J. Hortl. Sci.
Vol. 11(2): 91-103, 2017

FOCUS

Molecular mechanisms involved in biosynthesis and regulation of carotenoids in plants

P. Shilpa, K.V. Ravishankar, K.S. Shivashankara[1*], A.T. Sadashiva[2] and N. Sunil Kumar [3]
Division of Biotechnology
[1]Division of Plant Physiology and Biochemistry
[2]Division of Vegetable Crops
ICAR- Indian Institute of Horticultural Research, Bengaluru 560 089, India
*Corresponding author E-mail: shiva@iihr.res.in

ABSTRACT

Carotenoids are coloured compounds beneficial to plants and humans. Some of the major health benefits carotenoids provide include Vitamin A precursors and, antioxidants besides being involved in several physiological functions. Even though several carotenoids are synthesised by plants, only a few like beta/ alpha carotenes and cryptoxanthin serve as Vitamin A precursors. The rest are useful as antioxidants. To draw maximum benefits from carotenoids, we need to incorporate these in crop improvement programmes for enhancing available Vitamin A precursor carotenoids. Therefore, it is essential to study biosynthesis of carotenoids, their genetics and their control. In this review, we focus on factors regulating carotenoid biosynthesis, metabolism and storage in plastids. Transcriptional and genetic control of carotenoid production in plants is discussed in the review using several mutants too. Further, environmental regulation of carotenoid biosynthesis is also highlighted. Carotenoid-rich fruits and vegetables have greater economic value owing to their health-promoting effects. Besides,carotenoids have several industrial applications. Therefore, knowledge of regulation mechanism in carotenoid production in plants can help develop crop varieties or technologies, thus generating carotene-rich fruits and vegetables.

Key words: Carotenoid biosynthesis, regulation, plastid, fruit, transcription factor

INTRODUCTION

Carotenoids are naturally-occurring, lipophilic, C40 isoprenoid compounds of red, yellow and orange coloured pigments. They are usually found in all photosynthetic organisms (bacteria, algae and plants) as well as in some non-photosynthetic bacteria and fungi. Colour is an important factor that makes flowers, fruits and vegetables economically valuable. This, in turn, is directly related to accumulation of carotenoids. Orange colour from â-carotene, and red colour in tomatoes and watermelon from lycopene are some examples. Apart from the appealing colour of carotenoids in fruits such as tomatoes, water-melon and papaya, and in vegetables such as carrot, red-bell-peppers and green leafy vegetables such as spinach, broccoli and lettuce, these are also nutritionally important to humans. Consumption of carotenoid-rich fruits and vegetables has several health benefits. These are precursors for Vitamin A synthesis - deficiency of which leads to age-related macular degeneration. Carotenoids also act as free-radical scavengers owing to their antioxidant property, and help in prevention of several degenerative diseases, cardiovascular diseases and cancer (Fraser and Bramley, 2004; Fiedor and Burda, 2014). In plants, carotenoids have diverse functions: they serve as components of the light-harvesting apparatus during photosynthesis, they attract pollinators and seed-dispersal agents (Pandurangaiah *et al*, 2016). In view of the importance of carotenoids in plants and humans, focus is now on carotenoid research, particularly, in horticulture crops. There are several review articles on carotenogenesis and its regulation in plants (Fraser and Bramley, 2004; Walter and Strack, 2011; Giuliano, 2014). A major focus of carotenoid research is to find compositional variation

in carotenoids and regulation thereof at different levels in various plant species. The present review purports to be an overview of the recent progress in our understanding of regulation of carotenoid biosynthesis in plants.

