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Review

Prospects and progress in the production of valuable carotenoids: Insights from metabolic engineering, synthetic biology, and computational approaches

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ABSTRACT

Carotenoids are isoprenoid pigments synthesized exclusively by plants and microorganisms and play critical roles in light harvesting, photoprotection, attracting pollinators and phytohormone production. In recent years, carotenoids have been used for their health benefits due to their high antioxidant activity and are extensively utilized in food, pharmaceutical, and nutraceutical industries. Regulation of carotenoid biosynthesis occurs throughout the life cycle of plants, with vibrant changes in composition based on developmental needs and responses to external environmental stimuli. With advancements in metabolic engineering techniques, there has been tremendous progress in the production of industrially valuable secondary metabolites such as carotenoids. Application of metabolic engineering and synthetic biology has become essential for the successful and improved production of carotenoids. Synthetic biology is an emerging discipline; metabolic engineering approaches may provide insights into novel ideas for biosynthetic pathways. In this review, we discuss the current knowledge on carotenoid biosynthetic pathways and genetic engineering of carotenoids to improve their nutritional value. In addition, we investigated synthetic biological approaches for the production of carotenoids. Theoretical biology approaches that may aid in understanding the biological sciences are discussed in this review. A combination of theoretical knowledge and experimental strategies may improve the production of industrially relevant secondary metabolites.

1. Introduction

Carotenoids are a large group of isoprenoid pigments synthesized by phototrophic organisms and non-phototrophic species, except animals (Cazzonelli, 2011; Fraser and Bramley, 2004). Carotenoids are synthesized by an enormous range of organisms, including archaea, eubacteria (including cyanobacteria) and eukaryotes (algae, fungi, and plants), and serve a multitude of functions. In animals such as mammals, birds, fish, crustaceans, flies, and butterflies, bright coloring is due to the presence of carotenoids. These organisms cannot synthesize carotenoids but obtain them via their diet, including humans. Carotenoids may also be taken up from symbiotic or pathogenic microorganisms (Cazzonelli and Pogson, 2010). Among 1117 known natural carotenoids, 683 carotenoids were identified with a known source organism, (Yabuzaki, 2017) delivering a broad range of visible colors such as orange, yellow and red pigments to flowers and fruits that serve to attract pollinators (Siva, 2007; Cazzonelli and Pogson, 2010; Priya and Siva, 2014; Yuan et al., 2015). *'Carotene'* is derived from the Latin word *carota* and was coined in 1831 by Wackenroder, who isolated the orange pigment from carrot (*Daucus carota*). Later, in 1837, Berzelius

Abbreviations: DXS, 1-deoxy-xylulose-5-phosphate synthase; *IPI*, IPI Isomerase; *GGPS*, Geranyl geranyl pyrophosphate synthase; *PSY*, Phytoene synthase; *PDS*, Phytoene desaturase; *ZDS*, Z-Carotene desaturase; *ZISO*, Z-isomerase; *CrtISO*, Carotenoid isomerases; *LCY-ε*, Lycopene epsilon cyclase; *LCY-β*, Lycopene beta-cyclase; *β CRH*, β-Carotene hydroxylase; *ε CRH*, ε-Carotene hydroxylase; *ZEP*, Zeaxanthin epoxidase; *VDE*, Violaxanthin- de-epoxidase; *NCED*, 9-cis epoxy dioxygenases; MOMA, Minimization of Metabolic Adjustments; FSEOF, Flux Scanning Based on Enforced Objective Flux

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gave the name '*xanthophylls*' to the yellow pigments of leaves in autumn (Socaciu, 2008; Hurst, 2008). The history of research on the chemical nature of carotenoids was identified by Paul Karrer and Richard Kuhn in 1937 and 1938, respectively, which earned them the Nobel Prize (Davies, 2004). Traditionally, carotenoids are named based on the biological source from which they are isolated, for instance, zeaxanthin from *Zea mays* and lutein from *Macula lutea.* As a result, the name does not provide any structural information about the carotenoid. The names are based on the stem name 'carotene', which was preceded by the Greek letter prefixes (β,ε,α) that denotes the two end groups (Ruiz-Sola and Rodriguez-Conception, 2012). Identified carotenoid structures are broadly classified into two groups: carotenes (non-oxygenated molecules) and xanthophylls (oxygenated carotenoids) (Rodriguez-Concepcion and Stange, 2013; Zaripheh and Erdman, 2002).

In a plant cell, carotenoids are found in the membranes of plastids such as chloroplasts, which synthesize and store carotenoids to act as an accessory pigment for photosynthesis and protect chlorophyll from photodamage. Carotenoids have high blue light absorption and appear bright in color (red, yellow or orange). The color of carotenoids is masked by chlorophyll in photosynthetic tissues, but in later stages of plant development, these pigments contribute to the beautiful bright colors of fruits and flowers. In addition to their coloring property, carotenoids are known to contribute to plant cross-talk with pathogens, pests, and symbiotic organisms (Hirschberg, 2001). Plant physiologists indicate that carotenoids may have additional functions as regulators in specific developmental responses of plants.

As a result of oxidative cleavage, carotenoids produce apocarotenoids involved in plant growth and development. Apocarotenoids products such as the retinal derivatives vitamin A and retinoic acid play significant roles as nutritional and signaling molecules in animal development (Ohmiya, 2009; Giuliano et al., 2003). In plants, abscisic acid (ABA) plays a significant role in seed dormancy and plant adaptability towards abiotic stress. Strigolactone, another plant hormone, acts as a growth hormone inhibitor and is involved in the regulation of shoot branching (Liu et al., 2013). Apocarotenoids are also involved in allelopathic interaction and plant defense mechanisms (Bouvier et al., 2005). Carotenoid uptake is necessary for nutrient supplement and health benefits (Rao and Rao, 2007). Carotenoids such as *β*-carotene and α-carotene (pro-vitamin A) are essential dietary sources of vitamin A. Vitamin A deficiency is a common problem faced in many developing countries, inspiring researchers to develop nutritionally enriched carotenoid food crops such as rice and maize. Lycopene, an essential carotene (from tomato), has high antioxidant activity, decreasing the risk of cancers and defending against cardiac-related diseases (Bhakta and Siva, 2010). Lutein and zeaxanthin are xanthophylls known to prevent macular degeneration (Hadley et al., 2002; Krinsky et al., 2003; Lu and Li, 2008). Carotenoids and apocarotenoids are used as supplements in livestock and fish feed and as natural colorants in the food and cosmetic industries (Krishnamurthy et al., 2002; Siva, 2003; Umeno et al., 2005). Consequently, these pigments have been extensively studied by organic chemists, food chemists, biologists, physiologists, doctors, and environmentalists.

During a plant's life cycle, carotenoids are involved in germination, photomorphogenesis, fruit development and responses to external environmental factors (Cazzonelli and Pogson, 2010). Carotenoids are produced using biotechnological methods with plants that have been genetically engineered to withstand environmental stress (high visible light, UV-B radiation) to improve the nutritional content and produce high-value carotenoids at an industrial scale (Sandmann et al., 2006). This review provides an update on the carotenoid biosynthetic pathway, metabolic engineering of carotenoids in essential staple crops, and synthetic biological approaches for the production of valuable carotenoids.

