1.9	5.7

Table 2. Carotenoid content and composition in sweetpotato cultivars WS and W71.

Where ‘nd’, represents ‘not detected’.

3.1.2 Proposed Biosynthetic Pathway of Carotenoids based on HPLC Analysis of Carotenoids from Sweetpotato

The carotenoid biosynthetic pathway in sweetpotato tubers, was proposed on the basis of the results is shown in Figure 7. The tubers of the three cultivars WS, W71 and “Benimasari” thus contain sweetpotato-specific carotenoids with the 5,6-epoxy-β-ring, rather than the epoxy carotenoids common to higher plant leaves, i.e. violaxanthin and antheraxanthin, with the 3-hydroxy-5,6-epoxy-β-ring. Therefore, sweetpotato tubers are likely to contain a novel enzyme that converts carotenoids possessing a β-ring, such as β-carotene and β-cryptoxanthin, into metabolites with a 5,6-epoxy-β-ring, such as β-carotene-5,6-epoxide and β-cryptoxanthin-5′,6′-epoxide. Such a β-ring-epoxidation enzyme can be anticipated to have considerable homology to the ZEP (zeaxanthin epoxidase) enzyme, encoded by a ZEP paralog. A candidate gene has been cloned in our laboratory, awaiting functional analysis. Ipomoeaxanthin A is considered to be produced from β-carotene-5,6-epoxide by an unknown oxidizing enzyme. Meanwhile, carotenoids with an 5,8-epoxy-β-ring can arise non-enzymatically from precursors having an 5,6-epoxy-β-ring through an epoxy-furan transposition reaction.

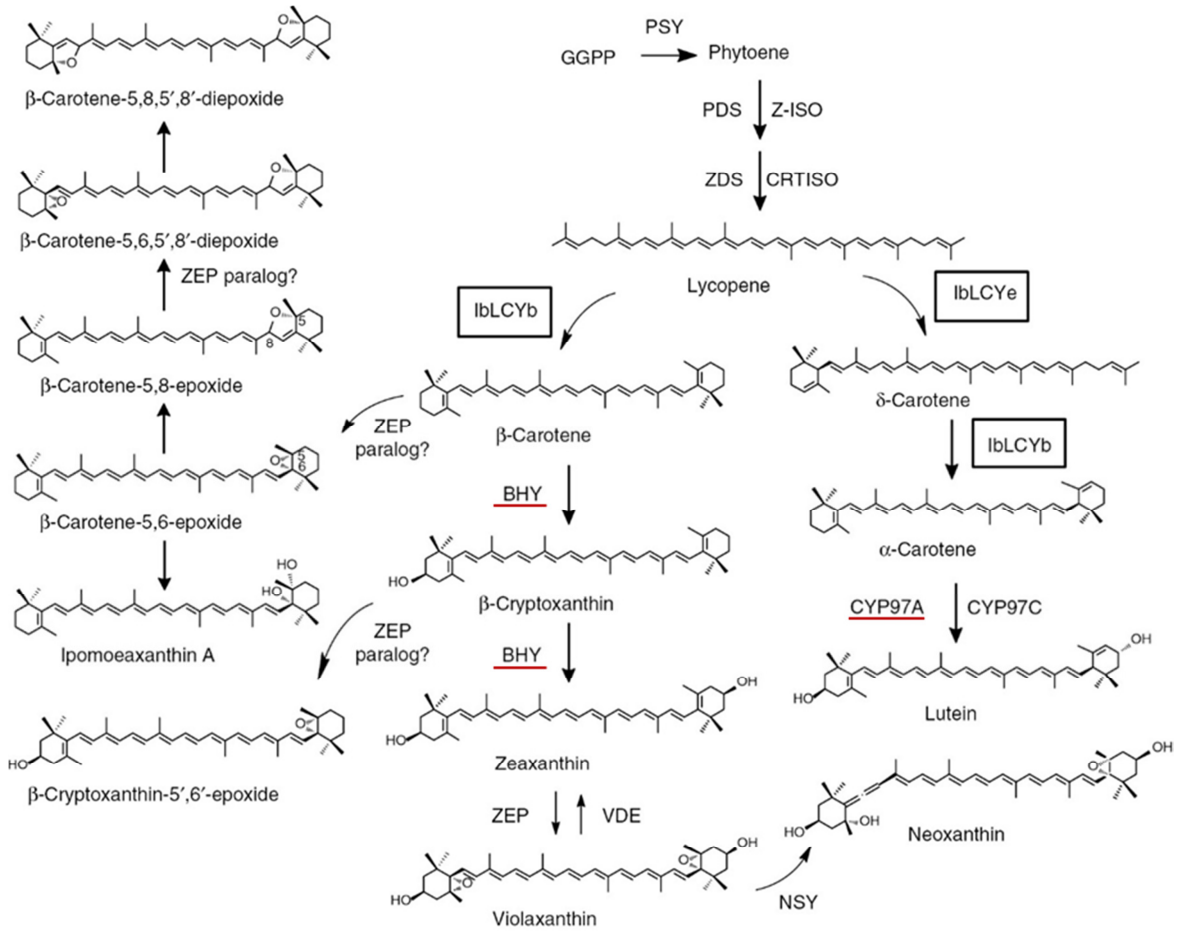


Figure 7. Proposed carotenoid biosynthetic pathway in the tubers of sweetpotato cultivars WS and W71. Ipomoeaxanthin A and β-cryptoxanthin-5′,6′-epoxide were found only in the tubers of WS and W71, respectively. It was considered that β-carotene-5,8-epoxide was non-enzymatically produced from β-carotene-5,6-epoxide, which was not observed in either WS or W71. Carotenoids containing the 5,6-epoxy-β-ring are stereospecific compounds, while those containing the 5,8-epoxy-β-ring occur as stereoisomers (C-8). Carotene cyclases and hydroxylases elucidated in this work are boxed and underlined resepectively.

3.1.3 Conclusion

The carotenoid analyses of sweetpotato provided a molecular map for carotenoids with varied composition especially the oxygenated and or hydroxylated products. These results will support molecular cloning and elucidation of carotenoids biosynthesis genes and their pathway engineering based on the proposed biosynthesis pathway in sweetpotato for future nutritional, medicinal and industrial gains

3.2 ISOLATION OF LYCOPENE CYCLASE GENES FROM SWEETPOTATO

3.2.1 Isolation of Lycopene β-cyclase (*IbLCYb*) Genes from WS and W71 Cultivars of Sweetpotato.

3′and 5′-RACE were carried out by SMARTer™ RACE cDNA Amplification Kit (Clontech, Mountain View, CA, USA) according to the supplier's instructions. Primers were designed for RACE-PCR based on the partial sequences of an *I. batatas* lycopene β-cyclase gene (*IbLCYb*; accession no. GQ283003) and an *I. batatas* lycopene ε-cyclase gene (*IbLCYe*; accession no. HQ828090) in the Genbank database.

Primer Name	Primer Sequence 5'-3'	Restriction enzymes used	Gene Cloning Experiment Type
IbLCYbF1	GCGTATGGCATTCTGGCAGAAGTGG	EcoRI	3′-RACE
IbLCYeF1	CGAAGTCCTGGTATGGGTGATATGC		3′-RACE
IbLCYbF2	CATGGATTGGCGAGACTCTCACC		3′-RACE nested PCR
IbLCYeF2	CAAGCTTGGGAAACGCTTTGGCC		3′-RACE nested PCR
IbLCYbR1	AAGCCAGTTGCATCGAGAACCACAG		5′-RACE
IbLCYeR1	TGAATACCCTGTAGCGGGATGAACC		5′-RACE
IbLCYbR2	CATGGATTGGCGAGACTCTCACC		5′-RACE nested PCR
IbLCYeR2	CAAGCTTGGGAAACGCTTTGGCC		5′-RACE nested PCR
IbLCYbF3	CGTCGACATGGATACTCTGCTAAA	BamHI	pETDuet Cloning
IbLCYbR3	GGCGGCCGCTTAATCTATATCCTGTAAC	SalI	pETDuet Cloning
IbLCYeF3	CGGATCCATGGAGTGCATCGGAGCTC	BamHI	pETDuet Cloning
IbLCYeR3	CGTCGACTTACAGAGTGAGATAAGT	SalI	pETDuet Cloning

Table 3. Primers used in cloning and isolation of *IbLCYb* and *IbLCYb* genes from W71 and WS cultivars of sweetpotato.

The primers are summarized in **table 3.** As the first PCR primers for 3′-RACE of *IbLCYb* and IbLCYe, IbLCYbF1and IbLCYeF1 were used, respectively. For the nested PCR amplification in 3′-RACE, respective nested gene-specific primers for IbLCYbF2 and IbLCYeF2 were used. Similarly, for the amplification of the IbLCYb and IbLCYe 5′-ends, IbLCYbR1 and IbLCYeR1 were used in the first PCR, respectively. For 5′-RACE, IbLCYbR2 and IbLCYeR2 were used as the nested PCR primers. Each RACE product was subcloned into the pGEM T-easy vector (Promega, Madison, WI, USA), followed by DNA sequencing. Full length cDNA sequences of *IbLCYb* and *IbLCYe* were obtained through the above PCRs. The full length cDNAs of the *IbLCYb*and *IbLCYe* genes were isolated by PCR amplification with a pair of primers: IbLCYbF3 and IbLCYbR3, and IbLCYeF3 and IbLCYeR3 respectively.

Two lycopene β-cyclase gene (*IbLCYb*) sequences were, isolated from sweetpotato cultivar WS, and named *IbLCYb1* and *IbLCYb2*. On the other hand, from W71, three lycopene β-cyclase genes, designated *IbLCYb3*, *IbLCYb4* and *IbLCYb5* were isolated. However, *IbLCYb3* and*IbLCYb4* were found to encode the same deduced amino acid sequences.

3.2.2 Isolation of Lycopene ε-Cyclase (*IbLCYe*) Genes from WS and W71 Cultivars

In the case of the lycopene ε-cyclase gene (*IbLCYe*), a single sequence, designated *IbLCYe*, was isolated from both WS and W71. Another putative lycopene ε-cyclase gene had previously been isolated from sweetpotato cultivar Nongdafu 14 (Ling et al., 2013) however, without functional identification of the gene product as ε-cyclase), which encoded a protein with an N-terminal region totally different from that of our IbLCYe, but the mature proteins were 97.9% identical.

3.2.3 Sequence Analysis and Alignments of *IbLCYb* and *IbLCYe* Genes

The nucleotide sequences of the sweetpotato carotene cyclase genes *IbLCYb and IbLCYe* cloned in the present study were submitted to DDBJ under accession number LC164788 (*IbLCYb*1), LC164789 (*IbLCYb*2), LC164790 (*IbLCYb*3), LC164791 (*IbLCYb*4), LC164792 (*IbLCYb*5), and LC164793 (*IbLCYe*). Sequence alignment of these isolated genes with other plant species is shown in figure 8 and 9. An inner deletion of 15 bases was noticed in *IbLCYb*5. The sweetpotato lycopene ε-cyclase reported by Ling et al., (2013), is not shown in this sequence alignment and phylogenetic analysis because its accession number was not available.

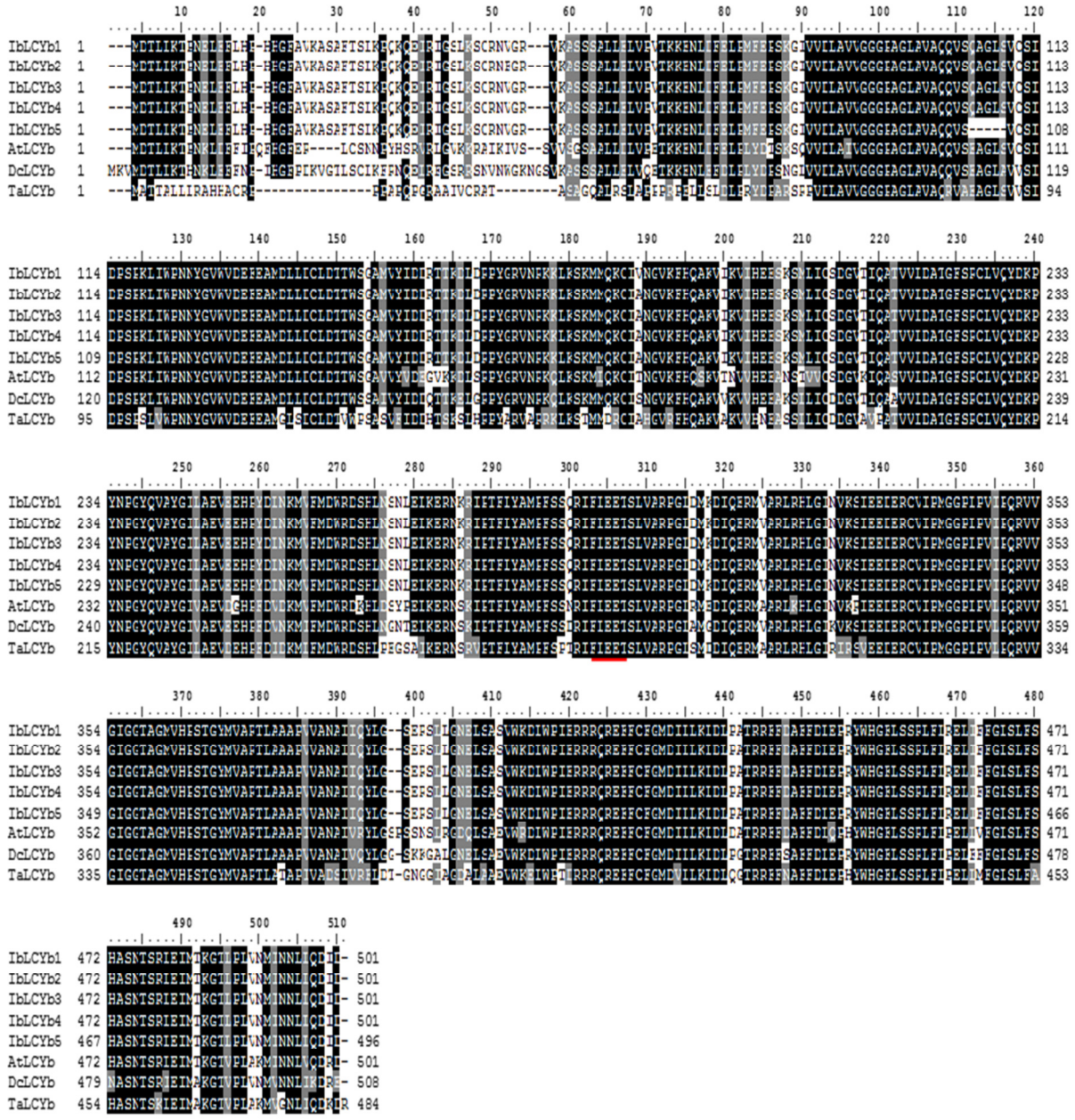


Figure 8. Alignment of *IbLCYb* with other plant LCYb proteins. The alignment was performed with MEGA7.0 (Tamura et al., 2013) using default settings. Dashes represent alignment gaps. Conserved amino acid residues in the alignment have a black background, while similar amino acids have grey background. Numbers on the right denote the number of amino acid residues. All conserved amino acid residues in the alignment have a black background, while similar amino acids have grey background. Conserved motif FLEET which is essential for establishing activity is underlined red. GenBank accession numbers of the aligned amino acid sequences from other plants are include: *Arabidopsis* LCYb (AtLCYb), U50739; carrot LCYb (DcLCYb), DQ192190; wheat LCYb (TaLCYb), N622196.1