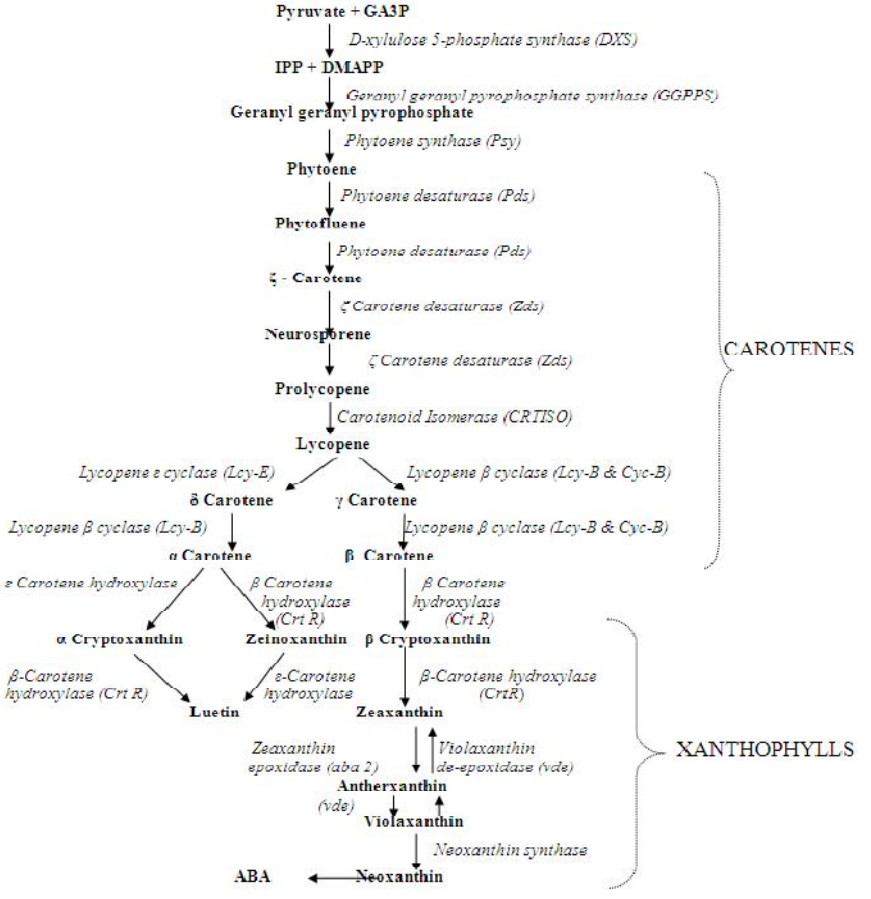
Carotenoid biosynthesis pathway

a Intermediate compounds and enzymes involved in the pathway

Synthesis of carotenoids needs some precursors belonging to the family of isoprenoids. Carotenoids are derived from two isoprene isomers, isopentenyl diphosphate (IPP) and its allylic isomer dimethylallyl diphosphate (DMAPP) (Nisar *et al*, 2015). Formation of isoprenoids or isopentenyl diphosphate (IPP) occurs via two pathways, the cytosolic mevalonic acid pathway (MVA) and the plastidic methyl-erythritol 4-phosphate (MEP) pathway (Rodriguez-Concepcion and Boronat, 2002; Eisenreich *et al*, 2004). In the MEP pathway, pyruvate and glyceraldehyde (mainly obtained by glycolysis) are used as substrates initially for the formation of deoxy-D-xylulose 5-phosphate (DXP) which is catalyzed by *DXP synthase* (*DXS*). Next, MEP is formed from reduction of DXP by the enzyme *DXP reductoisomerase* (*DXR*). The roles of *DXS* and *DXR* in the pathway are very important, as,these enzymes have been shown to affect carotenoid accumulation in plants (presumably through their control of IPP and DMAPP flux). In tomato, *DXS* gene expression exhibited developmental and organ-specific regulation and a strong correlation with carotenoid synthesis during fruit development (Lois *et al*., 2000). IPP is the fundamental C5 unit from which carotenoids are synthesized. DMAPP is sequentially formed from IPP, catatlyzed by IPP Isomerase. Further, by addition two IPP units, DMAPP is converted to Farnesyl diphosphate (FPP) and, later, FPP is further condensed to Geranyl geranyldi-phosphate (GGPP) - the first precursor to carotenoid biosynthesis.

Formation of Phytoene from two molecules of GGPP is the first rate-limiting step in the carotenoid biosynthesis pathway (Fig.1). Biosynthesis of phytoene

Fig. 1. Schematic representation of carotenoid biosynthesis pathway
(Source: Current Opinion in Plant Biology, 2001, 4:210–218)



from GGPP is a two-step reaction catalyzed by the enzyme, *phytoene synthase* (*PSY*). *PSY* is encoded by multi-gene families in most plant species, except *Arabidopsis* where PSY is encoded by a single gene. Three *PSY* genes are reported in cereal crops viz., maize, rice and wheat. Tomato contains two genes, *PSY1* and *PSY2*. The former encodes the fruit-ripening-specific isoform, whilst *PSY2* predominates in green tissues, including mature green fruit, and has no role in carotenogenesis in a ripening fruit (Fraser *et al*, 1999). *PSY* gene(s) has/have also been used to increase carotenoid content in other crops such as rice, carrot and tomato. By contrast, the bacterial *crtI* gene encodes a single desaturase, that converts phytoene into all-*trans* lycopene (Fraser *et al*,1992). Various members of *PSY* family of genes are expressed differentially in various organs of a plant and are regulated by environmental factors (Li *et al*, 2008a,b). *PSY3* in rice was recently found to control carotenoid biosynthesis in the root in response to abiotic stress (Welsch *et al*, 2008). A mutation in *PSY1*gene causes a yellow-flesh phenotype (the *r, r* mutant) and complete absence of carotenoids in the ripe fruit, an effect that can be mimicked with antisense *PSY1* transformation in tomato (Bird *et al*, 1991; Bramley *et al*, 1992). Further, Phytoene is converted into lycopene by a multi-step process involving desaturation of phytoene by two structurally and functionally similar membrane-bound enzymes, *phytoene desaturase* (*PDS*) and *æ-Zeta carotene desaturase* (*ZDS*) in plants. Quinones act as electron acceptors for *PDS* and *ZDS* desaturation reactions, as demonstrated in daffodil and *Arabidopsis* (Nisar *et al*, 2015). *PDS* and *ZDS* are two FAD-containing enzymes that require at least a plastoquinone (Mayer *et al*., 1992; Norris *et al*, 1995) and a plastid terminal oxidase (Carol and Kuntz, 2001) as electron acceptors. Next, two *cis-trans isomerases*, *Z-ISO* (Li *et al*, 2007) and *CRTISO* (Isaacson *et al*, 2002) are required to convert poly-*cis*-configured phytoene into the all-*trans* form lycopene. Recently, two distantly related *CRTISO*-like single-copy genes (*CrtISO-L1* and *CrtISO-L2*) have been discovered in tomato, *Arabidopsis* and grape. These enzymes are presumed to initiate a competing metabolic pathway, metabolizing carotenes upstream of all-*trans*-lycopene (Fantini *et al*, 2013). Isomerization of *cis* bonds to all-*trans* lycopene thus appears to be another regulatory step in carotenoid biosynthesis.