2. Structural diversity of carotenoids

Carotenoid diversity is generated through the introduction of cyclases, hydroxylases, ketolases and other enzymes in the linear backbone. Carotenoids with two terminal rings linked by the chromophore-bearing chain of conjugated double bonds are widely distributed (e.g., simple hydrocarbon carotenes (*β*-carotene)). Carotenoids that are hydroxylated at the terminal rings are known as xanthophylls, for example, zeaxanthin and lutein. In some carotenoids, oxygen can be introduced in the form of keto groups, with or without the addition of hydroxyl groups, such as astaxanthin and canthaxanthin. In marine sponges, such as mycobacteria and cyanobacteria, carotenoids contain aromatic rings in their structures, but the rings are rare and occasionally originate in the microorganisms. Synechoxanthin, a derivative of dicarboxylic acid from *Synechococcus* sp. PCC 7002, is a good example (Graham and Bryant, 2008). Torulene, a rare monocyclic carotenoid, is found in carotenogenic yeasts such as *Rhodotorula* or *Phaffia* species (Frengova and Beshkova, 2009). Spirilloxanthin has two methoxy groups and a fully desaturated polyene chain and is frequently found in the purple photosynthetic bacterium *Rubrivivax gelatinosus* (Steiger et al., 2000). Unusual shorter carotenoids, C_{30} staphyloxanthin from *Staphylococcus aureus* and glucosylated C_{50} carotenoid decaprenoxanthin from *Corynebacterium glutanicum*, were also recently identified (Heider et al., 2012).

3. Carotenoid biosynthetic pathway in plants: an overview

The enzymes that encode for carotenoid production are predominantly localized in the plastid region. Carotenoid enzymes such as isopentyl pyrophosphate isomerase (IPI), geranylgeranyl pyrophosphate synthase (GGPPS) and phytoene synthase (PSY) accumulate in leaf stroma. The thylakoid membrane possesses enzyme-like phytoene desaturase (PDS), zeta-carotene desaturase (ZDS), lycopene β cyclase (LCY-β) and lycopene ε cyclase (LCY-ε) (Pizarro and Stange, 2009). Shumskaya et al. (2012) reported that the carotenoid biosynthesis pathway occurs in plastids and that lipophilic carotenoids accumulate in envelope/thylakoid membranes and plastoglobuli. Joyard et al. (2009) reported that carotenoid biosynthetic enzymes were identified in the envelope membrane through proteomic analysis of chloroplast subfraction and spectral counting. Joyard et al. (2009) report that carotenoid producing enzymes such as phytoene synthase, phytoene desaturase, lycopene *β* cyclase, ε-ring hydroxylase, and *β*-ring hydroxylase are responsible for the production of *β*-carotene and lutein; zeaxanthin production occurs in the envelope membrane. Zeaxanthin epoxidase is present in both thylakoids and the envelope membrane, whereas the vioxanthin de-epoxidase is restricted to thylakoids. Similarly, neoxanthin synthase has been identified in envelope protein. Carotenoid biosynthesis also results in the production of carotenoid cleavage products such as ABA. Enzymes involved in such signaling products include CCD and NCED, which have also been identified in envelope protein. In 1974, Jeffrey et al. localized carotenoid biosynthesis in the spinach chloroplast envelope (Jeffrey et al., 1974; Shumskaya et al., 2012). However, the localization of carotenoid biosynthesis within the chloroplast, such as in thylakoids and envelope membrane/stroma, is still unclear, requiring in-depth research.

In plants, carotenoids are formed from the common five carbon (C5) unit; isopentyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) are synthesized by the 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway (Lichtenthaler, 1999; Giuliano et al., 2008). Pyruvate combines with glyceraldehyde-3-phosphate to act as a precursor for IPP formation, catalyzed by 1-deoxy xylulose 5-phosphate synthase (Estevez et al., 2001; Hirschberg, 2001). The three units of *IPP* and one molecule of DMAPP produce geranylgeranyl diphosphate

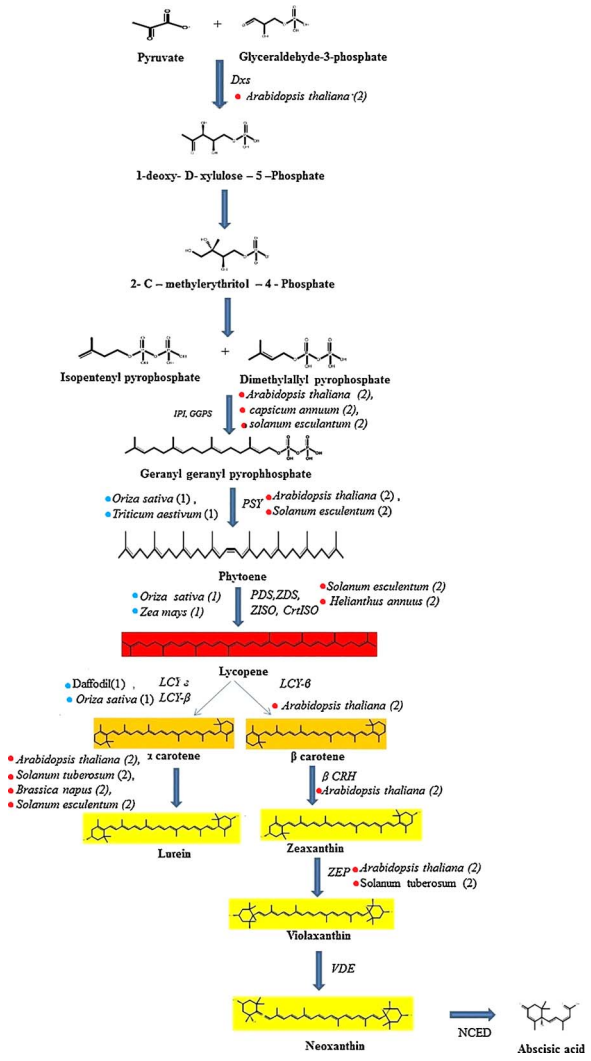

Fig. 1. Carotenoid biosynthesis pathway. Blue and red dots represent monocot and dicot plants, respectively, used in genetic engineering for specific genes. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(GGPP), a 20-carbon molecule catalyzed by GGPP synthase (GGPPS) (Zhu et al., 2010). In the carotenoid synthesis pathway, the first step is condensation of two GGPP molecules into 15-*cis* phytoene, catalyzed by phytoene synthase (PSY) (Cardenas-Conejo et al., 2015; Manoharan et al., 2017). Phytoene is converted into lycopene by the activity of two desaturases, phytoene desaturase (PDS) and zeta-carotene desaturase (ZDS) (Clotault et al., 2008). Two *cis-trans* isomerases, zeta- carotene isomerase (Z-ISO) and carotene isomerase (CRTISO), are essential for the conversion of poly-*cis*-configured phytoene into the *all-trans* form lycopene (Lu and Li, 2008). In the carotenoid biosynthesis pathway, lycopene acts as a branching point that is cyclized to produce *α*-carotene (*β, ε*-carotene) via lycopene *ε*-cyclase (LCY-ε) and lycopene *β*-cyclase (LYC-β) or lead to the production of *β*-carotene via lycopene *β*–cyclase (LCY-β) (Fig. 1).

The carotenoid biosynthetic pathway has been studied in bacterial and plant systems. The genes and enzymes involved in the carotenoid biosynthetic pathway are well studied in bacterial systems such as *Rhodobacter capsulatus, Erwinia uredovora* and *Erwinia herbicola* (Lee and Schmidt-Dannert, 2002). In plant systems such as tomato, the carotenoid biosynthesis has been studied through genetic engineering (Eugenia et al., 2006). Consequently, in this review, we discuss the genes and enzymes involved in carotenogenesis in plants.