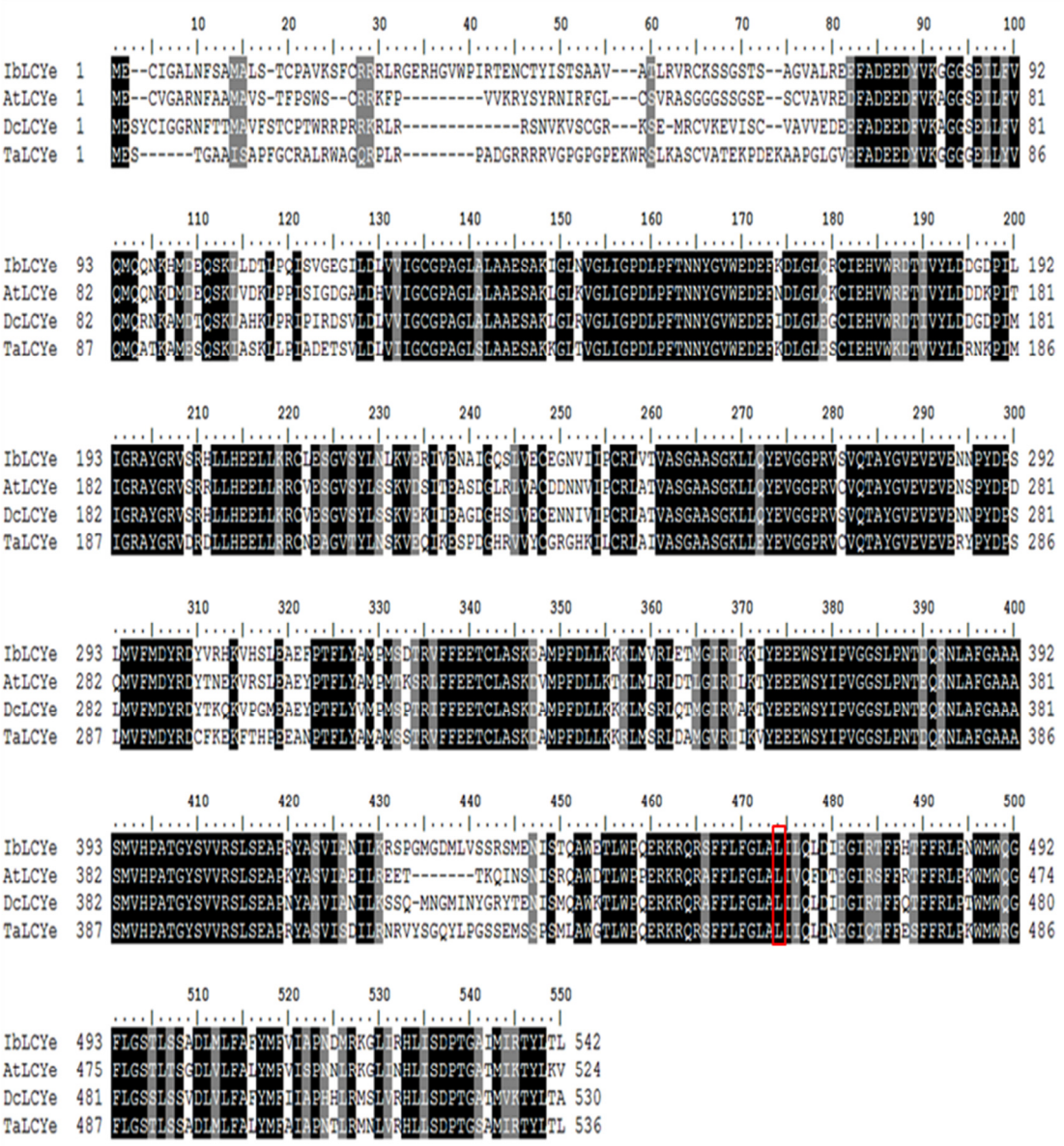


Figure 9. Alignment of IbLCYe with other plant LCYe proteins. The alignment was performed with MEGA7.0 (Tamura et al., 2013) using default settings. Dashes represent alignment gaps. Conserved amino acid residues in the alignment have a black background, while similar amino acids have grey background. Numbers on the right denote the number of amino acid residues. All conserved amino acid residues in the alignment have a black background, while similar amino acids have grey background. Amino acid residues shown inside red box are the L448 in *Arabidopsis* which are important conserved residues for determination of the number of ε-rings. GenBank accession numbers of the aligned amino acid sequences from other plants include: *Arabidopsis* LCYe (AtLCYe), U50738.1; carrot LCYe (DcLCYe), DQ192192.1; wheat LCYe (TaLCYe), EU649787.

3.2.4 Phylogenetic Analysis of IbLCYb and IbLCYe Proteins from Plants species

Phylogenetic analysis of the deduced proteins encoded by the isolated sweetpotato carotene cyclase genes, compared to other known carotene cyclases of higher plants is shown in Figure 10. *IbLCYb* (1–5) and IbLCYe shown in dotted boxes.

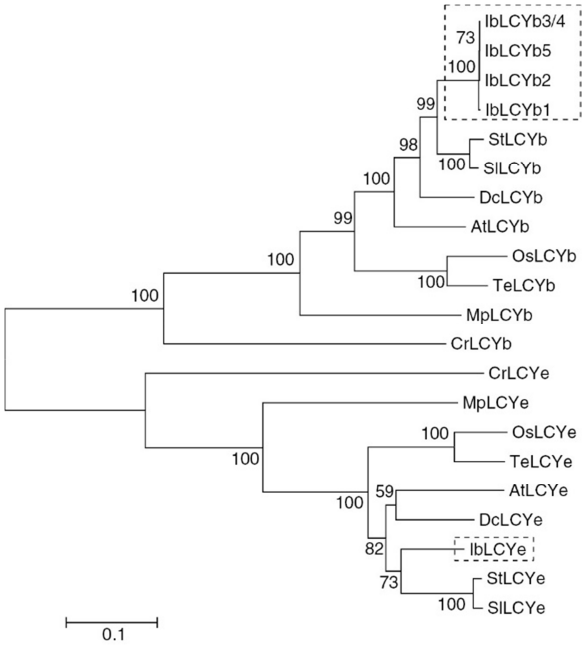


Figure 10. Phylogenetic tree of various lycopene β-cyclase (LCYb) and lycopene ε-cyclase (LCYe) proteins. GenBank accession numbers of the aligned cyclases include: Arabidopsis LCYb (AtLCYb), U50739; carrot LCYb (DcLCYb), DQ192190; wheat LCYb (TaLCYb), JN622196.1; rice LCYb (OsLCYb), XP_015627235; tomato LCYb (SlLCYb1), EF650013; potato LCYb (StLCYb), XP_006364433; liverwort LCYb (MpLCYb),AB794089; Chlamydomonas LCYb (CrLCYb), AY860818; Arabidopsis LCYe (AtLCYe), U50738.1

carrot LCYe (DcLCYe), DQ192192.1; wheat LCYe (TaLCYe), EU649787; rice LCYe (OsLCYe), XP_015622198; tomato LCYe (SlLCYe), Y14387; potato LCYe (StLCYe), XP_006353544.1; liverwort LCYe (MpLCYe), AB794090; Chlamydomonas LCYe (CrLCYe), AY606130.

3.2.5 Conclusion

IbLCYb and IbLCYe showed significant homology and individually formed single clades that were positioned close to the corresponding proteins from potato

(*Solanum tuberosum*) and tomato (*Solanum lycopersicum*), which, like sweetpotato, belong to the order Solanale.

3.3 FUNCTIONAL ANALYSIS OF ISOLATED *IbLCYb* AND *IbLCYe* CYCLASE GENES FROM SWEETPOTATO

3.3.1 HPLC analysis of Carotenoids formed in the Lycopene-producing *E. coli* t

E. coli BL21 (DE3) that already harbored pACCRT-EIB plasmid (shown in figure 11) were transformed with the sweetpotato *IbLCYb* or *IbLCYe* genes.

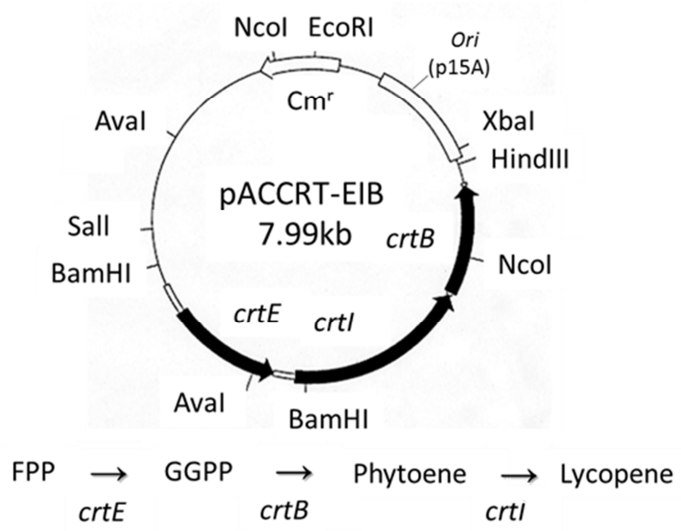


Figure. 11 pACCRT-EIB plasmid comprising gene cluster crtE,crtB and crtI from *Pantoea ananatis* for lycopene synthesis (Misawa et al., 1995; Misawa et al., 1990; Cunningham et al., 1993)

When plasmids, pETD-IbLCYb1, pETD-IbLCYb2, and pETD-IbLCYb4, were separately introduced into the lycopene-producing *E. coli* cells, β-carotene was detected in all the extracts (Figure 12 A–C). The enzymatic activity of *IbLCYb*4 (*IbLCYb*3) in the *E. coli* cells was weak compared with that of *IbLCYb*1 and *IbLCYb*2. In contrast, pETD-IbLCYb5 did not direct conversion of lycopene (Figure 12D). The introduction of plasmid pETD-IbLCYe led to efficient production of δ-carotene (Figure 11E), indicating that IbLCYe catalyzes the formation of δ-carotene by adding one monocyclic ε-ring to lycopene. Thus, this gene was shown to encode a lycopene ε-(mono) cyclase. MpLCYe derived from the liverwort *Marchantia polymorpha* was able to convert lycopene into ε-carotene with dicyclic

ε-rings by way of δ-carotene (Takemura et al., 2014), while IbLCYe appears to be strictly an ε-monocyclase (Figure 12).

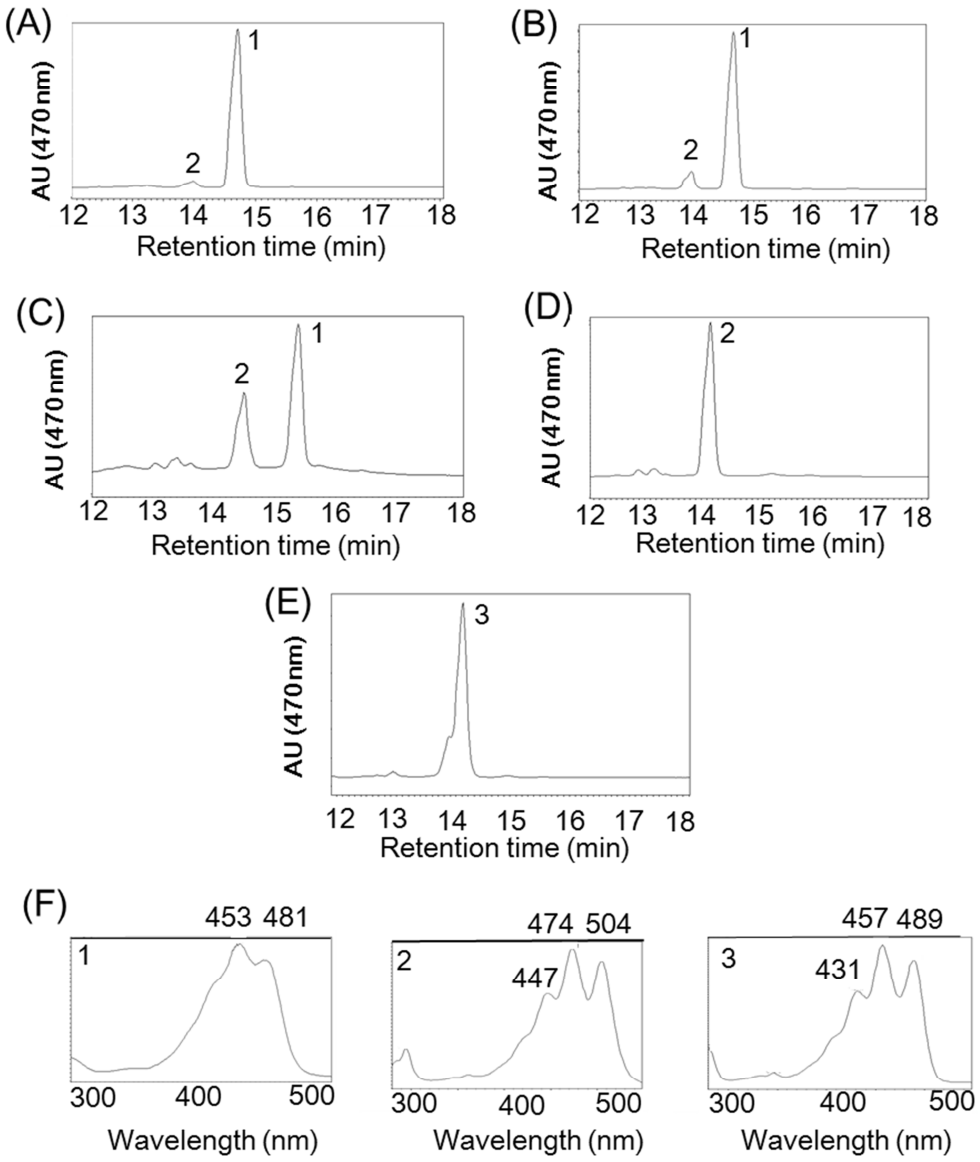


Figure 12. HPLC analysis of carotenoids formed in the lycopene-producing *E. coli* strain that had been transformed with the sweetpotato *IbLCYb* or IbLCYe genes.A TSKgel ODS-80Ts column (4.6×150 mm, 5 μm) was used in HPLC analysis.

(A–E) HPLC chromatograms of carotenoid extracts from *E. coli* that harbored pACCRT-EIB plus the respective sweetpotato gene: **(A)** pETD-*IbLCYb*1; **(B)** pETD-*IbLCYb*2; **(C)** pETD-*IbLCYb*4; **(D)** pETD-*IbLCYb*5; **(E**) pETD-IbLCYe. **(F)** Absorption spectra of individual peaks: **1**, β-carotene; **2**, lycopene; **3**, δ-carotene.