Cyclization of lycopene is a crucial branching point in carotenoid biosynthesis. In plants, all-*trans* lycopene is a substrate for the enzyme cyclase, which further derives a diverse group of carotenes [differentiated based on their different cyclic end-groups, either addition of a beta ring (â-ring) and/or an epsilon ring (å-type ring)]. These two competing steps of lycopene cyclization determine the proportion of lycopene channelled to the two branches of the carotenoid pathway, *viz.*, â- and á-carotenes. There are two types of *lycopene cyclases*: *lycopene å cyclase* (*LCY-E*), which convert lycopene into á-carotene; and, *lycopene â cyclase* (*LCY-B & CYC-B*), which convert lycopene into â-carotene (Pecker *et al*, 1996; Ronen *et al*, 1999). In fruits, carotenogenesis is carried out by two types of *lycopene â cyclases,viz*, chloroplast *lycopene â cyclase* (*LCY-B*), and chromoplast *lycopene â cyclase* (*CYC-B*). In higher plants, a chloroplast-specific *lycopene â-cyclase* enzyme (*LCY-B*) mediates conversion of lycopene into â-carotene in photosynthetic tissue (Ronen *et al*, 2000). Lycopene to â-carotene conversion in chromoplast is mediated by a paralog of *lycopene â- cyclase* called the chromoplast-specific *lycopene beta cyclase* gene (*CYC-B*). In tomato, *LCY-B* is expressed in leaves, flowers and fruits until the breaker-stage, whereas, *CYC-B* is expressed exclusively in flowers and in chromoplasts of fruits at breaker-and ripe-stages of fruit ripening. *CYC-B* retains the same catalytic function as *LCY-B*, but has only 55% amino acid sequence identity (Mohan *et al*,2016), but *CCS* (*Capsanthin Capsorubin Synthase*) of pepper has a high homology to *CYC-B* of tomato (Dalal *et al*, 2010) (Fig. 1).

Xanthophylls, *viz*., lutein, zeaxanthin and neoxanthin, are produced by hydroxylation of á-carotene and â-carotene. á-carotene is converted in lutein by two hydroxylation reactions catalyzed by *â ring carotene hydroxylases* and *å ring carotene hydroxylases*. On the other hand, â-carotene is converted into zeaxanthin by *â ring carotene hydroxylases*. Then, *Zeaxanthin epoxidase* (*ZEP*) hydroxylates the â rings of zeaxanthin in two consecutive steps to yield antheraxanthin and, then, violaxanthin. Violaxanthin is converted to neoxanthin by *neoxanthin synthase*, which is the final step in the carotenoid biosynthesis pathway (Fig. 1). Briefly, the carotenoid pathway starts with isoprenoid units, built together to form a series of carotenoids such

as phytoene, lycopene, carotene and, finally, xanthophylls, by the activity of various enzymes.