4. Plant carotenogenesis

4.1. IPP isomerase

The conversion of isopentenyl diphosphate (IPP) into dimethylallyl diphosphate (DMAPP) is a reversible isomerization reaction catalyzed by IPP isomerase (also called as IDI), which is the first step in regulating the biosynthesis of all isoprenoids. The mevalonic acid (MVA) pathway and the methylerythritol phosphate (MEP) pathway are responsible for the production of IPP/DMAPP. The cytosol-localized MVA pathway converts three acetyl-CoA to IPP via HMG-CoA and mevalonic acid intermediates (Hunter, 2007). The first step of the alternate MEP pathway was discovered by Rohmer in 1996 (Rodriguez-Conception and Boronat, 2002). This pathway is primarily found in prokaryotes and plastids. In 1999, Lichtenthaler described the 1-deoxy-D-xylulose-5-phosphate (DOXP) alternate pathway for IPP biosynthesis in higher plants that produces specific isoprenoids and IPP biosynthesis catalyzed by the *DXS* gene (Lichtenthaler, 1999; Rodríguez-Avila et al., 2011a). The plastid-localized pathway uses pyruvate and G3P for the synthesis of IPP and DMAPP (Cordoba et al., 2009). Two types of isomerases have been characterized, type 1 IPI and type II IPI. Type I IPI isomerase has two isoforms; a long-form localized in plastids and a short one located in cytosols. *IPP* is present in other organelles such as mitochondria and the endoplasmic reticulum. Type II IPI was discovered by Haruo Seto (Kaneda et al., 2001). This enzyme was found in gram-positive bacteria, some archaea, proteobacteria, and cyanobacteria. Type II IPI is different from type I IPI (Berthelot et al., 2012). *IPP* and *DMAPP* are the end products of these two pathways, and IPI regulates the *IPP/DMAPP* pool and flux in isoprenoid biosynthesis (Sivy et al., 2011; Withers et al., 2007). In *Arabidopsis*, the IPP2 enzyme has 284 amino acids with an N-terminal extension. This protein is related to mammalian and bacterial enzymes and has been suggested to localize this enzyme to the chloroplast. The *IPP*1 enzyme has 233 amino acids, lacks the N-terminal extension of *IPP*2 and is located in the cytosolic region. *IPP* isomerase appears to be an unlikely candidate for controlling or regulating isoprenoid biosynthesis. The activity of *IPP* isomerase has been studied using a genetically engineered *E. coli* strain, which has enhanced pigment production (Cunningham and Gantt, 1998). Moreover, improved accumulation of carotenoid pigments content was observed after introduction of *IPP* isomerase cDNA or additional copies of *E. coli* genes for this enzyme in a different plant, algae or yeast (Phillips et al., 2008). This confirms that *IPP* isomerase is involved in carotenogenesis.

4.2. GGPP synthase

The addition of three molecules of *IPP* and one molecule of *DMAPP* undergoes 1′-4 head-to-tail condensation to form the 20-carbon molecule geranylgeranyl diphosphate (*GGPP*) via *GGPP* synthase (Hirschberg, 2001). The reaction begins with the addition of a series of molecules such as DMAPP, GPP (geranyl diphosphate), FPP (famesyl diphosphate) and isoprenoid diphosphate to the IPP growing chain. DMAPP converts into FPP via FPP synthase (FPPS), and FPP leads to the production of GGPP via GGPP synthase (Huang et al., 2010). GGPP synthase acts as a branching point and is responsible for the growth and development of many plants (Okada et al., 2000). These compounds serve as substrates for the production of metabolites such as monoterpenes, sesquiterpenes, diterpenes, sterols, carotenoids, ubiquinones and prenylated protein. In 1992, GGPPS was first identified in the carotenogenic bacteria *Erwinia herbicola by* Math et al. and in plants by Kuntz et al. (Moise et al., 2014). Various GGPPS enzyme isoforms are located in the plastids of plants. The roles and regulation of these isoprenoids reveal the essential role of GGPPS isoforms in plants (Moise et al., 2014). In 1987, Dogbo and Camara isolated GGPP synthase from pepper (*Capsicum annuum*), and the cDNA coding for 369 amino acid-containing transit peptides of approximately 60 amino acids were sequenced (Fraser and Bramley, 2004). Immunolocalization experiments

confirmed the localization of GGPP synthase in the plastid (Kuntz et al., 1992). In stroma, the presence of GGPP synthase leads to carotenoid accumulation (Ruiz-Sola and Rodriguez-Conception, 2012). In 1997, Zhu et al. identified five different cDNA clones in *Arabidopsis* that were similar to the GGPP synthase of pepper. Of the five cDNA clones, two were determined to be *Ggps*1 and *Ggps*5 by functional complementation in *E. coli* (Coman et al., 2014). The *Ggps*1 that encodes for a 371-amino acid residue with a 76-amino acid N-terminal extension is related to bacterial enzymes. The subcellular location of five different GGPP synthase enzymes was studied using synthetic green fluorescent protein (sGFP) in *Arabidopsis* (Qin et al., 2007). RNA–blot and promoter-*β*-glucuronidase (GUS) analyses revealed that these GGPP synthase genes are expressed in an organ-specific manner in *Arabidopsis*, showing that different tissues express each GGPP synthase gene during plant developmental stages and that GGPP is specifically synthesized by organelles (Okada et al., 2000). Beck et al. (2013) studied 12 genes in *Arabidopsis* that encode GGPPS activity, subcellular localization and tissue-specific expression of an entire protein family in *Arabidopsis.* The results suggested that ten genes are responsible for functional proteins that can synthesize GGPP, and their subcellular localization and differential expression patterns suggest subfunctionalization in providing GGPP to specific tissues or in developmental stages or metabolic pathways. The *GGPPS*11 enzyme was essential for the production of most photosynthesis-related isoprenoids, and the *GGPPS*11 protein interacts with enzymes involved in the production of carotenoids, chlorophylls, tocopherols, phylloquinone, and plastoquinone (Ruiz-Sola et al., 2016). These studies emphasize the presence of GGPP synthase gene in the carotenogenic genes, suggesting the major role of this enzyme in carotenoid synthesis.

4.3. Phytoene synthase

Phytoene is the first carotenoid in the carotenogenesis pathway. In the carotenoid pathway, the condensation of two molecules of GGPP leads to the formation of the colorless compound phytoene, catalyzed by two-step reactions involving phytoene synthase (PSY) (Ruiz-Sola et al., 2016). The first step involves two molecules of geranylgeranyl diphosphate, leading to the production of prephytoene diphosphate, which is converted into phytoene (Yang et al., 2014; Yang et al., 2015). *PSY* plays a rate-controlling role in carotenoid synthesis. Consequently, it is highly regulated at transcriptional and post-transcriptional levels. The PSY gene is important for controlling carotenogenesis in different organs at various developmental stages and responds to environmental stimuli such as salt and drought stresses. At the transcriptional level, it responds to abscisic acid, temperature, photoperiod, developmental events, high light, and post-transcriptional feedback (Melendez-Martinez et al., 2015). Different types of PSY genes with tissue-specific expression and unique responses to environmental stimuli have been reported. Phytoene synthase was initially isolated from pepper (Bouvier et al., 2005). In *Oryza sativa,* three types of PSY genes were identified, *PSY1, PSY2,* and *PSY3*. *PSY1* and *PSY2* are involved in phytochrome control and exhibit different expression patterns throughout chloroplast development. The *PSY*3 gene is not affected by light and does not show tissue-specific differences. The gene is upregulated and exhibits increased ABA production under salt and drought stress conditions (Welsch et al., 2008; Moise et al., 2014). In tomato, two genes for PSY have been reported: *PSY*-1 encodes for fruit- and flower-specific isoforms, and *PSY*-2 encodes green tissue formation (Bartley and Scolnik, 1993; Fraser et al., 1999; Flowerika et al., 2016). In maize, three genes are reported: *PSY*1 (expressed in leaves and seed endosperm and characterized by a yellow color), *PSY*2 (expressed in leaves) and *PSY*3 (expressed in roots) (Gallagher et al., 2004; Li et al., 2008a,b; Vallabhaneni and Wurtzel, 2009; Melendez-Martinez et al., 2015). Of the three PSY genes, two are present in rice (*Oryza sativa*), poplar (*Populus trichocarpa*), and bread wheat (*Triticum aestivum*) whereas *Arabidopsis* exhibits only one *PSY* gene that encodes for a 423-amino acid polypeptide (Li et al., 2008a). Additionally, PSY is a rate-limiting enzyme involved in fruit ripening of tomato (Fu et al., 2016), canola seeds (*Brassica napus*) and marigold flowers (Shewmaker et al., 1999; Moehs et al., 2001). The PSY gene has been cloned in maize, pepper, *Arabidopsis* and *Narcissus* (Chaudhary et al., 2010; Howitt et al., 2009; Welsch et al., 2008). PSY acts as a key regulatory enzyme and is critical in the modification of carotenoid levels in crops by genetic engineering.