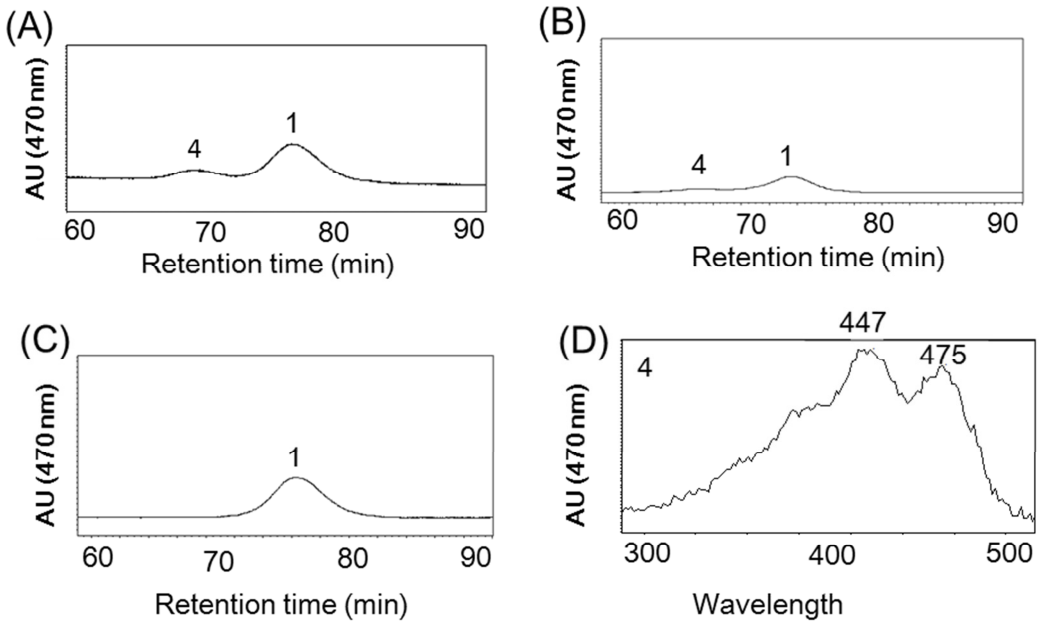


Figure 13. HPLC analysis of carotenoides formed in the lycopene-producing *E. coli* strain that had been transformed with both the sweetpotato *IbLCYb* and IbLCYe genes. A Nova-pak HR 6 µ C18 column (3.9×300 mm; Waters) was used in analysis.

(A–C) HPLC chromatograms of carotenoid extracts from *E. coli* that harbored pACCRT-EIB plus the respective sweetpotato genes: **(A)** pETD-*IbLCYb*1/IbLCYe; **(B)** pETD-*IbLCYb*2/IbLCYe; **(C)** pETDuet-*IbLCYb*4/IbLCYe; **(D)** Absorption spectra of individual peaks: 4, α-carotene; 1, β-carotene (Figure 12F).

The combined co-expression of *IbLCYb*1 or *IbLCYb*2 and IbLCYe (plasmid pETD-*IbLCYb*1/IbLCYe pETD-*IbLCYb*2/IbLCYe) in the lycopene-producing *E. coli* resulted in α-carotene formation, along with a considerable amount of β-carotene as shown in Figure 13 for HPLC analyses, whereas co-expression of *IbLCYb*4 and IbLCYe (plasmid pETD-*IbLCYb*4/IbLCYe) did not lead to such an activity and only β-carotene was detected.

3.3.2 Expression Analysis of the Isolated *IbLCYb* and *IbLCYe* Genes from sweetpotato cultivars WS and W71

Expression analysis of the carotene cyclase genes was performed in the leaves and tubers of the two sweetpotato cultivars (Figure 14). Both the *IbLCYb* and *IbLCYe* genes were highly expressed in the leaves of both cultivars, while in the tubers *IbLCYb* transcript levels were much higher than those of *IbLCYe*, providing an explanation why β-carotene and β-carotene-derived carotenoids occur abundantly in the tubers.

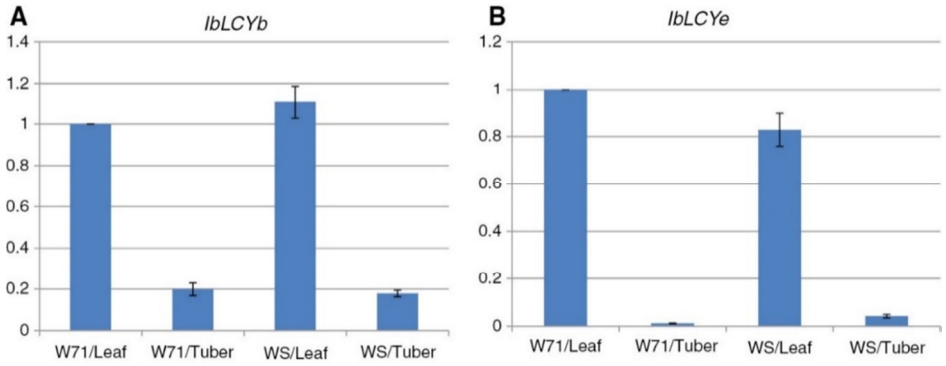


Figure 14. Transcript levels of the *IbLCYb* gene **(A)** and the *IbLCYe* gene **(B)** in the leaves and tubers of sweetpotato cultivars W71 and WS.

Primer Name	Gene	Direction	Sequence 5'-3'
IbLCYbRTF1	LCYb	Forward	AGCGTTGTGTTATCCCAATGG
IbLCYbRTR1		Reverse	TACCGCCAATTCCAACAACTC
IbLCYeRTF1	LCYe	Forward	TCATTCCATGCAGGCTAGTTACTG
IbLCYeRTR1		Reverse	CCCAACCTCATACTGCAACAATT
IbACT7F1	Actin	Forward	CCAAGAGCAGTGTTTCCCAGTAT
IbACT7R1		Reverse	TCTGTCCCATCCCAACCATAA

Table 4. Primers used in the real time PCR for expression analysis of lycopene cyclases in sweetpotato cultivars W71 and WS.

Basically, Lycopene β-cyclase (LCYb) individually can catalyse two β-rings at both ends of lycopene changing to form β-carotene. However, LCYe alone typically adds one ε-ring to the lycopene to make monocyclic δ-carotene which is then catalyzed by LCYb to α-carotene. Collective catalytic activity of lycopene β-cyclase (LCYb) and lycopene epsilon cyclase (LCYe) catalyzes the reaction which involves formation of one β and one ε-ring into lycopene to synthesize α-carotene. (Yamamizo et al., 2010). It has previously been reported that structurally LCYb and LCYe share high level of similarity ranging to 30% among there amino acid sequences (Hirshberg, 2001).These findings have suggested that both LCYb and LCYe share a common ancestor for their origin through gene duplication (Takemura et al., 2014). In tomato, two LCYb genes which separately exist in chloroplast and chromoplast have been reported that have 53% of identical amino acid sequences (Hirshberg, 2001; Bouvier et al., 1994). This corresponds to the present finding where sweetpotato also showed multiple copies of LCYb with

significant amino acid similarities as depicted in sequence analyses. Whereas, in case of Arabidopsis, only one LCYb gene has been reported (Cunnigham et al., 1996; Ronen et al., 2000, Alquezar et al. 2009) which is not similar finding to the present one in sweetpotato for LCYb. Contrarily, only one copy of LCYe gene found in all higher plants by now and highly regulated processes such as the control of gene expression levels are the factors determining the concentration and composition of carotenoids inside plant tissues (Ronen et al., 1999; Takemura et al., 2014). These reports support the present results for sweetpotato which also showed only one gene for LCYe and carotenoid gene expression in contrast with the actual amounts of carotenoids found in sweetpotato carotenoid chemical analysis. The allelic variation in the genes controlling carotenoids biosynthesis pathway is the important factor that determines the accumulation of carotenoids in a plant (Clotault et al*.,* 2010; Arizio et al., 2014). The amino acid sequences of all LCY β-type enzymes have a conserved motif called FLEET motif which is essential for establishing activity (Cunnigham et al., 1996; Moise et al., 2014). The present results for sweetpotato IbLCYb also showed conserved FLEET motif. As described previously, two ε- rings are commonly not found in majority of plants including algae (Goodwin, 1980) but lettuce is an exemption in which LCYe has been reported for making bicyclic ε-ε-carotene along with its hydroxylated derivative lactucaxanthin by cyclsing both Ψ-ends of lycopene (Phillip and Young, 1995; Takemura et al., 2014; Cunnigham and Gantt, 2001). However,sweetpotato ε- ring cyclase (IbLCYe) did not show any such activity instead it catalyzed the formation of mono-cyclic δ-carotene.

3.3.3 Conclusion

These results indicated that IbLCYb1, IbLCYb2, IbLCYb3, and IbLCYb4 possess lycopene β-cyclase activity, while IbLCYb5 was not functional. The non-functionality of IbLCYb5 might be due to the inner deletion of 15 bases making it a naturally existing mutant.Therefore, only the IbLCYb1-IbLCYb4 genes encode functional lycopene β-cyclase and IbLCYb1 and IbLCYb2 can synthesize α-carotene in the presence of IbLCYe.

3.4 ISOLATION OF β-CAROTENE HYDROXYLASE GENES FROM SWEETPOTATO

3.4.1 Isolation of Non-heme di-iron β-Carotene Hydroxylase genes (*IbBHY*) from WS and W71

By using the partial sequence data, expressed sequence tags and homology search, for sweetpotato β-carotene Hydroxylase genes (IbBHY), the gene spesific primers were desighned for 3'and 5'RACE to find the coding regions of sweetpotato *BHY(IbBHY)* genes. The primers used are shown in the table 3'and 5'RACE experiments were performed using SMART RACE cDNA Amplification Kit (Clontech). The first phase of amplification was performed by using GSP1 and GSP2 plus the universal primer provided in the kit for 3'RACE. For 5-'nested-RACE, NGSP1 with nested universal primer provided by the kit were used for 5'RACE. Similarly, NGSP2 with nested universal primer provided by the kit were used for 3'-nested RACE respectively. The products of each RACE were simultaneously cloned in pGEM®-T Easy Vector (Promega) and sequenced. After alignment and assembly of the 3' and 5'RACE products and the sequences of core fragment, the full length cDNA sequences of IbBHY were deduced. The full length cDNA sequences of IbBHY were isolated by using cDNA from both cultivars in separate reactions amplified by IbBHYF3 and IbBHYR3. The transit peptides were eliminated by amplifying the cDNA from both cultivars as template with the primers pair IbBHYF4 and IbBHYR3 by PCR at 95°C for 2 minutes, followed by 25 cycles of amplification at 95°C for 30seconds, 53°C for 30sec and 72°C for40 sec.

Primer Name	Primer Sequence 5'-3'	Restriction enzymes used	Gene Cloning Experiment Type
IbBHYR1	GAGGAGAGCGATGGCAGGGACAGCG		GSP1 (5'-RACE)
IbBHYR2	AGCTCGAACGGTCCTTCTCTTGG		NGSP1
IbBHYF1	CCACAAAGGTCTCGTTCCTGGCCTC		GSP2 (3'-RACE)
IbBHYF2	CGGAGCTGGGCTTGGAATCACAG		NGSP2
IbBHYF3	AGGATCCATGGCGGTCGGAATTTCC	BamHI	pETDuet Cloning
IbBHYR3	AGTCGACTTATCGTCCCTTACTCTTG	SalI	pETDuet Cloning
IbBHYF4	CGGATCCATGGCCGAGGAGATTGAGAAG	BamHI	pETDuet Cloning

Table 5. Primers used in cloning of *IbBHY* genes in sweetpotato cultivars W71 and WS. **GSP**, gene specific primer; **NGSP**, nested gene specific primer

Two lycopene β-carotene hydroxylase gene (*IbBHY*) sequences were, isolated one from sweetpotato cultivar W71, named *IbBHY1* and another from WS named IbLBHY2. Both were found to encode the different deduced amino acid sequences. Isolated full lengths cDNA size for each was 918bp with amino acid sequence length of 305amino acid bases.

3.4.2 Sequence Analysis and Alignments of *IbBHY* Genes

The open reading frame of IbBHY1 from W71 showed 70% sequence identity with beta-hydroxylase 1 (*Arabidopsis thaliana*) BCH1 acession number NP_194300.1 and beta-carotene 3-hydroxylase, chloroplastic-like of *Glycine max*. accession number NP_001241433.1, 94% with beta-carotene hydroxylase (*Ipomoea sp*. Kenyan) accession number BAI47578.1, and beta-carotene hydroxylase [*Ipomoea obscura*] accession number BAI47579.1, There were 75%, 77%, 83%, 80%, identity with beta-carotene hydroxylase (*Carica papaya*) accession number ADZ14893.1 , beta-carotene hydroxylase (*Gentiana lutea*) accession number BAE92729.1, beta-carotene hydroxylase (*Coffea arabica*)accession number ABA43903.1 and *beta-carotene hydroxylase (Solanum lycopersicum*) accession number NP_001234348.1 Japanese fruit beta-carotene hydroxylase (*Diospyros kaki*) accession number ACN86365.1 showed 74%, while, beta-carotene hydroxylase (*Solanum lycopersicum*), accession number CAB55626.2, beta-carotene hydroxylase 2 (*Solanum tuberosum*) accession number ADF28628.1 and beta-carotene hydroxylase BCH2 (*Arabidopsis thaliana*)

accession number NP_200070.1 showed 76%, 77% and 69% sequence matches with *IbBH1* of W71.

IbBHY2 from WS showed almost similar pattern of sequence homology with related plant species for β- ring hydroxylase. It showed 68% sequences identity with beta-hydroxylase BCH1 (*Arabidopsis thaliana*) accession number NP_194300.1, 95% with beta-carotene hydroxylase (*Ipomoea* sp. Kenyan) accession number BAI47578.1 , 86% with beta-carotene hydroxylase 2, chloroplastic [*Solanum tuberosum]*accession number XP_006360197.1 , 75% to that of beta-carotene hydroxylase [*Carica papaya*] accession number ADZ14893.1 , 84% with beta-carotene hydroxylase [*Coffea arabica*] accession number ABA43903.1, 81% with beta-carotene hydroxylase [*Solanum lycopersicum*] accession numberNP_001234348.1, 74% with beta-carotene hydroxylase [*Citrus sinensis*] accession numberNP_001275830.1 83% with chloroplast beta-carotene hydroxylase [*Cucurbita moschata*] accession number AEK86567.1 and 76% with beta-carotene hydroxylase 2, chloroplastic-like [*Nicotiana tabacum*] accession number NP_001312763.1 respectively. The sequence alignment of BHY species is shown in figure 15. Another putative β-carotene hydroxylase gene had previously been isolated from sweetpotato cultivar Shinhwangmi with an accession number of HQ828094 (429 bp) andHQ828095 (370 bp) by Kim et al., (2012) however, the isolated sequence by them was partial and no functional analyses they could performed.