Transgenic studies in plants for altering carotenoid content

Although conventional breeding approaches successfully increased carotenoid content in plants, gene transfer or genetic engineering methods help faster and easier introduction of carotenogenic genes into plants, in a less laborious way. There has been significant progress in development of transgenic crop varieties that produce higher levels of carotenoids and, more recently, there have been a number of key achievements in areas of branch-point modulation (shifting the flux towards particular molecules,and away from the others), *de novo* carotenogenesis (introduction of the entire carotenogenic pathway into plant tissues that lack carotenoids) and pathway extension (Farre *et al*, 2011). Plant carotenoids have been successfully engineered with either plant or bacterial genes, or, combinations of genes from the two sources. Because they display some particular features, bacterial genes have been used for engineering both early (phytoene synthesis, desaturation and isomerization) and late (lycopene cyclization, ketocarotenoid biosynthesis) biosynthetic steps (Rosati *et al*, 2010). Rice, one of the non-solanaceous crops, was choosen for carotenoid engineering. One of the major biotechnological breakthroughs has been molecular breeding of 'GOLDEN RICE' in both *japonica* and *indica* backgrounds, whose grain accumulates â-carotene (pro-vitamin A). Beyer *et al* (2002) introduced (in a single, combined transformation effort) the cDNA coding for *phytoene synthase* and *lycopene cyclase*-both from *Narcissus pseudonarcissus* and both under the control of the endosperm-specific glutelin promoter-together with a bacterial *phytoene desaturase* (*crtI*, from *Erwinia uredovora,* under constitutive 35S promoter control). This combination covers all the requirements for â-carotene synthesis an, as hoped, yellow â-carotene-bearing rice endosperm was obtained. Transgenic maize plants containing *CrtL* and *CrtI* genes expressed under the control of specific promoters showed increased levels of carotenoids, especially â-carotene (34-fold), contributing to the first transgenic maize developed to combat Vitamin A deficiency (Aluru *et al*, 2008). Jayaraj *et al* (2008) engineered the keto-carotenoid biosynthetic pathway in carrot tissues by introducing a â-carotene ketolase gene, isolated from the alga, *Haematococcus pluvialis*. Gene constructs were made with three promoters (double CaMV35S, *Arabidopsis*-ubiquitin, and *RolD* from *Agrobacterium rhizogenes*). Endogenous expression of carrot *â-carotene hydroxylases* was up-regulated in transgenic leaves and roots, and up to 70% of the total carotenoids were converted to novel keto carotenoids, with accumulation of up to 2,400ìg/g root dry-weight. As for Solanaceous crops, tomato is the best-investigated species within Solanaceae family due to its importance as a food crop, and nutritional value of its fruits accumulating lycopene. Fruit-specific expression of a bacterial *PSY* gene (*CrtB* from *Erwinia*) produced fruits with higher phytoene, â-carotene and total carotenoid levels, but with not increased lycopene content (Fraser *et al*, 2007). Large increases in fruit â-carotene and total carotenoids were achieved by manipulating the expression of *lycopene â-cyclase* genes: overexpressing *Arabidopsis*/ tomato *LCY-b* genes under the control of chromoplast-specific promoters resulted in higher *â*-carotene level, up to 7-fold (Rosati *et al*, 2000) and 32-fold (D'Ambrosio *et al*, 2004), respectively. In order to increase lycopene content in the fruits, antisense approach was used for silencing the *LCY-b* gene (Rosati *et al*, 2000). In a study on potato, a bacterial *phytoene synthase* gene under the control of *patatin* promoter increased *â*-carotene and total carotenoids (Ducreux *et al*, 2005). Diretto *et al* (2007) investigated modulation of carotenogenesis in potato leaves and tubers using bacterial phytoene desaturase, carotenoid *isomerase* and *lycopene â cyclase*. They observed an increase in metabolite as well as transcript levels in the transgenic plants. There was a 20-fold increase in expression of these genes simultaneously. The tubers showed enhanced â-carotene content and appeared deep yellow (golden) in colour. Peppers or hot chillis have peculiar caroptenoids that are different from those in other fruits. Capsanthin, capsorubin and capsanthin 5,6-epoxide are the red carotenoids exclusively accumulating in fruits of *Capsicum* spp. (Deli *et al*, 2001). The gene encoding *Capsanthin-capsorubin synthase* (*Ccs*) is involved in synthesis of these pigments. Pepper varieties can be classified according to fruit color: the two main isochromic families include red varieties-synthesizing capsanthin and capsorubin pigments; and, yellow ones accumulating a carotenoid

pool comprising antheraxanthin, violaxanthin (precursors of capsanthin and capsorubin) as well as zeaxanthin, á-cryptoxanthin, á-carotene and cucurbitaxanthin A (Guil-Guerrero *et al*, 2006). From a molecular and biochemical viewpoint, red-fruited varieties display high *Ccs* and high carotenoid gene-transcript levels, besides a high red-to-yellow (*R/Y*) isochromic pigment fraction as also high capsanthin-to-zeaxanthin (Caps/Zeax) ratios.