4.4. Synthesis of lycopene

The carotenoid pigment lycopene is produced from the colorless compound phytoene via two desaturation processes. The conversion of phytoene to all-*trans*-lycopene requires a complex set of reactions involving phytoene desaturase (PDS); 15-*cis*-ζ-carotene desaturase (ZDS); two isomerases, *ζ*-carotene isomerase (Z-ISO) and carotenoid isomerase (CRISTO); and light-mediated photoisomerization (Breitenbach and Sandmann, 2005; Park et al., 2002; Isaacson et al., 2004; Dong et al., 2007). The phytoene yields intermediates such as phytofluene, ζ-carotene, neurosporene and lycopene-containing conjugated double bonds. Enzymes such as phytoene desaturase (PDS) and Z-carotene desaturase (ZDS) catalyze the desaturation of non-colored phytoene into orange phytofluene, pale-yellow ζ-carotene, orange–yellow neurosporene and red lycopene upon exposure to light (Tanaka et al., 2008; Gomez-Garcia Mdel and Ochoa-Alejo, 2013). Carotenogenesis in organs (such as roots and etiolated leaves) under dark conditions suggested the contribution of *ζ*-carotene isomerase in transforming 9,15,9′-tri-*cis*-*ζ*-carotene into 9,9′di-*cis*-*ζ*-carotene. In maize and *Arabidopsis*, the *Z-ISO* gene was isolated, characterized and determined to be necessary for both light-exposed and dark conditions (Chen et al., 2010). ZDS catalyzes two desaturation steps of 9,9′-di-*cis*-*ζ*-carotene to produce 7,9,9′-tri-*cis*-neurosporene and 7,9,7′,9′-tetra-*cis*-lycopene and a carotenoid isomerase (CRISTO), which converts the latter compound into all-*trans*-lycopene (Sandmann, 2009). The PDS gene was identified in cyanobacteria as a carotenogenic enzyme, and the amino acid sequence in plants and cyanobacteria was unusually well conserved (Qin et al., 2007). The PDS cDNA was cloned in soybean, pepper, tomato, and maize, showing that inhibitors of PDS and PDS mutant phenotypes play major roles in carotenoid synthesis. ZDS activity was identified in candidate phytoene desaturase in *E. coli* strains that accumulate *ζ*-carotene. Similar to PDS, ZDS is vital for the synthesis of carotenoids and showed significant sequence homology with PDS. The Z-ISO enzyme is a chloroplast-localized transmembrane protein found in plants and cyanobacteria. The CRTISO gene was identified in cyanobacteria, tomato and *A. thaliana* using various methods for screening carotenoid strains and map-based cloning (Moise et al., 2014). *Arabidopsis and Synechococcus PCC7942* showed 79% and 65% identity and 566- and 474-amino acid similarity, respectively. The ZDS gene was identified in pepper cDNA and was distantly similar to plant PDS (Matthews et al., 2003). In *Arabidopsis,* ZDS encodes 558 amino acids. Both PDS and ZDS were well conserved in N-terminal transit peptide sequences for plastid targeting (Moise et al., 2014). In bacteria and fungi, only one gene, phytoene desaturase (*CRT*1), was involved in catalysis of phytoene into lycopene. During photomorphogenesis, PDS acts as a rate-limiting enzyme. The PDS mRNA was upregulated through the phytochrome-mediated pathway to generate 9,15,9′-tri-*cis*-*ζ*-carotene (Welsch et al., 2000). In *Arabidopsis* variegated mutants, plastid-targeted alternative oxidase (*PTOX*) is essential for phytoene desaturase (PDS) for desaturation in chloroplast electron transport (Yu et al., 2007). In soybean and capsicum, cDNA encoding for the PDS gene was cloned and expressed in a bacterial system for zeta-carotene production (Moise et al., 2014), and the PDS gene from tomato was cloned using an *E. coli* system for *ζ*-carotene formation (Pecker et al., 1992; Martel et al., 2011).

4.5. Lycopene cyclization

Porter and Lincoln proposed lycopene cyclization, which is the first

branching point in the carotenoid biosynthetic pathway (Porter and Lincoln, 1950; Moise et al., 2014). Cyclization of lycopene in the carotenogenic pathway leads to the production of carotenoids with two rings such as β-ring carotenoids (β-carotene and xanthophyll derivatives such as zeaxanthin, violaxanthin and neoxanthin), which occurs on one branch, or ε- ring carotenoids (α-carotene and lutein), which occurs on the other branch (Ruiz-Sola and Rodriguez-Conception, 2012). These isomerization reactions are catalyzed by carotene isomerase (CRTISO). The β-ionone ring formation was catalyzed by lycopene β-cyclase (LCY-β) at the end of the lycopene molecule, yielding β-carotene. The β-carotene was produced through the formation of one ε-ring by lycopene ε-cyclase (LCY-ε) (Krubasik and Sandmann, 2000). In tomato, two types of LCY-β's were differentially expressed, e.g., LCY-β1 was active in green tissues, whereas LCY-β2 was active only in chromoplast-containing tissues such as ripening fruit (Ronen et al., 2000; Zeng et al., 2015). In *Arabidopsis* and tomato, there is 30% identity between the amino acid sequences of LCY-β and LCY-ε. In plants and cyanobacteria, lycopene β-cyclase (LCY-β) catalyzes the production of bicyclic α- and β-carotene (Lu et al., 2016). In *Arabidopsis*, β-cyclase contains 501 amino acids, and ε-cyclase has 524 amino acids. When aligning the sequences of β- and ε-cyclases, the highly conserved but distantly different sequences in the β- and ε-cyclases suggest that the mature plant β- and ε-cyclases may retain 30 − 50 amino acids on the N-terminal sequence upstream of the position coincident with the start of cyanobacterial β-cyclase (Cunningham and Gantt, 1998). In tomato and tobacco, cDNA encoding lycopene β cyclase (*CrtL*) was cloned into the prokaryote *E. coli* (Pecker et al., 1996), and transcriptional down-regulation of lycopene β-cyclase leads to the accumulation of lycopene during fruit ripening (Cardenas-Conejo et al., 2015). The combination of LCY-ε and LCY-β activity is an overall major determinant of flux throughout the branch because it is important for the production of lutein, β-carotene, and xanthophyll cycle carotenoids (Cazzonelli and Pogson, 2010).