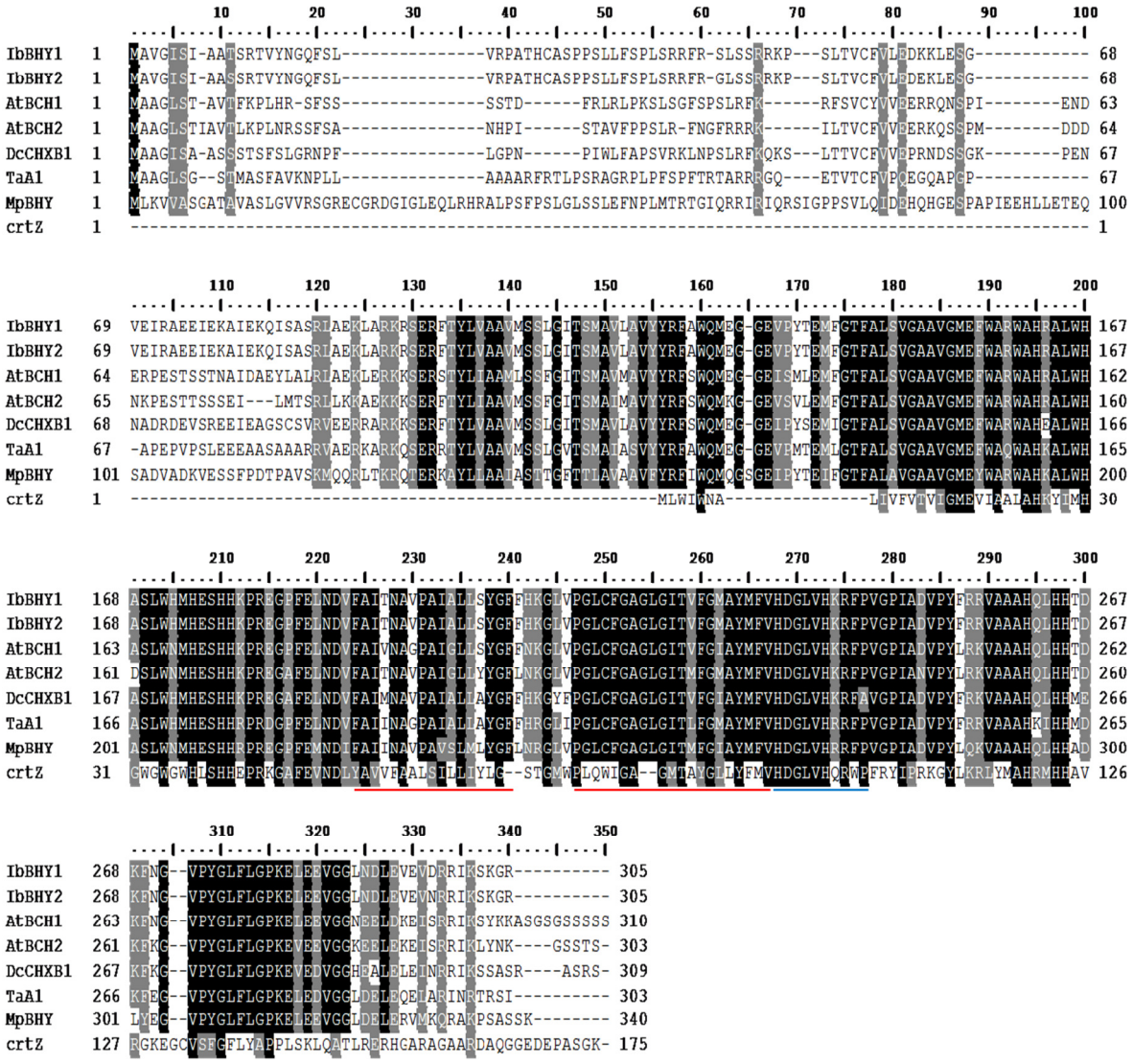


Figure 15. Alignment of BHY genes amino acid sequences

Based on the conservation level, sequence residues are indicated in black and grey. The bases underlined with the red and blue lines represent transmembrane helices and Motif 1 respectively. AtBCH1, *Arabidopsis thaliana* (accession number AY113923; AtBCH1, *Arabidopsis thaliana* (accession number AY117225) ; DcCHXB1, *Daucus Carota* (accession number DQ192193) ; TaA1, *Triticum aestivum* (Accession number JX_171670); MpBHY, *Marchantia polymorpha (acession number* AB981062); crtZ, *Pantoea ananatis* (accession number D90087).

3.4.3 Phylogenetic Analysis of IbBHY Proteins from Plants species

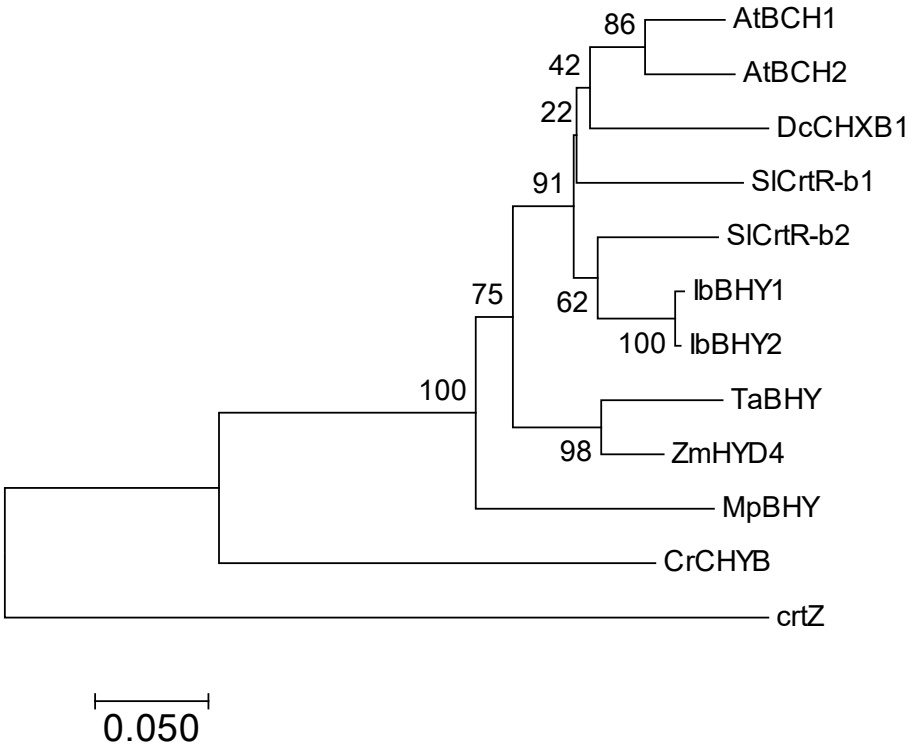


Figure 16. Neighbor joining tree for phylogenetic relationship among β-Carotene hydroxylases. Numbers attached to branches indicate bootstrap values supporting the final tree. Accession numbers include: Arabidopsis BCH1 (AtBCH1), AY113923; Arabidopsis BCH2 (AtBCH2), AY117225; Carrot CHXB1 (Dc CHXB1), DQ192193; Tomato CrtR-b1 (SlCrtR-b1), Y14809; Tomato CrtR-b2 (SlCrtR-b2), Y14810; Wheat BHY/TaBHY (TaHYD-A1), JX_171670; Maize HYD4 (ZmHYD4) AY844956; Liverwort BHY (MpBHY), AB981062; Chlamydomonas HYB (CrCHYB) and Pantoea ananatis crtZ (crtZ), D90087.

3.4.4 Conclusion

Both *IbBHY1* and *IbBHY2* closely brancjed with tomato β- Carotene hydroxylases at one end and with wheat and maize at the other. These results show that the sweetpotato occupies evolutionary space after bacteria, algae, lower vascular plants in evolutionary hierarchy after monocots.

3.5 FUNCTIONAL ANALYSIS OF ISOLATED *IbBHY* GENES FROM WS AND W71 CULTIVARS OF SWEETPOTATO

3.5.1 HPLC Analysis of Carotenoids formed in the β-Carotene producing *E. coli* Transformed with the Sweetpotato IbBHY Genes

In order to identify the β- Carotene hydroxylase genes isolated from sweetpotato *E. coli* expression system was used (Misawa et al., 1995).

The coding regions of sweetpotato IbBHY1 and IbBHY2 were amplified using cDNA from both of cultivars in separate PCR reactions and cloned into pETDuet vecto (Merck Millipore, Darmstadt, Germany). The plasmid constructs where were tagged as pETDuet-IbBHY1 and pETDuet-IbBHY2. When pET-IbBHY1 construct without transit peptide was used to transform β- carotene producing *E. coli*, zeaxanthin [(3*R*, 3′*R*)-β,β-carotene-

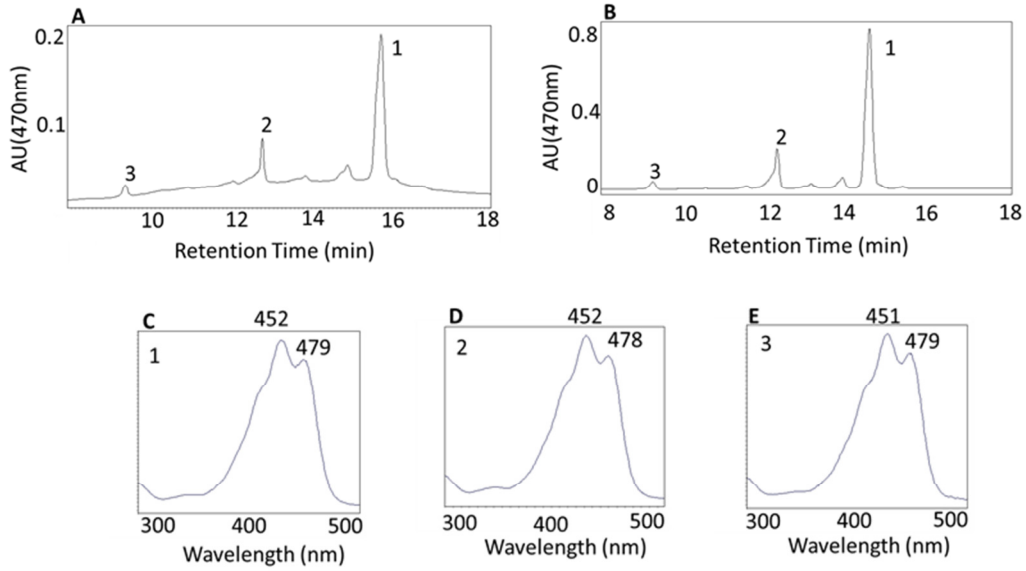


Figure 17. HPLC analyses of the carotenoids formed from β- Carotene. *E. coli* that produceβ-Carotene accumulating bacteria. **A**, pACHP-Beta plus pET-IbBHY1; **B**, pACHP-Beta plus pET-IbBHY2; **C-E** absorption spectra of individual peaks; **1**, β- Carotene; **2**, β- Cryptoxanthin and **3**, zeaxanthin.

3,3′-diol] along with monohydroxylated carotenoid β-cryptoxanthin [(3*R*)-β,β-caroten-3-ol] were detected by HPLC as shown in the figure 17.

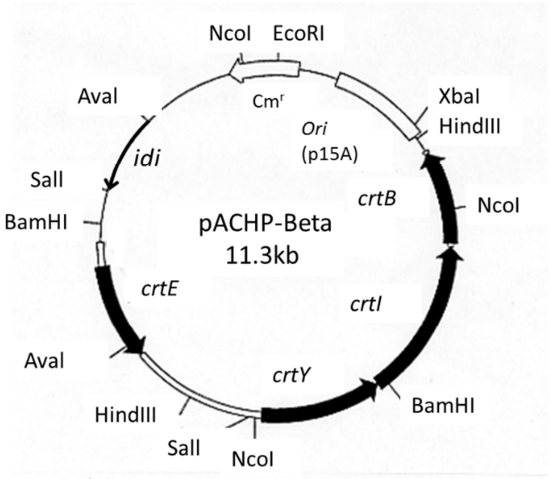


Figure 18. pACHP-Beta comprise the gene cluster *crtE, crtB, crtI and crtY* from *Pantoea ananatis* along with the *Haematococcus pluvialis idi* gene for β-carotene synthesis

pACHP-Beta (Figure 18) comprise a gene cluster *crtE, crtB, crtI, and crtY* along with an *idi* gene from *Haematococcus pluvialis* so they are efficient in synthesizing β- Carotene which can be used as substrate for β- Carotene hydroxylase enzymes genes isolated from a given species (Takemura et al., 2015).

As reported earlier, higher plants have two or more than two *BHY* genes. For example Arabidopsis (Sun et al., 1996; Tian and DellaPenna 2001; Takemura et al., 2015), citrus (Kim et al., 2001; Takemura et al., 2015), and tomato (Galpaz et al. 2006; Takemura et al., 2015) have been reported to possess two *BHY* genes. Similarly, six different *BHY* paralogs have recently been identified in maize (Vallabhaneni et al. 2009; Takemura et al., 2015). β-carotene hydroxylase was noticeably encoded by two of them, other two were pseudogenes, and the remaining two were found to be functionally unclear (Takemura et al., 2015; Vallabhaneni et al., 2009). On contrary, only one *BHY* gene was recently reported from a liverwort M. polymorpha genome (Takemura et al., 2015). More recently, only one *BHY* gene has been isolated from citrus fruits (Satsuma mandarin) and reported for its functional identification. It showed about 70 % identities to Arabidopsis BCH1 and BCH2 on its amino acid base sequences. Experiments on individual activity of CitHYb, did not show any carotene hydroxylation activity in the

β-carotene-accumulating *E. coli* BL21 (DE3) cells, or α-carotene- and β-carotene-producing *E. coli* BL21 (DE3) cells (Ma et al., 2016). Such type of results for *BHY* function in *Marcantia polymorpha* and citrus are similar to those observed in the present study on sweetpotato *IbBHY*. Carotenoid composition in flowering plants, however differ significantly in leaves, flowers and fruits since, the carotenoid biosynthesis genes are variably regulated in a tissue- in a development-specific way (Ma et al., 2016; Takemura et al., 2015; Galpaz et al. 2006; Li et al., 2010). However, the individual activity of all β- Carotene hydroxylases is to catalyze the hydroxylation of the β-ring of carotene to synthesise zeaxanthin through β- Cryptoxanthin. In this study the full length cDNA sequences of *IbBHY* were first time isolated along with their functional identification.

3.5.2 Conclusion

The present results revealed that *IbBHY* genes possess β-ring hydroxylation activity for β-carotene to produce zeaxanthin via β-cryptoxanthin. The results presented in this study will provide support in further interpretations of the mechanism of carotenoid biosynthesis in sweetpotato. Especially, it will provide insight for new procedures to improve carotenoid composition of sweetpotato.