Regulation of carotenoid biosynthesis

Transcriptional regulation

Carotenoid accumulation is determined mainly by transcription regulation of the genes involved in the carotenoid pathway. Transcriptional regulation of carotenoids is extensively studied in the model plant tomato during fruit ripening. In this crop, lycopene is accumulated via upregulation of the genes *DXS, PSY, PDS, CRTISO* (which are the upstream genes in the pathway) and downregulation of the downstream genes, *LCY-B, CYC-B and LCY-E. PSY* is one of the rate-limiting enzymes and is the most targeted enzyme involved in carotenogenesis (as, it provides the initial substrate phytoene, levels of which determine the level of carotenoids synthesized). *PSY1*, present in very low abundance in leaves, is moderate in petals, and extremely high in fruits at the pink and mature red-stages; *PSY2 is* reported to be expressed in all the tissues, with the highest level in yellow petals; and *PSY3* is found in the tomato genome and is predicted to regulate root carotenogenesis induced by abiotic stress, ABA and light. A tomato mutant related to lycopene biosynthesis is available, *viz.*, yellow flesh (locus r), a loss-of-function mutant of the *SlPSY1* gene, where there is lack of carotenoids; overexpression of the same restores carotenoid biosynthesis (Fray and Grierson, 1993). Gady *et al* (2012) identified a *PSY1* knockout mutant through Targeting Induced Local Lesions IN Genomes (TILLING). The mutant fruit is yellow in colour due to the absence of carotenoids proving, that, *PSY* is the candidate enzyme involved in the initial step of carotenoid biosynthesis. Light-induced synthesis of carotenoids is characterized by an increase in the expression of *PSY* and, also, increase in enzyme activity. *PSY* transcript levels have been reported to increase in response to light of the wavelength far-red (Welsch *et al*, 2000). The light-induced increase in *PSY* expression has been shown to be mediated by phytochrome (PHYs) photoreceptors. Light-induced activation makes the cytoplasmic localized PHYs to translocate to the nucleus where they interact with, and mediate, degradation of the Phytochrome Interacting transcription Factors (PIFs); these factors bind to G-boxes in the promoters of light-induced genes, and negatively regulate their expression (Leivar *et al*, 2009). Transgenic lines over-expressing *PSY1* gene significantly increased carotenoid content in the tomato fruit (Fraser *et al*, 2007). In tomato fruits, the MADS box transcription factor, RIN (Ripening Inhibitor), has been confirmed to regulate carotenoid accumulation by interacting with the SlPSY1 promoter (Martel *et al*, 2011). According to Luo *et al* (2013), SlSGR1 (STAY GREEN PROTEIN) regulates carotenoid accumulation through directly interacting with SlPSY1 during tomato ripening and, therefore, repression of SlSGR1 significantly increases lycopene and *â*-carotene accumulation.

Issacson *et al* (2002) elucidated the molecular mechanism of carotenoid isomerization by studying *tangerine* fruits in tomato. Fruit of *tangerine* are orange, and accumulate prolycopene (*cis*-lycopene) instead of all-*trans*-lycopene, which is normally synthesized in the wild type. Map-based cloning of the *tangerine* locus of tomato identified *CRTISO*, which encodes an authentic carotenoid isomerase that functions during carotenoid desaturation. In *Arabidopsis*, a gene designated *Pdh* encodes a polypeptide 75% identical to the *CRTISO* from tomato (86% identical in the predicted mature-polypeptide region). Evidence that *Arabidopsis CRTISO* ortholog is involved in carotenoid biosynthesis is described by Park *et al* (2002). It is interesting to note that *CRTISO* activity can be partially substituted by exposure to light in green tissues via photoisomerization (Isaacson *et al*, 2002; Park *et al*, 2002).

Cyclization of lycopene is a key branching-point in the carotenoid pathway. Lycopene cyclases are also important determinants of carotenoid content in different plants. The two cyclases, *viz.*, *â cyclases* and *å cyclases* are associated with differences in lycopene, *â*-carotene and, further, to xanthophylls by the former, and ä-carotene to lutein by the latter. Tissue-specific isoforms are involved in fine-tuning carotenoid composition in a few plants. In *Arabidopsis* and rice, only single-copy of these cyclases is expressed, whereas in plants like tomato, water melon and citrus, there are two types of cyclases: chloroplast-specific

and chromoplast-specific (Tadmor *et al*, 2005; Alque´zar *et al*, 2009; Devitt *et al*, 2010). Chloroplast-specific cyclases are expressed in leaves and in the green tissues of fruit. In contrast, chromoplast-specific cyclases are expressed in flowers and chromoplasts of the fruit. The chloroplast to chromoplast transition is a remarkable event during the fruit-ripening process, as, chlorophyll content decreases and carotenoid content increases. Downregulation of *â* cyclase leads to an accumulation of lycopene, whereas, its upregulation leads to synthesis of *â*-carotene from lycopene.

Expression of chromoplast-specific *â* cyclase has been found to correlate with accumulation of *â*-carotene and/or the downstream xanthophylls in tomato and citrus (Ronen *et al*, 2000; Alque´ zar *et al*, 2009). Increased level of *â*-carotene is due to the fruit-specific chromoplast lycopene *â* cyclase (CYC-B). This phenotype was named *Beta* (*B* gene). *Beta* is a partially dominant, single-locus mutation that imparts an orange colour to the fully-ripened fruit owing to accumulation of *â*-carotene at the expense of lycopene (Ronen *et al*, 2000). In wild tomatoes, the B gene is expressed at low levels, whereas in the *Beta* mutant, its transcription dramatically increases. Null mutations in the *B* gene are responsible for the phenotype of *og (old gold)*, indicating that *og* is an allele of *B*. Two recessive allelic mutations, *oldgold (og)* and *old-gold-crimson (og^c)*, have the same phenotype of deep red fruits, rich in lycopene but lacking â-carotene (Ronen *et al*, 2000). Lycopene å cyclase (LCY-E), in wild tomatoes is downregulated at the breaker-stage of ripening. In contrast, it increases approximately 30-fold during fruit ripening in *Delta* plants. This is due to a single, dominant gene, *Del*, in the tomato mutant *Delta*, which changes fruit colour to orange from accumulation of ä-carotene at the expense of lycopene (Ronen *et al*, 1999). LCY-E plays a key role in determining *â*-carotene/*á*-carotene ratio (Harjes *et al*, 2008). Lutein and zeaxanthin are produced next, by å-carotene hydroxylase and â-carotene hydroxylase. Further epoxidation of zeaxanthin by zeaxanthin epoxidase (ZEP) produces violaxanthin. This reaction is reversed by violaxanthin de-epoxidase (VDE) to give rise to the xanthophyll cycle in plants to adapt to high light-stress. Violaxanthin is converted into neoxanthin by neoxanthin synthase (NSY) (Shan Lu and Li, 2008). A study shows that a novel gene-product of *ABA4* is needed for neoxanthin synthesis (North *et al*, 2007).