The addition of oxygen moieties to cyclic carotenoids leads to the formation of xanthophylls. Similarly, the addition of hydroxyl groups to the β-carotene results in the production of zeaxanthin. α-Carotene leads to the production of lutein, catalyzed by β- and ε-hydroxylases (DellaPenna and Pogson, 2006). In 1996, Cunningham et al. identified a cDNA-encoding enzyme that catalyzes ε-ring formation in *Arabidopsis*. The ε-cyclase adds one ring to the symmetrical lycopene to form monocyclic δ-carotene (Lu et al., 2016). Enzymes such as zeaxanthin epoxidase (ZEP) and violaxanthin de-epoxidase (VDE) control the production of violaxanthin. Zeaxanthin epoxidase (ZEP) and β-cyclohexenyl xanthophyll epoxidase were identified in *Nicotiana plumbaginifolia* by transposon tagging (Sah et al., 2016). β-Cyclohexenyl xanthophyll epoxidase cDNA from pepper was cloned and purified. The pepper and *Nicotiana plumbaginifolia* sequence showed 88% amino acid identity, and the tobacco epoxides contain a 663-polypeptide. Violaxanthin de-epoxidase was obtained from romaine lettuce and contains a 348-amino acid polypeptide. VDE has been sequenced in tobacco and *Arabidopsis* (Zhao et al., 2016). Violaxanthin was converted into 9-*cis*-violaxanthin by neoxanthin synthase. The neoxanthin serves as the precursor for the synthesis of the plant hormone abscisic acid (ABA). The neoxanthin synthase (*NXS*) gene has been cloned in potato and tomato, and the amino acid sequence of NXS from tomato has 99% similarity with the lycopene β cyclase amino acid sequence (Al Babili et al., 2000; Bouvier et al., 2000; Li et al., 2016).

4.6. Carotenoid cleavage products and their apocarotenoids

Carotenoids are cleaved oxidatively to form apocarotenoids by carotenoid-specific cleavage oxygenases (*CCOs*) (Priya and Siva, 2014; Tran et al., 2012), including 9-cis epoxy carotenoid dioxygenases (NCEDs) and carotenoid cleavage dioxygenases (CCDs). The NCEDs are involved in biosynthesis of the plant hormone abscisic acid a cleavage product of 9′-*cis*-neoxanthin and/or 9-*cis*-vioxanthin, which was first proposed by Taylor and Smith in 1967 (Tran et al., 2012). The ABA-deficient mutant was first isolated in maize, viviparous mutant (vp14), and cleaves the 9′-*cis*-neoxanthin via NCED-producing xanthoxin (Liu et al., 2016a,b). The first CCD enzyme was characterized in *Arabidopsis thaliana* and named *AtCCD*1 (Schwartz et al., 2001). *AtCCD*1 was found to cleave several carotenoids at the 9, 10 and 9′, 10′ positions, generating a C14-dialdehyde and two C13-carotenoid end groups (Schmidt et al., 2006). The CCD genes are responsible for the color, flavor, and aroma of fruits and flowers of many plants. CCDs are involved in the synthesis of hormones, inhibiting lateral branching, controlling leaf senescence, root growth and flower development (Schwartz et al., 2004; Snowden et al., 2005; Sergeant et al., 2009). CCD genes and enzymes have been studied in species such as tomato, petunia flowers (Simkin et al., 2004a,b), *Cucumis melo* (Ibdah et al., 2006), *Crocus sativus* (Bouvier et al., 2003), *Rosa damascena* (Huang et al., 2009), Rice (Ilg et al., 2009), *Vitis vinifera* (Lashbrooke et al., 2013), *Chrysanthemum* (Ohmiya et al., 2006), and *Bixa orellana* (Rodríguez-Avila et al., 2011b; Sankari et al., 2016). NCED genes and enzymes have been studied in plant species including *Arabidopsis thaliana* (Iuchi et al., 2001; Lefebvre et al., 2006; Tan et al., 2003), *Citrus clementina* (Agusti et al., 2007), *Daucus carota,* Subspecies*, sativus* (Just et al., 2007), and *Solanum tuberosum* (Beltran et al., 2006).

5. Metabolic engineering of carotenoids to improve nutritional value

Carotenoids play a major role in the nutritional sector due to their antioxidant and anti-aging properties and have significant industrial applications in food coloring, cosmetics, and pharmaceuticals. These applications require molecular-level approaches for the production of carotenoids in plants and microorganisms. Cloning of carotenoid-producing genes involves exploring the controlled regulation of the pathway for opportunities for genetic manipulation of the carotenoid content in plants. The motivation for improving the carotenoid content in plants by genetic manipulation was an overproduction of carotenoids through natural sources or adoption of a chemically synthesized method for human health benefits. The main aim of improving the carotenoid content in food stuff is to reduce dietary carotenoid deficiency and avoid malnourishment in developing countries. Some metabolically engineered carotenoids and their apocarotenoids are listed in Table 1.

The primary reason for implementing biotechnological methods such as genetic engineering was to enhance the nutritional carotenoid value in staple crop plants. Half of the world's population consumes rice daily, which is a poor source of many essential micronutrients and vitamins. Various crop plants have been genetically manipulated by many researchers to increase the carotenoid content (Table 2). Several researchers are working to improve the provitamin content of rice. In 1997, Burkhardt et al., transformed japonica rice cv. Tapei 309 with a cDNA coding for PSY from daffodil under the control of endosperm-specific promoter. The transgenic rice plants expressed an active daffodil enzyme and lycopene accumulation occurred in the endosperm region of rice (Zhai et al., 2016). In transgenic tomato plants, constitutive expression of the PSY gene induced pleiotropic effects such as premature pigmentation of seed coats and cotyledons (Fraser et al., 2002) and reduced gibberellin levels, which redirected the GGPP into phytoene in carotenoid synthesis.

5.1. Metabolic engineering of plant carotenoids to improve stress resistance

To enhance the nutritional content of crop plants, genetic engineering of carotenoid biosynthesis might aid in development of plants with special qualities such as stress tolerance, herbicide resistance, photoprotection and capability of synthesis of novel carotenoids in crop plants. The exposure of plants to high light conditions and UV radiation induces photo-oxidative stress that alters the plant morphology and

Table 1
Metabolic engineering of carotenoid products.

Carotenoids, apocarotenoid, molecular formula	Enzyme responsible for synthesis of carotenoid and EC number	Strategy	Function of the gene	Applications	References
Phytoene ($C_{40}H_{64}$)	PSY (EC-2.5.1.32)	Conala (*Crt-β* over expression)	a, b	A	Shewmaker et al. (1999)
		tomato (*Crt-β* over expression)	b	B	Fraser et al. (2002)
		Arabidopsis thaliana (upregulation of *PSY)*	b,a	C	Von Lintig et al. (1997)
Lycopene ($C_{40}H_{56}$)	PDS (EC-1.3.99.31)	Tomato fruit (*Crt-β* overexpression, *LCY-β* silencing)	C	A	Gerszberg et al. (2015)
	Z-ISO (EC-5.2.1.12) ZDS (EC–1.3.5.6) CRTISO (EC-5.2.1.13)	Tomato fruit (*LCY-β* expression)	D	D	Fraser et al. (2002) Rosati et al. (2000)
β-carotene ($C_{40}H_{56}$)	LCY-β, (EC-5.5.1.19)	Canola seed (*Crt-β, I, Y* overexpression)	d,e	A	Shewmaker et al. (1999)
α-carotene ($C_{40}H_{56}$)	LCY-ε (EC – 5.5.1.18)	Potato tuber (*Crt-β, I, Y* overexpression, *LCY-ε* silencing)	d,e,a	D	Ravanello et al. (2003)
		Rice seed (*PSY, CrtI* overexpression),	d,a	E	Romer et al. (2000)
		Tomato plant (*CRY*2 overexpression)	d	F	Rosati et al. (2000)
		Tomato fruit (*CrtI* overexpression, *LCY-β*, *CHY* overexpression)			
Lutein ($C_{40}H_{55}O_2$)	β-ring hydroxylase (EC – 1.14.99) ε-ring hydroxylase (EC – 1.14.99.45)	Arabidopsis plant (*LCY-ε* overexpression)	f	GA,H	Pogson and Rissler (2000)
Zeaxanthin ($C_{40}H_{55}O_2$)	β carotene hydroxylase (EC – 1.14.13.129)	Potato tuber(*ZEP* silencing) Rice seed (*PSY, CrtI* overexpression)	G	G,H	Romer et al. (2002)
β-cryptoxanthin ($C_{40}H_{55}O$)	β-carotene hydroxylase (EC – 1.14.13.129)	Tomato fruit (*β-LCY* and *β-Chy* overexpression)	d,g,h	D,A	Dharmapuri et al. (2002)
Violaxanthin ($C_{40}H_{55}O_4$)	Violaxanthin de epoxidase (vde) (EC 1.10.99.3)	Arabidopsis seeds (upregulation of *PSY*)	D,f,ID,f,i	G	Lindgren et al. (2003)
		Potato tubers (overexpression of *Crt-β*)			Ducreux et al. (2005)
Neoxanthin($C_{40}H_{55}O_4$)	Neoxanthin synthase (EC 5.3.99.9)	Arabidopsis (over expression *NSY*/ABA4 gene)	J	I	North et al. (2007)
Bixin ($C_{25}H_{30}O_4$)	Lycopene cleavage dioxygenases aldehyde dehydrogenases (1.2.1.5), norbixin methyl transferases (2.1.1.-)	Engineered *E.coli* (*LCD, ADH, nBMT*)	K	G,J	Bouvier et al. (2003) Siva et al. (2010) Anantharaman et al. (2015) Anantharaman et al. (2016)
Crocetin ($C_{20}H_{24}O_4$)	Zeaxanthin cleavage dioxygenases (1.14.99.42), aldehyde oxydoreductase, Glucosyl transferases	Crocus sativus (expression of *PSY, PDS, LCY-β*)	G	G	Castillo et al. (2005) Bouvier et al. (2003)
Abscisic acid ($C_{15}H_{20}O_4$)	Nine cis epoxy carotenoid dioxygenases (1.13.11.51)	Arabidopsis thaliana (*NCED*3)	L	K,L	Luchi et al. (2001) Priya and Siva (2015)