3.6 ISOLATION OF CYTOCHROME P450 HYDROXYLASE GENES FROM SWEETPOTATO

3.6.1 Isolation of Cytochrome P450 Hydroxylase Genes *IbCYP97A* and *IbCYP97B* from WS and W71 Cultivars

To isolate full length cDNA sequences of sweetpotato cytochrome P450 Hydroxylase genes *IbCYP97A* and *IbCYP97B* homology search was made for *Ipomoea* genome. For this purpose sweetpotato wild relative and the most probable progenitor of sweetpotato (*Ipomoea batatas* L.) was searched to find the EST and the homologous sequences to the already known *CYP97A* and *CYP97B* genes. So, *Ipomoea trifida* and closely related species were aligned and worked out for primer designing for full length cDNA isolation of *IbCYP97A* and *IbCYP97B*. The primers thus synthesized and used are shown in table 6.

Primer Name	Primer Sequence 5'-3'	Restriction enzymes used	Gene Cloning Experiment Type
IbCYP97AF1	AGGATCCATGGCAGCTAGCTCCGTT	BamHI	pRSF Cloning
IbCYP97AR1	TGCGGCCGCTACACTGAAGATCTCGC	NotI	pRSF Cloning
IbCYP97AF2	AGATTGACATTTGGCCCC		Sequencing primer
IbCYP97AR2	GGCTGTGGATATAGTCGC		Sequencing primer
IbCYP97BF1	GCCCGGGATGAATTTGTTAGACAATG	SmaI	pRSF Cloning
IbCYP97BR3	GGGTACCTCAGCACATATCCAACCTC	KpnI	pRSF Cloning

Table 6. Primers used for full length cDNA isolation of *IbCYP97A* and IbCYP97B genes in sweetpotato cultivars W71 and WS.

Coding region of each gene were amplifies by PCR using pair of primers cloned in cloning vectors and sequenced.

3.6.2 Sequence Analysis and Alignment of *IbCYP97A* and *IbCYP97B* Genes

Using the sweetpotato cDNA as template from two cultivars, the coding regions were amplified by PCR and subsequently cloned into cloning (pBluescriptII K^+) and expression (pRSF) vectors. Two *IbCYP97A* full lenghth sequences from W71, named *IbCYP97A1* and *IbCYP97A2* were isolated. Four, *IbCYP97A* full length cDNA suquences were isolated from WS and named *IbCYP97A3*, *4*, *5*, *6*.

Whereas, only one *IbCYP97B* full length cDNA sequence each from W71 and WS were isolated and named as *IbCYP97B1* and *IbCYP97B2* respectively. The isolated full length sequences were cloned in pRSF expression vector. The plasmid constructs were tagged as, pRSF-IbCYP97A1, pRSF-IbCYP972, pRSF-IbCYP97A3, pRSF-IbCYP97A4, pRSF-IbCYP97A5, pRSF-IbCYP97A6, pRSF-IbCYP97B1 and pRSF-IbCYP97B2 and sequenced respectively before *E. coli* transformation and functional complementation experiments.

Isolated *IbCYP97A1*, *IbCYP97A2*, *IbCYP97A3*, *IbCYP97A4*, *IbCYP97A5* and *IbCYP97A6* were 1878bp long with significant difference among all from one another. The open reading frames encoded amino acid with each 625bp size. On the other hand each full length sequence isolated for *IbBHY1* and *IbBHY2* was found to be 1563bp with encoded amino acid size of 520bp. Blast search was performed for detailed sequence homology analyses. From Blast search, *IbCYP97A1* was found to be 79% identical with cytochrome P450, family 97, subfamily A, polypeptide 3 of *Arabidopsis thaliana* AtCYP97A3 accession number,NP_564384.1. 82% identity with cytochrome P450-type monooxygenase 97A29 of *Solanum lycopersicum* accession number NP_001234049.1, 82% with P450 carotenoid beta-ring hydroxylase (*Lycium barbarum*) accession number AIX87503.1. P450 carotenoid beta-ring hydroxylase *Lycium ruthenicum* accession number AIX87527.1 showed 80% ,homology , Cytochrome P450 97B2 from *Cajanus cajan* ccession number KYP47625.1, 77% a and chloroplast cytochrome P450 monooxygenase 97A3 from *Daucus carota* accession number AFU10536.1 showed 77% sequence homology with IbCYP97A1 isolated from W71 cultivar of sweetpotato.

For IbCYP97A2 and with mostly similar pattern of sequence similarities showed significant sequence identities with minor variations to that of *IbCYP97A1*. For example, Cytochrome P450-type monooxygenase 97A29 *Solanum lycopersicum* accession number NP_001234049.1,80% ,P450 carotenoid beta-ring hydroxylase accession number AIX87503.1from *Lycium barbarum* , P450 carotenoid beta-ring hydroxylase accession number AIX87527.1 from *Lycium ruthenicum* all showed 80% sequence homology while, cytochrome P450-type monooxygenase 97A3 *Bixa orellana* AMJ39485.1, showed 75% , cytochrome P450, family 97A3,

subfamily A, polypeptide 3 *Arabidopsis thaliana* accession number NP_564384.1 showed 79% sequence homology with the IbCYP97A2 isolated from W71 cultivar of sweetpotato.

Cytochrome P450-type monooxygenase 97A29 (*Solanum lycopersicum*) accession number NP_001234049.1 showed 81% resemblance with IbCYP97A3. And though, *Lycium barbarum* , P450 carotenoid beta-ring hydroxylase accession number AIX87527.1 from *Lycium ruthenicum* showed 80% sequence homology same way as to that of IbCYP97A2 but, it differed in case of cytochrome P450-type monooxygenase 97A3 *Bixa orellana* (accession number AMJ39485.1) which showed 76% sequence similarity with IbCYP97A3. For IbCYP97A4 P450-type monooxygenase 97A29 from *Solanum lycopersicum*, P450 carotenoid beta-ring hydroxylases from *Lycium barbarum*, and *Lycium ruthenicum* showed same similarity percentage of 80% which was similar to other IbCYP97A sequences. However, it showed a 79% identity with cytochrome P450 CYP97A41 (*Salvia miltiorrhiza*) and75% identity with cytochrome P450-type monooxygenase 97A3 (*Bixa orellana*) accession number AMJ39485.1.

Cytochrome P450 97B2 (*Cajanus cajan*) accession number KYP47625.1 was 81% identical to IbCYP97A4. CYP97 family CYP97A3, subfamily A, polypeptide 3 *Arabidopsis thaliana* accession number NP_564384.1 was found to be 79% similar to IbCYP97A5. Chloroplast cytochrome P450 monooxygenase 97A3 from *Daucus carota* accession number AFU10536.1 was 77% similar with IbCYP97A5. but, it differed in case of cytochrome P450-type monooxygenase 97A3 *Bixa orellana* (accession number AMJ39485.1) which showed 75% sequence similarity with IbCYP97A3.

IbCYP97A5 and IbCYP97A6 were almost same in homology with significant similarity with other IbCYP97A amino acid sequences. IbCYP97B1 and IbCYP97B2 show similar sequences matches with minor variations same way as found in IbCYP97A sequences.

CYP97B2, chloroplastic from Glycine max accession number NP_001235534.1was 86% similar with both IbCYP97B1 IbCYP97B2. Similarly, CYP97B3 family 97, subfamily B, polypeptide 3 *Arabidopsis thaliana* accession number NP_193247.2 showed 83% sequence match with IbCYP97B1 but 82%

similarity in case of IbCYP97B2. *Cajanus cajan* accession number, KYP52637.1 ; CYP 97B2, chloroplastic showed 85% sequence matches with both IbCYP97B1 and IbCYP97B2. Cytochrome P450 97B2, chloroplastic *Ananas comosus* accession number OAY64819.1 was observed to be 83% identical with both IbCYP97B1 and IbCYP97B2.

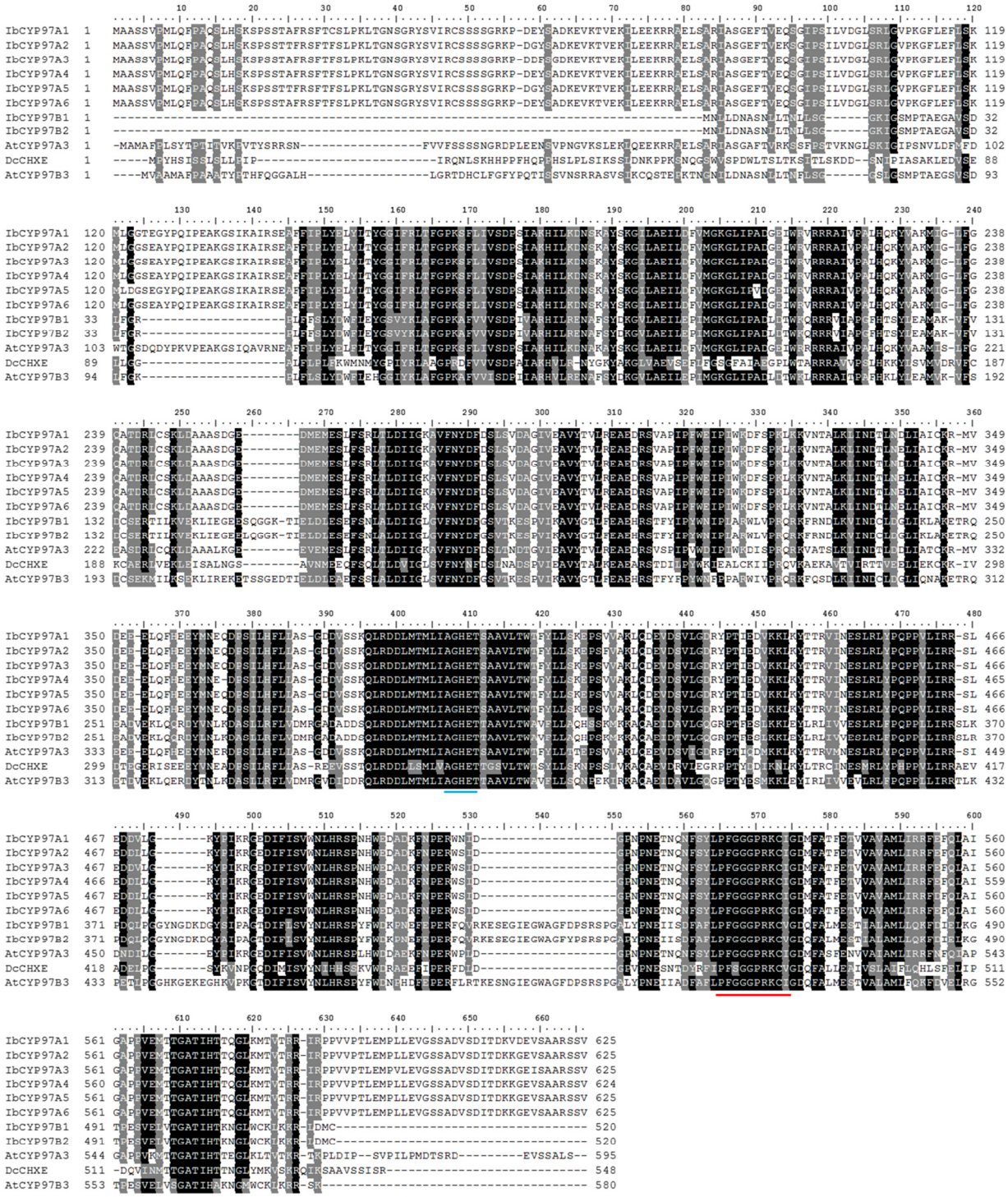


Figure 19. Amino acid sequences alignment of CYP97A and CYP97B genes.

Accession number used are: Arabidosis CYP97A3 (AtCYP97A3), NM_102914; Carrot CHXE (DcCHXE), DQ192196 and Arabidosis CYP97B3 (AtCYP97B3), NM_117600. The predicted oxygen binding pockets and heme- binding domain are underlined with blue and red lines respectively.

3.6.3 Phylogenetic Analysis of CYP97A and CYP97B Proteins from Plants Species

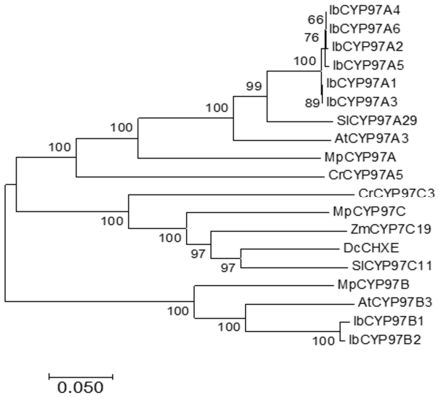


Figure 20. Neighbor joining tree for phylogenetic relationship among CYP97A and CYP9 B carotene hydroxylases. Numbers attached to branches indicate bootstrap values supporting the final tree. Accession numbers include: Tomato CYP97A29 (SlCYP97A29), EU849605; Arabidopsis CYP97A3 (AtCYP97A3), NM_102914; Liverwort CYP97A (MpCYP97A), AB981063; Chlamydomonas CYP97A5 (CrCYP97A5), EF587911; Chlamydomonas CYP97C3 (CrCYP97A5), EF587910; Liverwort CYP97A (MpCYP97C), AB981065; Maize CYP97C19 (ZmCYP9719), GU130217; Carrot CHXE (DcCHXE), DQ192196; Tomato CYP97C11 (SlCYP97C11), EU849604; Liverwort CYP97B (MpCYP97B), AB981064; Arabidopsis CYP97B3 (AtCYP97B3), NM_117600

3.6.4 Conclusion

Both *IbCYP97A* and *IbCYP97B* isolated from Sweetpotato showed significant homology with known homologues and individually formed single clades that were positioned close to the corresponding proteins from tomato (*Solanum lycopersicum*), *Arabidopsis thaliana* and *Marcantia polymorpha* which suggested that these genes from these species might have a single closely related ancestor through which they evolved.