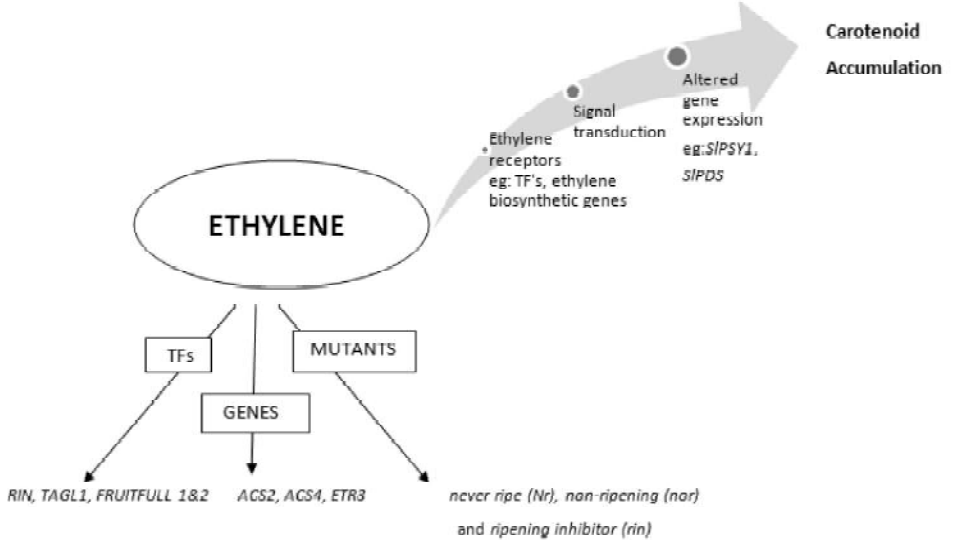
Environmental regulation

Light plays an important role too in carotenoid biosynthesis. Light intensity, duration, direction, spectral quality are all key factors for modulating plant carotenoids during fruit development. Photomorphogenic mutants have been reported in a number of species, including *Arabidopsis*, sorghum, brassica, tobacco, tomato and pea (Levin *et al*, 2006). The tomato *high pigment 1* (*hp1*) and *high pigment 2* (*hp2*) are regulated by light, which controls plastid development in these mutants. Light-signalling proteins are responsible for *hp1* and *hp2* phenotypes in tomato. Two regulatory genes, *UV-DAMAGED DNA-BINDING PROTEIN1* (*DDB1*) and *DE-ETIOLATED1* (*DET1*), control light-signalling pathways. Mutations in these genes are responsible for high-pigment mutants (*hp1* and *hp2*) in tomato (Shan Lu and Li, 2008). Carotenoid accumulations in these mutants is linked to early plastid-biogenesis, number and size to provide a large compartment for carotenoid biosynthesis and deposition (Liu *et al*, 2004). The red-ripe fruits of these mutants are characterized by an intense red colour mainly from increased levels of carotenoids, primarily lycopene. These *hp* mutants have proved to be excellent tools in the study of complex interactions between light and plant development. Some of these have also been harnessed in breeding programs. Similarly, like *hp1* and *hp2*, *high pigment 3* (*hp3*) mutant in tomato is caused by a mutation in the gene of *ZEP*, which confers an enhanced level of carotenoid accumulation by increasing the size of plastid compartment in the cells, to enable greater biosynthesis and higher storage capacity (Galpaz *et al*, 2008). Apart from these mutants, light-regulated carotenoid synthesis is observed through LREs (Light Responsive Elements) and G box elements present in the promoter region of the genes involved in the pathway. The most common type of LREs present in genes activated by light are ATCTA element, and, G1 (CACGAG) and G2 motifs (CTCGAG) (von Lintig *et al*, 1997). Therefore, light acts as an inducer of photomorphogenesis, and carotenoid biosynthesis through photoreceptors and activation of known transcription factors (Pizarro and Stange, 2009). PSY activity is regulated by red and far-red light as a consequence of phytochrome activation, leading to increase in PSY activity only under red light conditions (Schofield and Paliyath, 2005).

Temperature has a significant influence on growth and development of tomato fruits. It has been reported that high temperature (35°C) can specifically inhibit accumulation of lycopene by stimulating conversion of lycopene into └ carotene (Hamauzu *et al*, 1998). Biosynthesis of lycopene is affected by environmental conditions. If the temperature of the fruits exceeds 30°C, synthesis of lycopene is inhibited in tomato (Brandt *et al*, 2006). Another study by Dumas *et al* (2003) showed that temperatures below 12°C strongly inhibited lycopene biosynthesis, while, temperatures above 32°C stopped this process altogether in tomato.