Function of genes: (a) Enhanced/Increased carotenoid, (b) enhanced/increased phytoene, (c) enhanced lycopene (d) *β*- carotene accumulation (e) α-carotene accumulation, (f) enhanced lutein, (g) Zeaxanthin accumulation (h) *β*- crypoxanthin (i) increased violaxanthin (j) neoxanthin accumulation (k) lycopene induced bixin synthesis (l) Abscisic acid accumulation.
Applications: (A) antioxidant (B) protection against UV damage (C) enhance protection against free radical damage (D) anticancer agent (E) UV-induced radical damage (F) coronary heart disease (G) food colorants (H) age-related macular disorder (I) precursor ABA synthesis (J) cosmetic industries (K) seed dormancy (L) fruit maturation/senescence.

Table 2
Genetic manipulations to increase the carotenoids in plant species.

Plant species	Gene manipulation	Promoter	Estimated carotenoid level	References
Arabidopsis	Endogenous phytoene synthase	1	43-fold increase in *β*-carotene	Lindgren et al. (2003)
	DXS	2	112–131% increase in total carotenoids	Estevez et al. (2001)
	PSY	2	10-fold, 100-fold increase in total carotenoids in seed- derived calli and roots respectively	Maass et al. (2009)
Canola	*Crt-β*	1	50-fold (1600 μg/g) α-and *β*-carotene	Shewmaker et al. (1999)
	Crt-I and *Crt-Y*	4	6-fold (10 mg/g to 60 mg/g)	Diretto et al. (2007)
	ε-LCY	2	43.30%, 44.16%, 11.46% and 0.84% of Lutein, *β*-carotene, violaxanthin and *β*-crypoxanthin was increased	Yu et al. (2008)
	idi, crt-ε, crt-β, crt-l and crt-Y (P. ananatis) crt-Z, crt-W (Brevundimonas sp.)	2	30-fold increase in total carotenoids (412–657 μg/g)	Fujisawa et al. (2009)
Corn	*PSY*	4	146.7 μg/g in total carotenoids	Zhu et al. (2008)
	Crt-β and crt-1	4	34-fold increase in total carotenoids(33.6 μg/g)	Aluru et al. (2008)
	PSY (zea mays), CrtI (E. uredovora)	5	112-fold increase in total carotenoids (163.2 μg/g) and 169 −fold increase in *β*-carotene(59.32 μg/g)	Naqvi et al. (2009)
Potato	*Zep (Arabidopsis)*	6	5.7-fold increase in total carotenoids	Romer et al. (2002)
Sweet potato	*DXS*	7	2-fold increase in carotenoids, 6-fold increase in phytoene	Morris et al. (2006)
	Lyc-ε	7	2.5-fold, 14-fold increase in total carotenoids and *β*-carotene respectively	Diretto et al. (2006)
	Crt-Y and/or *Crt-l*	7,8	20-fold, increase in carotenoid and 3600-fold, increase in *β*-carotene	Diretto et al. 2007
	PSY	6	6.3-fold increase in carotenoid, 19-fold increase in *β*-carotene	Ducreux et al. 2005
	Crt-O (Synechocystis sp)	2	10–12% increase in total carotenoid	Gerjets and Sandmann (2006)
	CHY-β	9	117 l μg/g (dry weight)incease in total carotenoids and 34.43 lg/g (dryweight) in *β*- carotene	Kim et al. (2012a,b)
Soy bean	*PAC (phytoene synthase-2A carotene desaturase)*	1	146 μg/g of total carotenoids (62-fold increase) and 112 μg/g of β-carotene (77%)	Kim et al. (2012a,b)
Rice	*PSY* and β-*LCY*	4,2	1.6 μg/g increase in total carotenoids	Ye et al. (2000)
	PSY gene and Crt1 (Erwinia uredovora)	10	17–23-fold increase in *β*-carotene and total carotenoids(37 μg/g)	Paine et al. (2005)
Lotus japonicas	*crtW* (*Agrobacterium aurantiacum*)	2	1.5-fold increase in total carotenoids (387 μg/g)	Suzuki et al. (2007)
Tomato	Bacterial phytoene synthase gene *crtβ* from *Erwinia*	11	2-fold total fruit carotenoids, 3-fold increase in *β*-carotene	Fraser et al. (2001)
	E.uredovora PDS gene (*crt*1)	2	3-fold increase in total carotenoid	Misawa et al. (1993)
	PSY1	2	1.14- fold increase in total carotenoids	Fray et al. (1995)
	βLcy and *β-Chy*	11	12-fold increase in *β*-carotene. Increased in *β*-cryptoxanthin and zeaxanthin	Dharmapuri et al., (2002)
Tomato	*Lcy-β*	11	3.8-fold *β*-carotene	Rosati et al. (2000)
	Crt-I	12	2-fold increase in *β*-carotene	Romer et al. (2000)
	LCY-β	12	> 7-fold increase in *β*-carotene	D'Ambrosio et al. (2004)
	PSY	11	2–4 fold increase in fruit carotenoids	Fraser et al. (2002)
	DXS (*Bacteria*)	2	1.6-fold increase in total carotenoids and 2.4 and 2.2-fold increase in phytoene and *β*-carotene	Eugenia et al. (2005)
	CRY2	2	1.7-2.0- fold increase in total carotenoids	Giliberto et al. (2005)
Tobacco	*Crt-W* and *Crt-Z* (*Paracoccus sp.*)	8,2	10-fold increase in total carotenoids	Ralley et al. (2004)
Wheat	*PSY* (*zea mays*), *CrtI* (*E.uredovora*)	4,2	10.8-fold increase in total carotenoids	Cong et al. (2009)

Promoters used: 1) seed specific over expression, 2) CaMV35S 3) Coexpression 4) Endosperm- specific promoter 5) Wheat LMW glutelin and barley D-hordien promoter 6) Tuber specific primer 7) Patatin promoter 8) Glutelin Glu01 (Glu) promoter 9) Silencing of *CHY*-β10) Fruit specific 11) Overexpression 12) Constitutive expression of CaMV35S.