3.7 FUNCTIONAL ANALYSIS OF ISOLATED P450 HYDROXYLASE GENES *IbCYP97A* AND *IbCYP97B* FROM SWEETPOTATO

3.7.1 HPLC Analysis of Carotenoids formed in the β-Carotene producing *E. coli* Transformed with the Sweetpotato P450 Hydroxylase I*bCYP97A* and *IbCYP97B* Genes

The coding regions of sweetpotato *IbCY97A* and *IbCYP97B* were cloned into pRSF vector The constructs were used to check the activity of the isolated *IbCYP97A* and *IbCYP97B* genes The plasmid constructs where used to transform β- Carotene producing *E. coli* and individual trans-formants were tagged as pACHP-Beta-pRSF-IbCYP97A1, pACHP-Beta-pRSF-IbCYP97A2, pACHP-Beta-pRSF-IbCYP97A3 pACHP-Beta-pRSF-IbCYP97A4 pACHP-Beta-pRSF-IbCYP97A5 pACHP-Beta-pRSF-IbCYP97A6 pACHP-Beta-pRSF-IbCYP97B1 and pACHP-Beta-pRSF-IbCYP97B2

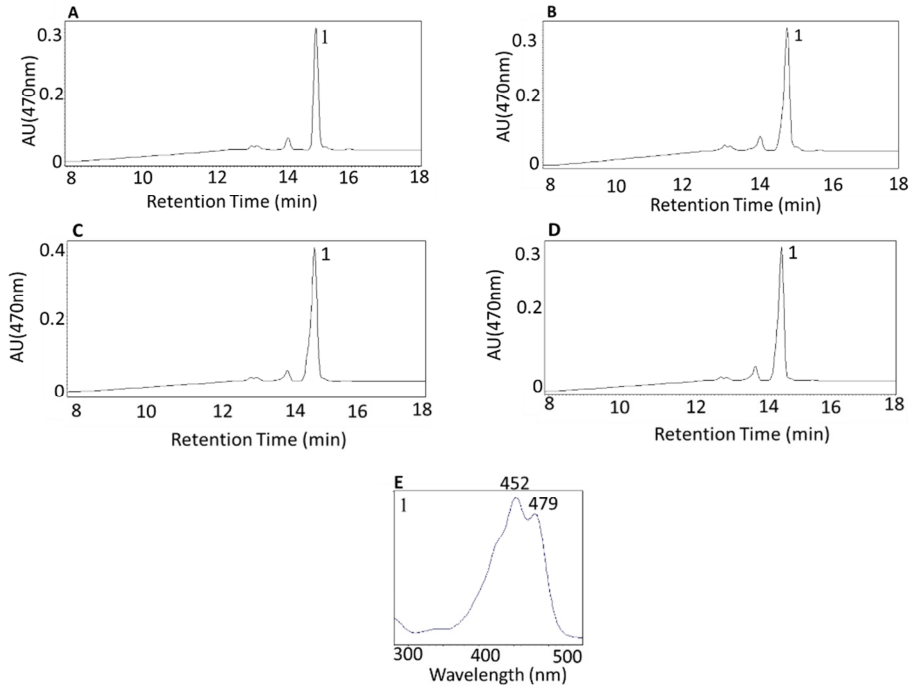


Figure 21a. HPLC analysis of carotenoids formed in the β- Carotene producing *E. coli* strain that had been transformed with the sweetpotato IbCYP97A genes. **A,** pACHP-Beta- pRSF-IbCYP97A1; **B**, pACHP-Beta- pRSF-IbCYP97A2; **C** pACHP-Beta- pRSF-IbCYP97A3; **D**, pACHP-Beta-

pRSF-IbCYP97A4. Whereas, **E** represents the absorption spectrum of individual peak for β-Carotene.

When plasmid constructs of pACHP-Beta-pRSF-IbCYP97A1 to pACHP-Beta-pRSF-IbCYP97A6 were used to transform β- carotene producing *E. coli*, no activity was detected. β-Carotene was normally produced with no other carotenoids among all of the transformed *E. coli* when detected by HPLC as shown in figure 21a and 21b.

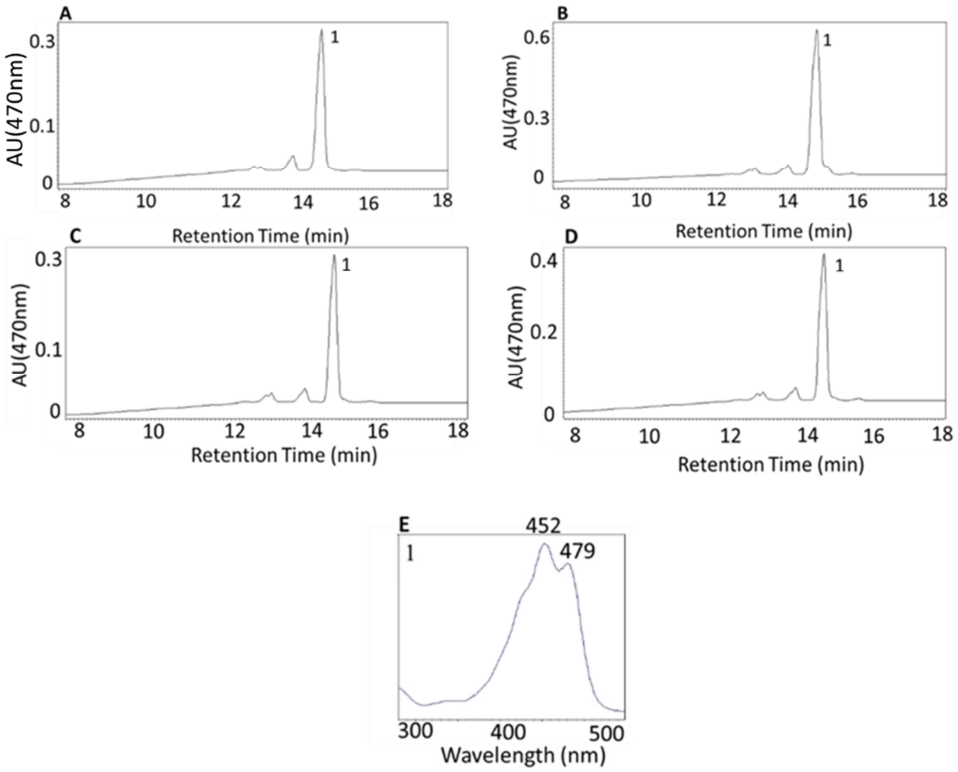


Figure 21b HPLC analysis of carotenoids formed in the β- Carotene producing *E. coli* strain that had been transformed with the sweetpotato IbCYP97A and IbCYP97B genes. **A,** pACHP-Beta-pRSF-IbCYP97A5; **B**, pACHP-Beta- pRSF-IbCYP97A6; **C** pACHP-Beta- pRSF-IbCYP97B1; **D**, pACHP-Beta- pRSF-IbCYP97B2. Whereas, **E** represents the absorption spectrum of individual peak for β- Carotene.

Similarly, for pACHP-Beta-pRSF-IbCYP97B1 to pACHP-Beta-pRSF-IbCYP97B2-Constructs, were used to transform β- carotene producing *E. col*i, no activity was detected. β-Carotene was normally produced with no other carotenoids among all of the transformed *E. coli* when detected by HPLC as shown in the figure 21b.

3.7.2 HPLC Analysis of Carotenoids-formed in the α-Carotene producing *E. coli* Transformed with the Sweetpotato P450 Hydroxylase *IbCYP97A* or *IbCYP97B* Genes

In another experiment, IbCY97A and IbCYP97B were fed separately to α-Carotene producing *E. coli* that have two plasmids one for lycopene which is pACCRT-EIB and another from *Marchantia polymorpha* (Takemura et al., 2015).

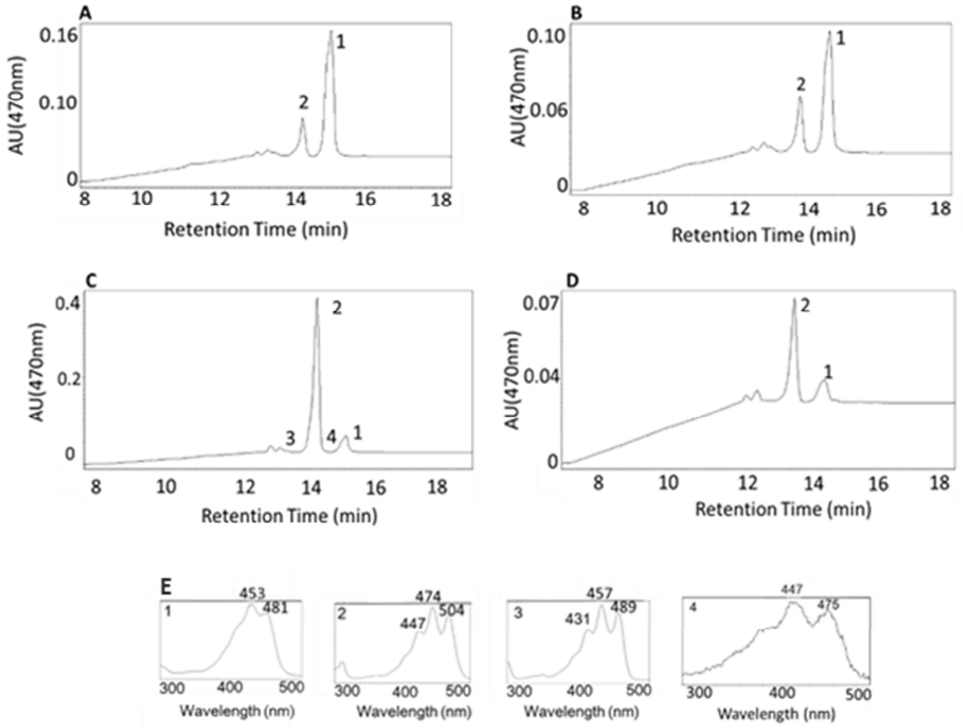


Figure 22. HPLC analysis of carotenoids formed in α- Carotene producing *E. coli* strain that had been transformed with the sweetpotato IbCYP97A genes. (Results for IbCYP97A4 and 5 not shown). A-D, shows the plasmid construct for pACCRT-EIB+MpLCYb/MpLCYe -pRSF-IbCYP97A1; B, pACCRT-EIB+MpLCYb/MpLCYe-pRSF-IbCYP97A2; pACCRT-EIB+MpLCYb/MpLCYe -pRSF-IbCYP97A3;D, pACCRT-EIB+MpLCYb/MpLCYe pRSF-IbCYP97A6. Whereas, E represents the absorption spectrum of individual peak for1, β- Carotene. 2, lycopene; 3, δ- Carotene and 4, α-Carotene.

The first set of experiments used the plasmid constructs which were made and tagged as pACCRT-EIB+MpLCYb/MpLCYe -pRSF-IbCYP97A1 to pACCRT-EIB+MpLCYb/MpLCYe-pRSF-IbCYP97A6.

Similarly, for IbCYP97B case, pACCRT-EIB+MpLCYb/MpLCYe-pRSF-IbCYP97B1 and pACCRT-EIB+MpLCYb/MpLCYe -pRSF-IbCYP97B2 constructs were used to transform α- carotene producing *E. coli*, *E. coli* were cultured with addition of IPTG for carotenoid extraction and analyses by HPLC. However, the HPLC results showed that the constructs had no enzymatic activity for α- carotene as a substrate (Figure 23 A-B).

In a separate experiment, in order to check the effect of IPTG on the growth and activity, of *E. coli* transformed without IPTG along with the control which had the IPTG added into the. *E. coli* were cultured and carotenoids extracted. It was revealed that in *E. coli* transformed with pACCRTEIB+MpLCYb/MpLCYe-pRSF-IbCYP97A(s) without IPTG had showed changed in the carotenoid composition. The extracted carotenoids were analysed by HPLC. The results indicated peaks for β-Carotene, lycopene, δ- Carotene and α- Carotene (Figure 22). From these results, it was assumed that IbCYP97A(s) interpreted the enzymatic activity of lycopene cyclases. Similar results were obtained in case of IbCYP97B(s) constructs when used to transformα- Carotene producing bacteria (Figure 23C-D). Due to such an interpretation, the extracts showed variable carotenoids composition, instead of a single carotenoid. These results are different from those previously reported for Arabidopsis CYP97A3 (Kim and DellaPenna, 2006), tomato, CYP97A29 (Stigliani et al., 2011) and rice CYP97A4 (Lv et al., 2012). In higher plants, CYP97A and CYP97C synthesize carotenoids whereas the function of CYP97B has not yet been elucidated completely except for only two reports. In the one report it was described that *Arabidopsis* CYP97B3 showed the activity of β-carotenoid hydroxylase (Takemura et al., 2015; Kim et al., 2010). While, in the other one, β-carotenoid hydroxylation activity along with possible ε-carotenoid hydroxylation activity *in Porphyra* $PuCHY_1$ was described (Yang et al., 2014). Takemura et al., (2015) isolated liverwort MpCYP97A and MpCYP97B followed by their functional analysis for hydroxylating activity for β- and α-carotene, but their results showed no individual activity by both genes they investigated. CYP97B carotene hydroxylase is also present in higher plants however, their biosynthetic role is not clear (Kim et al., 2009; Yang et al., 2014). CYP97A has been found to preferably catalyse hydroxylation of β-rings

(Moise et al., 2014). CYP97C together with the CYP97A β-hydroxylases the formation of lutein but preferentially hydroxylating the ε-ring of substrates of whose β-ring have already been hydroxylated (Quinlan et al., 2007; Kim and DellaPenna, 2006). It has also been identified that different carotenoid hydroxylases function in coordination to synthesize xanthphylls (Quinlan et al., 2012; Takemura et al., 2015; Moise et al., 2014).

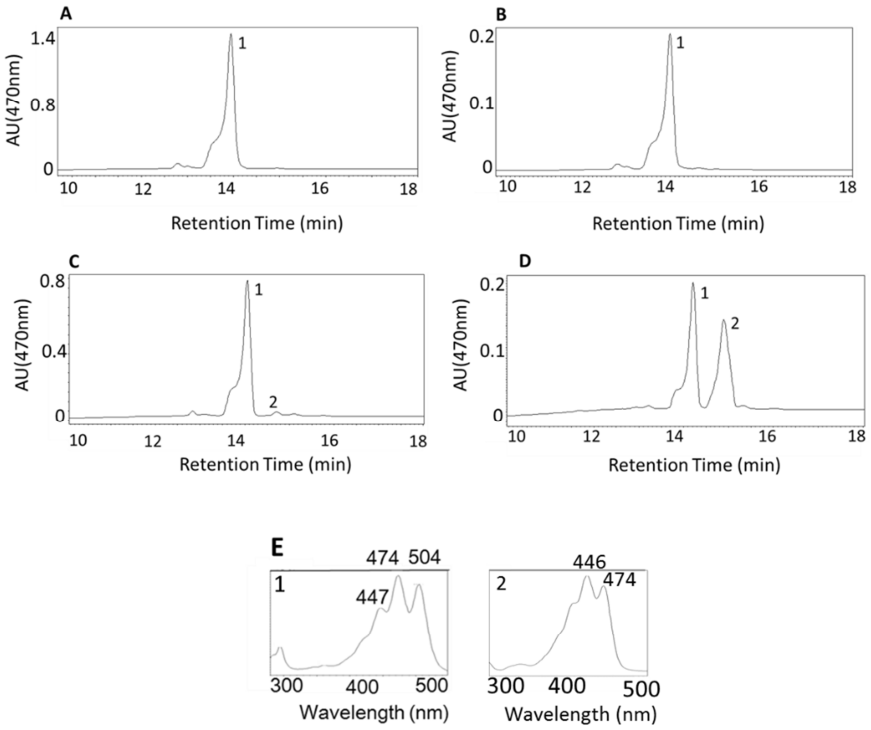


Figure 23. HPLC analysis of carotenoids formed in α- Carotene producing *E. coli* strain that had been transformed with the sweetpotato IbCYP97B genes. **A**, pACCRT-EIB+MpLCYb/MpLCYe -pRSF-IbCYP97B1 (+IPTG); **B**, pACCRT-EIB+MpLCYb/MpLCYe -pRSF-IbCYP97B2 (+IPTG); **C** pACCRT-EIB+MpLCYb/MpLCYe -pRSF-IbCYP97B1 (-IPTG); **D**, pACCRT-EIB+MpLCYb/MpLCYe pRSF-IbCYP97B2 (-IPTG). Whereas, **E** represents the absorption spectrum of individual peak for**1**, β- Carotene.; **2**, lycopene; **3**, δ- Carotene and **4**, α- Carotene.