Carotenoid accumulation in plants is regulated by hormones such as ethylene, jasmonates (JA), ABA,etc. Ethylene plays a central role in fruit-ripening. Effect of ethylene in regulating carotenoid accumulation during fruit development in tomato has been increasingly reviewed. Ethylene production strongly correlates with rapid accumulation of └-carotene and lycopene, and expression of *SlPSY1* and *SlPDS*. This shows that the process is dependent on ethylene (Marty *et al*, 2005). Many transcription factors have been shown to affect carotenoid accumulation in the fruit of tomato through regulation of ethylene biosynthesis and signalling. In fruit tissues, MADS box transcription factor, ripening inhibitor (RIN), has been confirmed to regulate carotenoid accumulation by interacting with *SlPSY1* promoter (Martel *et al*, 2011) (Fig. 2). RIN interacts directly with the promoters of ethylene biosynthesis genes, *ACC SYNTHASE2* (*ACS2*) and *ACS4*, also the ethylene perception gene, *ETHYLENE RECEPTOR 3* (*ETR3/NR*) (Fig. 2). The corresponding mutant of RIN, *rin*, fails to synthesize climacteric ethylene and accumulates lycopene in the fruit (Vrebalov *et al*, 2002). *TAGL1* and *FRUITFULL 1* and *2* (which belong to MADS box transcription factor)

Fig. 2. Schematic representation of role of ethylene in carotenoid accumulation



are positive regulators of ethylene biosynthesis (Vrebalov *et al*, 2009). *ETHYLENE RESPONSE FACTOR 6* (*SlERF6*), a tomato Ethylene Response Factor, is also found to be a negative carotenoid modulator. Reduced expression of *SlERF6* by RNAi enhanced both ethylene release and carotenoid accumulation during fruit ripening in tomato (Lee *et al*, 2012). The role of ethylene in carotenoid formation is further demonstrated by a tomato ethylene-insensitive mutant, Never ripe (*Nr*), which exhibits a ripening-defective fruit phenotype, and fails to accumulate lycopene (Lanahan *et al*, 1994). JA is also of importance in positively controlling carotenoid accumulation. In tomato, lycopene content is greatly reduced in the fruits of JA-deficient mutants, and, is increased in transgenic lines having enhanced JA levels. Exogenous MeJA treatment of an ethylene-insensitive tomato mutant (*Nr*) can dramatically enhance lycopene accumulation in the fruit. ABA has also been documented to control carotenoid biosynthesis by regulating plastid development. A study of ABA-deficient tomato mutants, namely, *hp3*, *flacca* (*flc*), and *sitiens* (*sit*), shows an inverse correlation between ABA levels and plastid number. In these mutant fruits, both plastid number and lycopene levels are enhanced (Galpaz *et al*, 2008) indicating that ABA, a carotenoid

derivative, may be implicated in build-up of storage capacity in plastids (Liu *et al,* 2015). Deficiency of ABA in *hp-3* mutant led to enlargement in plastid compartment size, probably by increasing plastid division. By doing so, it enabled greater pigment biosynthesis, and 30% more storage capacity for carotenoids in the mature fruit (Galpaz *et al*, 2008).

Some studies are reported on the role of transcription factors in regulation of carotenoid biosynthesis. Lee *et al* (2012) characterized one candidate for impact on trans-lycopene and ^{L}-carotene accumulation, *viz.*, *SlERF6*, revealing that it indeed influenced carotenoid biosynthesis and other ripening phenotypes. Reduced expression of *SlERF6* by RNAi enhanced both carotenoid and ethylene levels during fruit ripening, demonstrating an important role for *SlERF6* in ripening through integrating the ethylene and carotenoid synthesis pathways. A number of transcription factors impacting ripening and, thus, carotenoid accumulation, have been identified including *RIN-MADS* (Vrebalov *et al,* 2002), *CNR SQUAMOSA* promoter binding protein (Manning *et al,* 2006), *TAGL1* MADS box (Vrebalov *et al,* 2009), *LeHB-1 HBzip* (Lin *et al*, 2008) and *SlAP2a*, an *AP2* gene (Karlova *et al*, 2011). More specific carotenoid regulators have been identified in non-fruit tissues. In *Arabidopsis,* modulating mRNA levels of ethylene response factor (ERF) *RAP2.2* (which is capable of binding the *PSY* promoter), resulted in small carotenoid alterations in root calli (Welsch *et al*, 2007). *AtRAP2.2* belongs to AP2/EREBP family of transcription factors and binds to upstream regulatory elements of the genes *PSY* and *PDS*. *AtRAP2.2* recognizes *cis*-acting element ATCTA, which mediates a strong basal promoter activity (Welsch *et al*, 2007). A putative tomato NAC transcription factor named *SlNAC4* acts as a positive regulator of fruit carotenoid synthesis in tomato. *SlNAC4* plays an important role in response to abiotic stress, but, transgenic repression demonstrates that *SlNAC4* also participates in normal fruit-ripening as a positive regulator by modulating the climacteric ripening hormone, ethylene, and carotenoid pigmentation (Zhu *et al,* 2013) (Table 1) .