causes DNA dimerization (Fu and Shen, 2017). In *Arabidopsis*, overexpression of the *chyβ* gene (occurring in the zeaxanthin biosynthetic pathway) results in a 2-fold increase in the size of the xanthophyll pigment cycle pool; the plants also become tolerant to high light and high temperature. Moreover, *chyβ* overexpression reduced leaf necrosis, production of the stress indicator anthocyanin and lipid peroxidation (Davison et al., 2002). Similarly, in tobacco, the transformation was conducted with the beta-carotene hydroxylase gene under the control of a constitutive promoter, which revealed increased zeaxanthin production and UV stress protection by preventing cell damage (Gotz et al., 2002). This method was used extensively in genetic manipulation of fruit and flower pigmentation, crop plants for production of secondary metabolites for human nutritional needs and other important applications. In sweet potato, Kim et al. (2012a,b) observed that when downregulating β-carotene hydroxylase, the gene expression was altered between the transgenic and non-transgenic calli. The transgenic cultures of sweet potato increased the β-carotene and total carotenoids to 34.43 and 117 μg/g dry weight respectively, and the calli increased the ABA content and induced salt stress. Similarly, in sweet potato (*Ipomoea batatas* L. Lam. Cv. Sinhwangmi), the orange (*Or*) gene was responsible for carotenoid accumulation. The *IbOr* gene was expressed in storage roots of orange-fleshed sweet potato calli. Overexpression vectors such as *IbOr*-Wt and *IbOr*-Ins were transformed into white-fleshed sweet potato (cv. Yulmi (Ym)). Both the transformants showed higher expression levels of β-carotene, lutein, and total carotenoids. When compared to the Ym calli, the transgenic *IbOr* calli showed higher antioxidant activity and increased tolerance to salt stress (Kim et al., 2013). Goo et al. (2015) investigated *IbOr* gene in transgenic sweet potato under different environmental stresses, and the carotenoid level was elevated up to 2.7-fold compared to the non-transgenic control. Chen et al. (2011) observed that the β-lycopene cyclase gene *Se*LCY from *Salicornia europaea*, when transformed into *Arabidopsis*, improved salt stress tolerance.

6. Synthetic biology approaches for carotenoid production

The term synthetic biology was coined by Waclaw Syzbalsky in 1974, meaning to create organisms with new genetic properties (Benner, 2010). Synthetic biology is a developing field that combines engineering principles with plant biology to change the behavior of an organism or engineer them to achieve new functionalities. This emerging field has a significant influence on future agriculture and traditional crop improvement, facilitating novel secondary metabolites production in plants (Liu and Seward, 2015). Synthetic biology

approaches are used to redesign biological systems for innovative applications (Nguyen et al., 2012). The past two decades are referred as the 'post-genomic era' because gene, protein and metabolite data were accrued and specific techniques were established to investigate cellular metabolism. Metabolic engineers are studying individual pathways to manipulate the total cell; this provoked the concept of 'system metabolic engineering' (Kholodenko and Westerhoff, 2004; Park and Lee, 2008; Stafford and Stephanopoulos, 2001). Metabolic engineering was widely used for conventional methods such as deletion or overexpression of genes, introducing heterogeneous genes to control gene expression and modify regulatory networks throughout the cell (Blazeck and Alper, 2010; Klein-Marcuschamer et al., 2007; Tyo et al., 2010).

To generate the 'omic' technologies in larger datasets, mathematical biology has addressed an important component of the workflow. Mathematical approaches such as co-expression analysis, Times series network interference (TSNI), Bayesian analysis and ordinary differential equations (ODE) have been widely utilized. Co-expression analysis normally uses clustering approaches, as biological entities such as genes or metabolites may function similarly if they are functionally related. The degree of similarity could be evaluated by pairwise similarity functions such as the Pearson or Spearman correlation coefficients (Persson et al., 2005). Fraser et al. (2009) demonstrated that correlation analysis between metabolites could be used to identify metabolic changes through a perturbation engineered for the carotenoid pathway. Data were generated for Psy-1 overexpression in fruit from the mature green developmental stages to the ripe fruit stage (Fraser et al., 2007). Nearly 120 metabolites were determined in a quantitative manner using GC–MS and targeted profiling techniques. The metabolites were processed with a control and the heat map showed some correlation between metabolites. The results suggested that the positive correlations occurred among carotenoids and other isoprenoids, except non-plastid derived isoprenoids. The negative correlation between carotenoid biosynthesis and most primary metabolites were observed except for the Calvin cycle. Sectors of metabolism can be recognized that are not directly related to the target pathway but may have the potential to alter levels of targeted products.

Bayesian networks employ a graphical model for probabilistic determination of relationships (Bansal et al., 2007). This approach is achieved using steady-state or time-series dynamic datasets because it is based on the probabilities of the outputs tested through experimentation. In *Catharanthus roseus* cell cultures, this approach was used to determine gene-to-metabolite networks for terpenoid indole alkaloid biosynthesis to identify new objectives for future pathway engineering (Rischer et al., 2006). The ODEs were based on a deterministic approach whereby the relationship of the entities in the network to each other was determined, not based on the estimation of probabilities (Bansal et al., 2007). This approach was used to predict the behavior of the network under diverse conditions and realistic models. Based on knowledge of the components in the pathway, this approach has been extensively utilized in a focused manner. Using an ODE approach, Rios-Estepa et al. (2008) employed a kinetic mathematical model to identify the pattern of monoterpenoid accumulation in peppermint. The TSNI approach is similar to ODE and uses differential equations to characterize the rate of synthesis of network entities as a function of the concentration of every other network component, typically following perturbation (Bansal et al., 2007).

Numerous researchers in the field of metabolism are using synthetic biology approaches. The entire synthetic biosynthetic pathway can be constructed for the production of new compounds. For biosynthesis of plant products, microorganisms act as alternative hosts because of their rapid growth, cost effectiveness and simple purification process to convert them into valuable products. The microbial synthesis of plant natural products can occur through 'precursor-mediated product synthesis', by which researchers can alter the existing host pathway to integrate it into a heterologous pathway or use '*de novo* synthesis' to achieve new biosynthetic routes. Heterologous synthesis is necessary for metabolic engineers to obtain maximum yield production (Chang and Keasling, 2006; Chemler and Koffas, 2008). Using synthetic biology approaches, the terpenoid pathway flux in microbial hosts has been achieved with a 'chromosomal promoter engineering' strategy to express endogenous MEP genes from a bacteriophage T5 promoter in an *E. coli* strain harboring β-carotene biosynthetic genes to enhance the β-carotene production related to the parental strain (Yuan et al., 2006). A similar method has been used to enhance lycopene production in *E. coli* using 'global transcription machinery engineering' on the *rpo*D gene encoding σ^{70}, a primary sigma factor (Alper and Stephanopoulos, 2007; Moses et al., 2013). Similarly, carotenoid production has been enhanced in *E. coli* via expression of the heterologous MVA pathway using IPP and DMAPP intermediates in the MEP pathway. The MVA pathway is grouped to two modules: the first module catalyzes acetyl-CoA to mevalonic acid via an enzyme from *Enterococcus faecalis,* and the second module converts mevalonic acid into IPP and DMAPP via a *Streptococcus pneumonia* enzyme. Using this combination, the *E. coli* strains contained 465 mg/L of β-carotene (Yadav et al., 2012).