3.7.3 Conclusion

Present results for activity analysis in case of *IbCYP97A* and IbCYP97B revealed that these genes have no individual effect in hydroxylation of carotenes.Such

reults coincide with previous reports on some other plant species However, further experimentas are essential for complete elucidation of P450 cytochrome based IbCYP97 families. For example, it would be important to test the combined activity of IbBHY, IbCYPA(s) IbCYP97B(s) and or more necessarily IbCYPC(s) to fully understand the molecular bases for the lutein and other carotenoid hydroxylation products in sweetpotato.

3.8 ANALYSIS OF CAROTENOIDS FROM TRANSGENIC SWEETPOTATO CARRYING BACTERIAL β-CAROTENE KETOLASE (*CRTW)* GENES

3.8.1 Sweetpotato W71 Tuber Transformation with the *Agrobacterium* EHA101 Cells carrying *crtW* Construct and Culturing

The *crtW* gene constructs were prepared by the following the method reported earlier by Hasunuma et al., (2008) with modifications. pUC19 high copy cloning vector for replication *in E. coli*, suitable for “blue-white screening” technique was used to manipulate with the β-amylase promoter (Maeo et al., 2001).

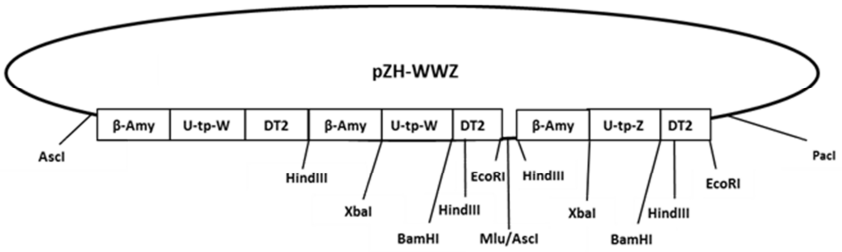


Figure 24. β-amylase promoter construct of *crtW* gene showing arrangement of promoter, gene and terminator.

After performing all construction with the auxiliary plasmid (modified pUC19), the gene cassette part were excised with 8 base recognition enzymes PacI and SalI and ligated into the pZH2B binary vector (Kuroda et al., 2010). Since the multi-cloning site of the auxiliary plasmid is the same as the basic binary vector, it is possible to put in and out promoters etc. using the same restriction enzyme site (Hajdukiewicz et al., 1994).

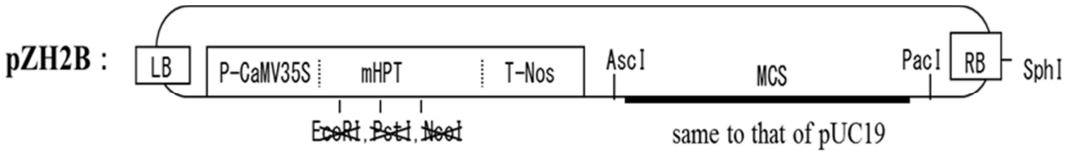


Figure 25. Binary vector pZH2B with summarized arrangements of promoter, gene, and terminator. The pZH2B binary vector harboring the *crtW* construct was used to transform the *Agrobacterium* strain EH101 (Cm^r) by electroporation as described earlier (Fujisawa et al., 2009). *Agrobacterium* transformation and culturing of sweetpotato W71 tuber was performed by following the protocol reported by Otani et al., (2003) with some modifications that will be reported later in another article.

It was reported that sweetpotato contained not only β-carotene but also several epoxy carotenoids unique to the sweetpotato tubers *e.g.*, β-carotene-5,8-epoxiside and β-carotene-5,8,5',8' -diepoxiside (Maoka et al., 2007). Therefore, it was insighted that new structural carotenoids with epoxy and keto groups can be produced by expressing the ketolase *crtW* gene in sweetpotato tubers. In this study marine-bacterial genes that include the *crtW* gene encoding carotenoid 4,4'-ketolase (Shindo et al., 2008) were introduced into sweetpotato cultivar W71 under the control of the CaMV promoter. As the results, novel carotenoids with epoxy and keto groups **1**, **2**, and **3** were obtained along with a series of ketocarotenoids. The the structural elucidation of these novel epoxy-keto carotenoids was elucidated by HPLC-PDA analysis and were identified based on UV-vis, ESI-MS, ^{1}H-NMR, and CD spectral data and confirmed by COSY and NOESY experiments.

3.8.2 HPLC-PDA Analysis and Identification of Novel Carotenoids in Transgenic Sweetpotato by UV-vis, ESI-MS, ^{1}H-NMR, and CD Spectral Data.

Bacterial genes that include the *crtW* gene encoding carotenoid 4,4'-ketolase were expressed in the tubers (tuberous roots) of sweetpotato, *Ipomoea batatas*.(L.) Lam. Consequently, three novel carotenoids with epoxy and keto groups **1**, **2**, and **3** were found to be produced in sweetpotato. The structures of **1**, **2**, and **3** were determined as 5',8' -epoxy-5',8' -dihydro-β,β-caroten-4-one (named ecinenone 5',8' -epoxide), 5 ',6' -epoxy-5',6'-dihydro-β,β-caroten-4-one (named echinenone 5',6'

-epoxide), and 3' -hydroxy-5',6' -epoxy-5',6' -dihydro-β,β-caroten-4-one (named 3' -hydroxyechinenone 5',6'-epoxide), respectively, based on spectral data.

The fraction eluted with n-hexane contained β-carotene (yield 5.1 mg). The fraction eluted with acetone:*n*-hexane (5:95) contained β-carotene 5,8-epoxide (yield 0.3 mg). The fraction eluted with acetone:*n*-hexane (1: 9) contained echinenone(yield 1.1 mg) and a new carotenoid **1**(yield 0.1 mg) . The fraction eluted with acetone:*n*-hexane (3:7) contained canthaxanthin (yield 1.2 mg). The fraction eluted with acetone:*n*-hexane (5:5) contained 3'-hydroxyechinenone, (yield 0.2 mg) a new carotenoid **3**, (yield 0.1 mg) (3*S*)-asteroidenone, (yield 0.2 mg) (3*S*)-adonirubin (yield 0.5 mg), and (3*S*,3'*S*)-astaxanthin (yield 0.2 mg). They were identified based on UV-vis, ESI-MS, ^{1}H-NMR, and CD spectral data. The new carotenoid **1** was isolated from the fraction eluted with acetone:*n*-hexane (1:9) by HPLC (Silica gel with acetone: *n*-hexane (2:8) at a flow rate of 2 mL/min). The new carotenoid **3** was isolated from the fraction eluted with acetone:*n*-hexane (5:5) by HPLC (Silica gel with acetone: *n*-hexane (2:8) at a flow rate of 2 mL/min).

The yield of new carotenoid **2** , Echinenone 5',6'-epoxide was only 0.01 mg from 10 g the tubers of the *crtW* gene-expressed sweetpotato. UV-vis λ max (Ether) 450 nm; HR-ESI MS; *m/z* 566.4122 (M^+, calcd for $C_{40}H_{54}O_2$, 566.4124); products ions of MS/MS *m/z* 548 (M-18), 474 (M-92); Due to the small amount of the sample, ^{1}H-NMR could not be measured. Therefore, these spectral data were compared with semi-synthetic echinenone 5',6'-epoxide. These spectral data were identical to semi-synthetic echinenone 5',6'-epoxide and this compound was identified to be echinenone 5',6'-epoxide by co-chromatography with the semi-synthetic one on HPLC (Silica gel with acetone:*n*-hexane (2:8). Synthesis of echinenone 5,6-epoxide from echinenone was performed as follows:

m-Chloroperbenzoic acid at 3.6 mg in 2 mL of dichloromethane was added to a solution of 1 mg of echinenone in 2 mL dichloromethane. The solution was stood for 20 min at room temperature in darkness. After that, the reaction mixture was partitioned with a 5% solution of $NaHCO_3$ and ether. The ether layer was removed and evaporated. The reaction products were submitted for preparative HPLC. Echinenone5',6'-epoxide; yield 0.8 mg. UV-vis λ max (Ether) 450 nm; HR-ESI MS; *m/z* 566.4122 (M^+, calcd for $C_{40}H_{54}O_2$, 566.4124).

3.8.3 Identification and Confirmation of Novel Carotenoids from Transgenic W71 by UV-vis, ESI-MS, ^{1}H-NMR, CD Spectral data and COSY and NOESY Experiments.

The new carotenoid **1** showed an absorption maximum at 430 nm. Its molecular formula was determined as $C_{40}H_{54}O_2$ by high-resolution ESI-MS. The ^{1}H NMR data for **1** in $CDCl_3$ showed that this compound was obtained as a mixture of C-8' epimers. Due to the small amount of samples available (about 0.1 mg), separation of their epimers was not feasible. Spectroscopic data for Echinenone 5',8'-epoxide (**1**) is given in the table 7. The ^{1}H NMR signals of H-2 to H-20 were identical to the H-2 to H-20 part of echinenone i.e., β,β-caroten-4-one (Englert, 1995).

UV-vis λ max (Ether) 430 nm; HR-ESI MS; *m/z* 556.4122 (M^+, calcd for $C_{40}H_{54}O_2$, 566.4124); products ions of MS/MS *m/z* 548 (M^+-18), 474 (M^+-92); ^{1}H NMR of 8*R* isomer ($CDCl_3$, 500 MHz) δ 1.10 (H_3-17', s), 1.15 (H_3-16', s), 1.20 ($2H_3$-16, 17, s), 1.43 (H_3-18', s), 1.75 (H_3-19', s), 1.85 (H_2-2, t, *J*=7.5 Hz), 1.87 (H_3-18, s), 1.96 (H_3-20', s), 1.98 (H_3-20, s), 2.00 (H_3-19, s), 2.51 (H_2-4, t, *J*=7.5 Hz,), 5.16 (H-8', br. s,), 5.17 (H-7', br. s), 6.20 (H-10', d, *J*=11 Hz,), 6.22 (H-14', d, *J*=11Hz), 6.23 (H-7, d, *J*=16Hz), 6.28 (H-10, d, *J*=11 Hz), 6.30 (H-14, d, *J*=11Hz,), 6.30 (H-10, d, *J*=11Hz), 6.32 (H-12', d, *J*=15Hz), 6.37 (H-8, d, *J*=16 Hz), 6.43 (H-12, d, *J*=15Hz), 6.51 (H-11', dd, *J*=15, 11 Hz), 6.63 (H-15 and H-15', m), 6.64 (H-11, dd, *J*=15,11 Hz). ^{1}H NMR of 8*S* isomer ($CDCl_3$, 500 MHz) δ 1.11 (H_3-17', s), 1.18 (H_3-16', 3), 1.20 ($2H_3$-16, 17, s), 1.46 (H_3-18', s), 1.80 (H_3-19', s), 1.85 (H_2-2, t, *J*=7.5 Hz,), 1.87 (H-18, s), 1.96 (H_3-20', s), 1.98 (H_3-20, s), 2.00 (H_3-19, s), 2.51 (H_2-4, t, *J*=7.5 Hz,), 5.07 (H-8' br. s), 5.23 (H-7', d, *J*=1.5 Hz,), 6.20 (H-10', d, *J*=11 Hz,), 6.22 (H-14', d, *J*=11Hz), 6.23 (H-7, d, *J*=16Hz), 6.28 (H-10, d, *J*=11 Hz), 6.30 (H-14, d, *J*=11Hz,), 6.30 (H-10, d, *J*=11Hz), 6.32 (H-12', d, *J*=15Hz), 6.37 (H-8, d, *J*=16 Hz), 6.43 (H-12, d, *J*=15Hz), 6.51 (H-11', dd, *J*=15, 11 Hz), 6.63 (H-15 and H-15', m), 6.64 (H-11, dd, *J*=15,11 Hz). The ratio of 8*R* and 8*S* isomer was 6:4.

Table 7. Spectroscopic data for Echinenone a new carotenoid **1**, 5', 8'-epoxide in the tubers of the *crtW* gene-expressed sweet potato. Yield 0.1 mg from 100 g obtained as mixture of *8' R* and *8'S* isomers.

The ^{1}H signals of the remaining part (H-2$'$ to H-20$'$) in the major epimer of **1** were identical to those parts of (5*R*,8*R*,5$'$*R*,8$'$*R*)-aurochrome (5,8, 5$'$,8$'$ -diepoxy-5,8, 5$'$,8$'$ -tetrahydro-β,β-carotene) (Englert, 1995). All *trans* geometry of the polyene chain was revealed by chemical shift values and their coupling constants. (Englert, 1995) This structure was confirmed by COSY and NOESY experiments, as shown in figure 26. Therefore, the structure of this compound was determined to be (5$'$*R*, 8$'$*R*)-5$'$,8$'$ -epoxy-5$'$,8$'$ -dihydro-β,β-caroten-4-one and named echinenone 5$'$,8$'$-epoxide. On the other hand, ^{1}H signals of the minor epimer of H-2 to H-20 and H-2$'$ to H-20$'$ were identical to those of echinenone (H-2 to H-20) and (5*R*,8*S*,5*R*,8$'$*S*)-aurochrome. (Englert, 1995) Therefore, the structure of the minor isomer was determined to be (6$'$*R*,8$'$*S*)-5$'$,8$'$ -epoxy-5$'$,8$'$ -dihydro-β,β-caroten-4-one. The ratio of 8$'$*R* and 8$'$*S* epimers of **1** was estimated to be 6:4 from the intensities of ^{1}H NMR signals.

It is well-known that 5,8-epoxy-carotenoids are derived from the corresponding 5,6-epoxy-carotenoids *via* epoxide-furanoid rearrangements by acid catalysis.(Schiedt and Liaaen-Jensen, 1995). Therefore, it was assumed that the intact form of **1** in sweetpotato was echinenone 5$'$,6$'$-epoxide (5$'$,6$'$-epoxy-5$'$,6$'$-dihydro-β,β-caroten-4-one), and echinenone 5$'$,8$'$ epoxide was converted from echinenone 5$'$,6$'$-epoxide during the isolation procedures. In order to test this hypothesis, sweetpotato was extracted under alkaline medium conditions and the obtained carotenoids were analyzed. Consequently, echinenone 5$'$,6$'$-epoxide (**2**) could be identified along with β-carotene 5,6-epoxide under alkaline medium conditions extract of carotenoid of sweetpotato. The spectroscopic data for echinenone 5$'$,6$'$-epoxide (**2**) is shown in table 8.This result clearly indicated that echinenone 5$'$,8$'$-epoxide (**1**) was present as echinenone 5$'$,6$'$-epoxide (**2**) in sweetpotato.