Table 1. List of some transcription factors and type of regulation involved in carotenoid synthesis

Transcription factor	Family	Type of regulation
Sl ERF6	MADS RIN	Positive regulation
TAGL1	MADS Box	Positive regulation
LeHB-1	HB Zip	Positive regulation
SlAP2a	APETALA2/ERF	Negative regulation
RAP2.2	ERF	Positive regulation
SlNAC4	NAC	Positive regulation

Carotenoid catabolism

A metabolic equilibrium between biosynthesis and catabolism of carotenoids is essential for maintaining the content and composition of carotenoids in photosynthetic tissues (Beisel *et al*, 2010). Carotenoid degradation is brought about by a group of enzymes known as *Carotenoid Cleavage Dioxygenases* (*CCDs*). Thus, catalytic activity of *carotenoid cleavage dioxygenases* (*CCDs*), which leads to enzymatic turnover of C_{40} carotenoids into apocarotenoids, is critical in regulating carotenoid accumulation. In *Arabidopsis*, the *CCD* gene family consists of nine members and can be divided into two groups: four *CCDs* (CCD1, 4, 7 and 8) and five *9-cis-epoxycarotenoid dioxygenases* (*NCED* 2, 3, 5, 6 and 9). These enzymes cleave different carotenoids and some exhibit unique substrate specificity (Auldridge *et al,* 2006). Different *CCDs* and *NCEDs* recognize different carotenoid substrates and cleave at different sites, producing various apocarotenoids (Walter and Strack, 2011). I*n vitro* expression of *CCD1* in *E. coli* from a number of horticultural crops such as tomato fruit saffron flower, and melon fruit indicates that *CCD1* cleaves ^{L}-carotene and other carotenoids into a range of volatiles. *CCD1* transcription is associated with carotenoid levels in some horticultural crops. Although a negatively correlated change between *CCD1* or *CCD4* and carotenoid content has been observed in various fruits and vegetables, regulation of *CCD* expression is not well-understood (Yuan *et al*, 2015). Investigation on the activity of *SlCCD1B* and its homolog, *SlCCD1A*, provides direct evidence for involvement of *CCD* in carotenoid degradation. *In vitro* assays showed that *SlCCD1A* and *SlCCD1B* target double bonds in all *trans* configured and *cis*-

configured carotenoids, acyclic carotenes and apocarotenoids, contributing to the production of a vast majority of tomato isoprenoid volatiles (Ilg *et al*, 2014). Fruit specific RNAi-mediated suppression of *SlNCED1* produces deep-red fruits with reduced *SlNCED1* transcription and ABA biosynthesis, besides increased accumulation of lycopene and *b*-carotene (Sun *et al*, 2012).

Besides catabolism and turnover of carotenoids, the ability of a cell to store and sequester carotenoids plays a significant role in determining the level of carotenoid accumulation in a given cell. Nearly all of carotenoid biosynthesis enzymes are located in the plastid, but all their genes are encoded by the nuclear genome (Cazzonelli *et al*, 2009). Carotenoids in plants are synthesized *de novo* in nearly all types of plastids, but accumulate in large quantities in chloroplasts and chromoplasts (Howitt and Pogson, 2006). Carotenoids in the chloroplast are integrated with chloroplast binding proteins and, in chromoplast, carotenoids are associated with polar lipids and carotenoid associated proteins, to form carotenoid-lipoprotein sequestering substructures to effectively sequester and retain a large quantity of carotenoids (Vishnevetsky *et al*, 1999). Chromoplast development and differentiation plays a crucial role in carotenoid biosynthesis, as, chromoplasts provide a site not only for active carotenoid biosynthesis, but also for carotenoid storage. Size and structure of the plastids is also important for accumulation of carotenoids. Thus, carotenoid accumulation is a net result of biosynthesis, turnover and, finally, stable storage of end-products (Shan Lu and Li, 2008).

CONCLUSION

An understanding of carotenoid biosynthesis and regulatory mechanism has been of interest from several years, as, carotenoid content is one of the most important quality traits in fruits and vegetables with industrial, health and nutritional attributes. The high value of naturally-occurring carotenoids (which are economically important) has triggered special interest in uncovering the several levels/ modes of carotenoid regulation. Several attempts have been made to improve plant carotenoid content and composition. Although significant progress has been made in understanding carotenoid metabolism in plants, some aspects such as signalling mechanism involved in plastid biogenesis, cross talk between carotenoid biosynthesis pathway and other pathways, and ways in which such interactions influence carotenoid content and overall growth and development of the plant, need to be studied. An in-depth investigation of these aspects will enhance knowledge to help grow plants rich in carotenoids. Therefore efforts should be made to improve the yield of these valuable components by developing promising varieties and hybrids with high agronomical and economic importance.

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(MS Received 29 September 2016, Revised 19 December 2016, Accepted 23 December 2016)