Diretto et al. (2007) studied the tissue-specific expression of a synthetic bacterial mini-pathway containing three *Erwinia* genes, such as phytoene synthase *(CrtB),* phytoene desaturase *(CrtI)* and lycopene beta-cyclase *(CrtY)*, under the control of a tuber-specific promoter that led to the production of golden tubers in potato (*Solanum tuberosum*), which contains 3600-fold and 20-fold increases in beta-carotene and other carotenoids, respectively (Liu and Stewart, 2015). The overproduction of the artemisinin precursor artemisinic acid is a good example of engineering secondary metabolite biosynthesis using a synthetic biology approach. The genes and their enzymes that are involved in the artemisinic biosynthetic pathways were taken from *Saccharomyces cerevisiae*, *Artemisia annua,* and *E. coli*, accumulated into two operons and transformed into an *E. coli* host strain. Consequently, some optimization steps were needed to achieve efficient compound production (Ro et al., 2006). Ajikumar et al. (2010) demonstrated the modular synthetic biology strategy in the isoterpenoid pathway. This pathway was engineered for biosynthesis of taxadiene, which is a precursor for the anticancer drug Taxol, achieving approximately 15,000-fold yields in *E. coli.* The major aim of this approach was to identify the side products and overproduction of interesting products. Comas et al. (2016) studied the synthetic biology approach in maize. They combined gene expression and quantitative metabolomics with mathematical modeling to study strategies for improving the carotenoid production yield in endosperm synthesized via alternative biosynthetic pathways in synthetic maize lines. Sensitivity analysis and simulation were used to predict the function of gene activities to further increase the carotenoid production in each line. These prediction systems are applicable across four maize lines, which will increase *Zmlycb/Gllycb*-enhancing β-carotene accumulation (Comas et al., 2016).

This synthetic biology approach is useful for improving the activities of key enzymes for production of desired metabolites for synthetic metabolic pathway construction. Knowledge of plant metabolism, biochemical pathways, and their secondary metabolites are needed to metabolically engineer plants.

7. Computational biology-aided experimental biology for carotenoid production

Bioinformatics and computational biology are essential components of new technical and scientific developments; the computational tools used for carotenoid production are given in Table 3. It is vital to design computational strategies prior to metabolic engineering, to aid in the production of secondary metabolites (de Lorenzo et al., 2006). In addition, computational biology bridges the gap between *in vitro* and *in vivo* studies (Duschak, 2015). Computational tools have aided in understanding the phylogenetics of the carotenoid pathway (Velasco et al., 2007; Ibdah et al., 2006; Alboresi et al., 2008; Lao et al., 2011;

Table 3
Computational tools to aid in carotenoid synthesis.

Aid of Computational tools in Experimental Biology	Computational Tools	References
Comparative expression profiles		Koul et al. (2016)
Metabolic engineering (In silico gene knock out, Gene Amplification Targets, incorporation of enzymes into a functional circuit using DNA construct) and Protein Designing	MOMA	Liu et al., (2016a,b); Asadollahi et al. (2009) and Copeland et al. (2012)
	FSEOF TinkerCell GenoCAD	
Conserved motif analysis	ClustalW, ClustalX	Moreno et al. (2013) and Lao et al. (2011)
Sequence similarity using Molecular phylogenetic analysis	PhyML,	Velasco et al. (2007); Ibdah et al. (2006); Alboresi et al. (2008); Lao et al. (2011) and Klassen et al. (2010)
	Clustal W, BioEdit, Mega Software, Blastp	
Physical and Chemical Characterization of	ProtParam	Lao et al. (2011)
Secondary structure prediction And 3D structure prediction	PSIPRED, 3D-JIGSAW	Lao et al. (2011)
Spilicing and Exon Prediction	NCBI-splign, GENESCAN	Lao et al. (2011)
ADMET predictions		Sathya and Gopalakrishnan (2013)
Codon Bias and Codon optimization	Gene Designer	Copeland et al. (2012)
DNA visualization, organization, assembly, and alignment features. Geneious users can annotate sequences to highlight features such as promoter sites, origins of replication, or restriction sites	Geneious	Copeland et al. (2012)
Identification of gene deletions and mutations	Stoichiometric model and metabolic network models	Wang et al. (2014)

Klassen et al., 2010; Priya and Siva, 2014) and in identification of motif region sequence similarity splicing and exons (Moreno et al., 2013; Lao et al., 2011). The computational field has also aided in understanding the molecular mechanism at genomic and proteomic levels. Metabolic engineering techniques have been aided by tools such as TinkerCell (Chandran et al., 2009) and GenoCAD (Coll et al., 2016). These tools help in identifying gene knockout methods, gene amplification, and DNA construct strategies (Liu et al., 2016a,b; Asadollahi et al., 2009; Copeland et al., 2012). At the proteomics level, analysis of the secondary and tertiary structure of the protein using in silico tools such as PSIPRED (McGuffin et al., 2000) and Modeller v9.7 (Sali et al., 1995) has been conducted to investigate carotenoids (Lao et al., 2011). Copeland et al. (2012) and Priya et al. (2016) used relative codon bias (RCB) and codon adaptation index (CAI) strategies to analyze gene expression and mutation levels in carotenoids. The development of new techniques has provided opportunities for food production with the nutraceutical requirements for human well-being (Virmani et al., 2013). Interest in carotenoids and specific analytical techniques has been essential in identifying various issues in food composition and processing, bioavailability, bioactivity, and modifications during storage of these components.

8. Carotenoid biotechnology perspectives

Using biotechnological approaches, we can improve carotenoid production yields without using the agronomic performance of plants; carotenoid engineering is beneficial for the nutritive significance of vital crops. Biotechnological perspectives allow for multidimensional approaches and strategies to enhance the nutritional value of various secondary metabolites of different plants such as rice, tomato, maize and banana (Ye et al., 2000; Paine et al., 2005; Aluru et al., 2008; Tanumihardjo, 2010).

Manipulation of the light signal transduction pathway has a major role in altering gene products via alteration in numerous steps and/or pathways; this work also utilizes endogenous plant genes. Application of technology such as tilling allows for the opportunity to breed these high-carotenoid traits into crop plants without the use of GM technology (Slade and Knauf, 2005). The quantitative trait locus (QTL) method is an important approach used to identify the genes responsible for carotenoid accumulation in agricultural plants. Analytical methods are also useful for identification of carotenoids and their metabolites in biological fluids. Additional biotechnological approaches are required for improvement of carotenoid compounds.

Metabolic engineering techniques were integrated with biological knowledge at organizational and functional levels. System biology tools allow for analysis of the candidate pathway genes in a genome and characterization of the germplasm diversity. For a system-level approach, genome-scale metabolic models with the computational tools have been widely used to predict the effects of network modification on cellular function. It is now possible to computationally simulate the important cellular components, from a genome through the expression and function of proteins (Fong, 2014). These simulations might be useful in designing microbial strains with modified functions.

In a future scenario, the combination of molecular, genetic modification and computational approaches such as genomic, metabolic and proteomic knowledge may play a chief role in understanding the nutritional importance of carotenoids and their pigments. Therefore, the molecular-level computational analysis provides a powerful tool to enhance the design and analysis of aspects of metabolic engineering. Researchers can employ these techniques to increase and improve the nutritional content of foods and impact millions of people worldwide.

9. Conclusion

Understanding the molecular mechanisms of biosynthetic carotenoid pathways in plants and cloning of genes involved in these pathways has resulted in the increased production of high-nutritional-value compounds in transgenic plants. However, the biosynthetic pathways of several carotenoids have not been identified. Metabolic engineering and system biology has resulted in significant developments in understanding the pathways, but improvement is necessary due to economic constraints. To aid in this, the computational field has bridged the gap between metabolic engineering and synthetic biology. Advancement in mathematical tools and bioinformatics methods will aid in the design of metabolic engineering strategies that may improve secondary metabolite production. Metabolic engineering studies, gene expression, and profiling have been performed, and, with the development of computational tools such as CAI and RSC, similarity prediction between the known and unknown genes has become possible. Integration of system biology and metabolic engineering may enable

identification of novel pathways. The coming decade promises great excitement and challenges in the field of industrial carotenoid compound production using engineered organisms.

Conflict of interest

All authors declare no competing interest.

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