Echinenone5$'$,6$'$-epoxide; yield 0.8 mg. UV-vis λ max (Ether) 450 nm; HR-ESI MS; *m/z* 566.4122 (M^{+}, calcd for $C_{40}H_{54}O_2$, 566.4124); products ions of MS/MS *m/z* 548 (M^{+}-18), 474 (M^{+}-92); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 0.94 1.10 (H_3-16$'$, s), 1.10 (H_3-17$'$, s), 1.15 (H_3-18', s), 1.20 ($2H_3$-16-17, s), 1.85 (H_2-2, t,

J=7.5 Hz), 1.87 (H_3-18, s), 1.94 (H_3-19', s), 1.96 (H_3-20', s), 1.98 (H_3-20, s), 2.00 (H_3-19, s), 2.51 (H_2-4, t, *J*=7.5 Hz,), 5.88 (H-7', d, *J*=16Hz), 6.20 (H-10', d, *J*=11 Hz,), 6.22 (H-14', d, *J*=11Hz), 6.23 (H-7, d, *J*=16Hz), 6.28 (H-10, d, *J*=11 Hz), 6.29 (H-8', d, *J*=16Hz), 6.30 (H-14, d, *J*=11Hz,), 6.30 (H-14, d, *J*=11Hz), 6.32 (H-12', d, *J*=15Hz), 6.37 (H-8, d, *J*=16 Hz), 6.43 (H-12, d, *J*=15Hz), 6.51 (H-11', dd, *J*=15, 11 Hz), 6.63 (H-15 and H-15', m), 6.64 (H-11, dd, *J*=15,11 Hz).

Table 8. Spectroscopic data for new carotenoid 2 (Echinenone5',6'-epoxide) in the tubers of the *crtW* gene-expressed sweet potato. Yield 0.8 mg from 100 g.

The semi-synthetic echinenone 5',6'-epoxide was obtained as a mixture of enantiomers and showed no optical activity.

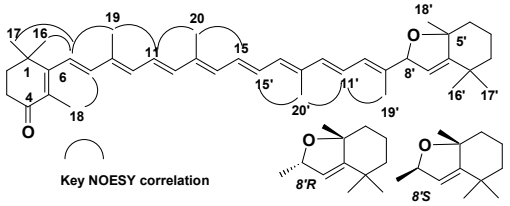
The new carotenoid **3** showed an absorption maximum at 452 nm. Its molecular formula was determined as $C_{40}H_{54}O_3$ by high-resolution ESI-MS. Spectroscopic data for 3'-Hydroxyechinenone 5',6'-epoxide (**3**) is given in table 9. The ^{1}H NMR data for **3** in $CDCl_3$ also showed the presence of an echinenone moiety (H-2 to H-20). Schiedt and Liaaen-Jensen, 1995; (Britton et al., 2004). The ^{1}H NMR signals of the remaining part (H-2' to H-20') were identical to those of violaxanthin (5,6, 5',6'-diepoxy-5,6, 5',6' -tetrahydero-β,β-carotene-3,3'-diol) as reported in literature (Englert, 1995). All *trans* geometry of the polyene chain was revealed by chemical shift values and their coupling constants (Englert, 1995). This structure was confirmed by COSY and NOESY experiments, as shown in Figure 26.

UV-vis λ max (Ether) 452 nm; HR-ESI MS; *m/z* 583.4150 (MH^+, calcd for $C_{40}H_{55}O_3$,583.4151); products ions of MS/MS *m/z* 565 (MH^+-18), 491 (MH^+-92); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 0.98 (H_3-16', s), 1.15 (H_3-17', s), 1.19 (H_3-18', s,), 1.20 ($2H_3$-16, 17, s), 1.23 (H2'-ax, dd, *J*= 14, 13 Hz), 1.66 (H-2'eq and H-4'-ax, overlapped), 1.85 (H_2-2, t, *J*=7.5 Hz), 1.88 (H_3-18, s), 1.93 (H_3-19', s), 1.97 (H_3-20', s), 1.98 (H_3-20, s), 2.00 (H_3-19, s), 2.37 (H-4'eq, ddd, *J*=14, 5, 1.5 Hz,), 2.51 (H_2-4, t, *J*=7.5 Hz), 3.91 (H-3', m), 5.89 (H-7', d, *J*=16 Hz), 6.21 (H-10', d, *J*=11 Hz), 6.23 (H-7, d, *J*=16 Hz,), 6.25 (H-14, d, *J*=11 Hz), 6.28 (H-10, d, *J*=11 Hz,), 6.29 (H-8', d, *J*=16 Hz), 6.30 (H-14', d, *J*=11 Hz,), 6.37 (H-8, d,

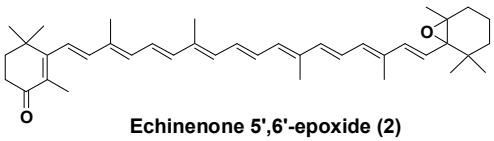
J=16 Hz,), 6.38 (H-12', d, *J*=15 Hz,), 6.43 (H-12, d, *J*=15 Hz,), 6.62 (H-11', dd, *J*=15, 11 Hz), 6.64 (H-11, dd, *J*=15, 11 Hz,), ~6.64 (H-15 and H-5', overlapped); CD (diethyl ether) λ (Δε) 245 (+2.5), 250 (0), 280 (-5.0), 295 (0), 315 (+4.0), 342 (0), 350 (-1.5).

Table 9. Spectroscopic data for new carotenoid 3 (3'-Hydroxyechinenone 5',6'-epoxide) in the tubers of the *crtW* gene-expressed sweetpotato. Yield 0.1 mg from 100 g.

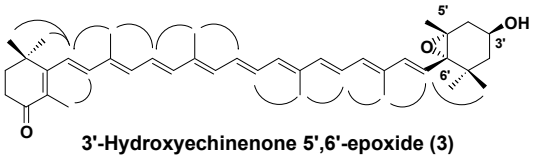
Therefore, the structure of this compound was determined as 3'-hydroxy-5',6'-epoxy-5',6'-dihydro-β,β-caroten-4-one and named 3'-hydroxyechinenone 5',6'-epoxide. The CD spectrum of **3** showed almost the same Cotton effect of (3*S*, 5*R*, 6*S*)-cryptoxanthin 5,6-epoxide (Britton et al., 2004). Therefore, a 3'*S*, 5'*R*, 6'*S* configuration was assigned to new carotenoid **3.**



Echinenone 5',8'-epoxide (1)



Echinenone 5',6'-epoxide (2)



3'-Hydroxyechinenone 5',6'-epoxide (3)

Figure 26. Structures and key NOESY correlation of new carotenoids **1**, **2**, and **3** formed in transgenic sweetpotato cultivar W71.

3.8.4 Proposed biosynthetic pathway of the Novel Carotenoids Produced in the Tubers of the *crtW* Gene-expressed Sweetpotato

By introducing the ketolase *crtW* gene in sweetpotato, a series of keto carotenoids, echinone, canthaxanthine, 3'-hydroxyechinone, (3S)-asteroidenone, (3S)-adonirubin, and (3S, 3'S)-astaxanthin were produced along with the new

epoxy-carotenoids 1-3. Meanwhile, β-carotene content was decreased from 75.8% of the total carotenoids in the sweetpotato wild type to 50.6% in the ketolase gene-expressed sweetpotato. Similarly, content of β-carotene epoxides was decreased from 10.0% to 2.5%. These reults clearly indicated that β-carotene and β-carotene epoxides were used as substrates of *crtW,* and converted to a series of keto carotenoids and epoxy-keto carotenoids, respectively. Based on these results a biosynthestic pathway biosynthetic pathway of new carotenoids **1**, **2**, and **3** in the tubers of the *crtW* gene-expressed sweetpotato was also proposed and shown in figure 27.

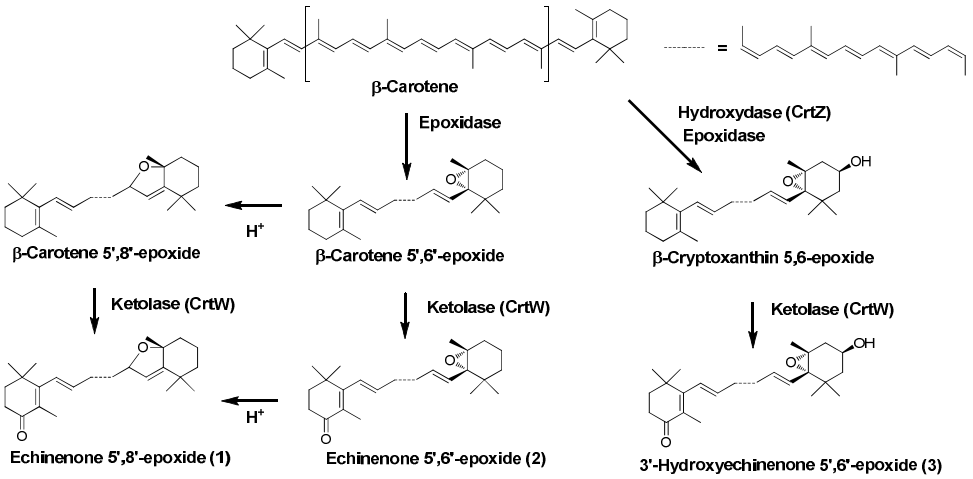


Figure 27. Proposed biosynthetic pathway of new carotenoids **1**, **2**, and **3** in the tubers of the *crtW* gene-expressed sweetpotato.

3.8.5 Conclusion

Many higher plants possess carotenoid epoxidase genes. Therefore, several epoxy carotenoids such as violaxanthin, antheraxanthin, neoxanthin, β-cryptoxanthin epoxides, and β-carotene epoxides are found in higher plants. However, higher plants do not ordinarily possess keto carotenoid due to the absence of the carotenoid ketolase gene (Britton et al., 1998). Therefore, epoxy-keto carotenoids, eichinenone 5',8'-epoxide (echinenone 5',6'-epoxide), and 3'-hydroxyechinenone 5',6'-epoxide are not present in higher plants.

4. GENERAL CONCLUSION AND FUTURE PROSPECTS

The oxygenated and or hydroxylated carotenoids products found in weetpotato suggested valuable information and a compressive molecular map for carotenoids with varied composition These results will support not only the further molecular cloning and elucidation of carotenoids biosynthesis genes and their pathway engineering based on the carotenoids biosynthesis pathway proposed in this study but also the molecular breeding of sweetpotato for future nutritional, medicinal and industrial gains. Lycopene cyclases isolated from both cultivars showed significant homology and individually formed single clades that were positioned close to the corresponding proteins from potato (*Solanum tuberosum*) and tomato (*Solanum lycopersicum*), which, like sweetpotato, belong to the order Solanale. The presence of multiple copies of *IbLCYb* revealed significant genetic variation present in the individual genome of W71 and WS. These results suggest that the cyclase genes might be distributed across different chromosal sets in the hexaploid sweetpotato genome. Moreover, the *IbLCYb5* with an inside deletion support that there may be other naturally existing mutations in sweetpotato which can be explored further for comparative analysis and understanding of the molecular genetic variation in sweetpotato. Both *IbBHY1* and *IbBHY2* closely branched with tomato β- Carotene hydroxylases at one end and with wheat and maize at the other. These results show that the sweetpotato occupies evolutionary space after bacteria, algae, lower vascular plants in evolutionary hierarchy after monocots. The present results revealed that *IbBHY* genes possess β-ring hydroxylation activity for β-carotene to produce zeaxanthin via β-cryptoxanthin which are pro vitamin A carotenoids. These results will provide comprehension in further genetic engineering for the enhanced β-Carotene hydroxylated carotenoids for the improvement of pro-vitamin A carotenoids in sweetpotato production by RNAi based silencing or genome editing approaches Moreover, these results will help better in further interpretations of the mechanism of xanthophylls biosynthesis in sweetpotato. Present results for *IbCYP97A* and *IbCYP97B* showed almost similar function to that reported in other plant species when tested individually using either α-Carotene or β-Carotene as substrates. However, further experiments are essential for complete elucidation of P450 cytochrome based

IbCYP97 families for understanding the molecular mechanism by which these genes interact with other counterparts for the catalysation of of lutein formation in sweetpotato. For example, it would be important to test the combined activity of *IbBHY* with *IbCYPA*(*s*), *IbCYPb*(*s*) and or more necessarily *IbCYPC*(*s*) in separate control experiments to fully understand the molecular bases for the lutein and other carotenoid hydroxylation products in sweetpotato. The presence of several epoxy carotenoids such as violaxanthin, antheraxanthin, neoxanthin, β-cryptoxanthin epoxides, and β-carotene epoxides in higher plants is due to the existence of carotenoid epoxidase genes. However, higher plants do not ordinarily possess keto carotenoid due to the absence of the carotenoid ketolase gene *crtW.* In the present study, *crtW* gene was transferred into sweetpotato as a foreign gene as a result epoxy-keto carotenoids, eichinenone 5',8'-epoxide, echinenone 5',6'-epoxide and 3'-hydroxyechinenone 5',6'-epoxidewere generated in sweetpotato which are new carotenoids produced for the first time in sweetpotato. Futher, the results will help in planning and engineering for enhanced astaxanthin which has immensely increasing market demands because of its multipurpose uses. The novel carotenoids can also be further tested for their use in medicine, cosmetic or chemical industry for economic uses. These results will be the strong base for future based metabolic and pathway engineering of sweetpotato and their evaluation for potential use in pharmaceutical, and cosmetic industry.

APPENDIX

Buffers, Solutions and Media

SOC Media

Tryptone 2% (w/v)
Yeast extract 0.5% (w/v)
NaCl 10 mM
KCl 2.5 mM
$MgCl_2$ 10 mM
Glucose 20 mM
(For 1L: 20 g/L Tryptone; 5 g/L Yeast Extract; 4.8 g/L $MgSO_4$; 3.603 g/L dextrose; 0.5g/L NaCl; 0.186 g/L KCl
First prepare a solution containing the first four reagents, sterilize at 121 °C, and then add sterile $MgCl_2$ and glucose)

2YT Media

Tryptone 1.6%
Yeast Extract 1%
NaCl 0.5%
(For 1L: Measure approximately 900 ml of distilled H_2O. Add 16 g Bacto Tryptone, 10 g Bacto Yeast Extract and 5 g NaCl. Adjust pH to 7.0 with 5N NaOH. Adjust to 1L with distilled H_2O (optional: add bottom agar 12 g/L), Sterilize by autoclaving)

1x TAE buffer

Tris, 40mM
Glacial Acetate 20mM
EDTA 1mM
For a 500-milliliter stock solution of 0.5 M EDTA, weigh out 93.05 grams of EDTA disodium salt (FW = 372.2). Dissolve in 400-milliliter deionized water and adjust the pH with sodium hydroxide (NaOH). Top up the solution to a final volume of 500 milliliters.

Fo 50x stock solution of TAE: weigh 242 grams of Tris base (FW = 121.14) and dissolve in approximately 750 milliliters of deionized water.Carefully add 57.1milliliters of glacial acid and 100 milliliters of 0.5 M EDTA (pH 8.0). After that, adjust the solution to a final volume of 1 liter.

1X TE buffer
Tris, 10 mM adjust pH 8.0 with HCl
EDTA 1mM (EDTA: 0.5M Ethylene diamine tetra-acetic acid, pH 8.0)

1× STE Buffer
100 mM NaCl
20 mM Tris-HCl (pH 7.5) 10 mM EDTA

IPTG: 0.1M Isopropyl-β-D-thiogalactopyranoside in sterile water

X-gal
5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
2% (w/v) in dimethylformamide (DMF)

100 mg/ml Ampicillin .5 g in 5 ml sterile distilled water. Dissolve and filter sterilize. Store in aliquots at -20 C

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