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Regulation of Carotenoid Metabolism in Tomato

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ABSTRACT

Carotenoids serve diverse functions in vastly different organisms that both produce and consume them. Enhanced carotenoid accumulation is of great importance in the visual and functional properties of fruits and vegetables. Significant progress has been achieved in recent years in our understanding of carotenoid biosynthesis in tomato (*Solanum lycopersicum*) using biochemical and genetics approaches. The carotenoid metabolic network is temporally and spatially controlled, and plants have evolved strategic tactics to regulate carotenoid metabolism in response to various developmental and environmental factors. In this review, we summarize the current status of studies on transcription factors and phytohormones that regulate carotenoid biosynthesis, catabolism, and storage capacity in plastids, as well as the responses of carotenoid metabolism to environmental cues in tomato fruits. Transcription factors function either in cooperation with or independently of phytohormone signaling to regulate carotenoid metabolism, providing novel approaches for metabolic engineering of carotenoid composition and content in tomato.

Key words: carotenoid metabolism, *Solanum lycopersicum* (tomato), fruit, transcription factor, plastid, phytohormone

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INTRODUCTION

Carotenoids are a class of 40-carbon hydrocarbon compounds that can be broadly divided into two subgroups: xanthophylls and carotenes. They are generally orange, red, and yellow pigments synthesized via the general isoprenoid biosynthetic pathway in both dark- and light-grown tissues in plants, such as endosperm, maize kernels, leaves, roots, flowers, and fruits.

Carotenoids participate in a wide range of physiological processes, including plant growth, development, and responses to environmental stimuli. In non-green tissues, carotenoids confer distinctive colors on specialized plastids known as chromoplasts. As accessory pigments in photosynthesis, carotenoids can augment light harvesting and attract pollinators and seed dispersers to brightly colored flowers and fruits (Ronen et al., 2000). In addition, xanthophylls, well-known antioxidants, have been proven to quench the excited status of singlet chlorophyll in photosystem II when exposed to excessive radiation (Muller et al., 2001; Robert et al., 2004). Carotenoids are essential components of human diets, providing precursors for biosynthesis of vitamin A, which is a well-known carotenoid derivative with widespread biological functions (Krinsky and Johnson, 2005). Lycopene, the most abundant carotenoid in ripe tomato, is regarded as a bioactive component with regard to treating chronic diseases and lowering risk of cancer and cardiovascular disease (Sandmann et al., 2006; Ford and Erdman, 2012). Besides their functions as pigments and nutrients, carotenoids are also the precursors of many important volatile flavor compounds in plants, conferring the sensory attribute that can be detected by consumers (Vogel et al., 2010). Carotenoids in plants produce a range of compounds named apocarotenoids under oxidative cleavage, giving rise to volatile compounds that constitute the aromatic components of flowers, fruits, and leaves, as well as the well-known phytohormones such as abscisic acid (ABA) and strigolactones, upon abiotic stress (reviewed by Rolland et al., 2012).

The positive effect of carotenoids in the human diet has triggered numerous attempts to engineer plant products with enhanced carotenoid accumulation, which is not only of agricultural importance but also of scientific interest in terms of chemical, biological, and genetic regulation. Despite detailed knowledge of the carotenoid biosynthetic pathway, information about the regulation of carotenoid metabolism is sparse. Carotenoid metabolism is dependent upon a number of factors besides modulation of the pathway gene expression. A single factor, either an environmental or developmental cue, may activate and regulate a dedicated carotenoid pathway network branch via rate-limiting enzymes. Elucidation of carotenoid metabolism and its regulatory

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network will allow rational engineering of carotenoid to boost plant protection and improve crop quality. Tomato contains a complex mixture of bioactive components, serving as a dietary source of nutrients, such as a mixture of carotenoids, including lycopene, β-carotene, and lutein. The mechanisms controlling carotenoid metabolism during fruit ripening are systematic and sophisticated. The sequenced genomes of tomato have unveiled approximately 35 000 protein-coding genes, which provide the foundation for investigating interactions between the transcription factor network, phytohormone signaling pathways, and other downstream factors affecting carotenoid metabolism (The Tomato Genome Consortium, 2012). In addition, the availability of mutants with single-gene mutation and knockdown transgenic lines influencing carotenoid accumulation makes tomato the ideal system to study carotenoid metabolism (Klee and Giovannoni, 2011). In this review, we summarize the current status of studies on the regulatory network of carotenoid biosynthesis, catabolism, and storage by transcription factors and phytohormones in tomato.

BIOSYNTHESIS AND CATABOLISM OF CAROTENOID

Significant progress has been made in understanding carotenoid biosynthetic reactions, the isogenes for specific biosynthetic steps, and their regulations. The composition and content of carotenoids are determined partially by genetic fluctuations. The engineering of carotenoid biosynthetic and catabolic isogenes, as well as their regulations by transcription factors, are addressed in this section (Figure 1).

Carotenoid biosynthesis depends on the supply of building blocks, isopentenyl diphosphate (IPP), and its isomer dimethylallyl diphosphate (DMAPP), in higher plants (Chappell et al., 1995). Plants have two distinct routes for IPP and DMAPP biosynthesis: the mevalonic acid (MVA) pathway in the cytosol and the methylerythritol 4-phosphate (MEP) pathway in plastids (Lichtenthaler, 1999; Eisenreich et al., 2001). The reaction chains of carotenoid biosynthesis occur in plastids, and carotenogenesis is dependent on precursors produced via MEP pathway (Milborrow and Lee, 1998). Furthermore, the MEP pathway is also associated with the synthesis of isoprene, diterpenes, the side chains of the chlorophylls, other key photosynthesis-related compounds (plastoquinones, phylloquinones, and tocopherols), hormones (gibberellins, ABA, and strigolactones), and monoterpenes (Lichtenthaler et al., 1997; Milborrow and Lee, 1998; Matusova et al., 2005).

The biosynthesis of linear C40 lycopene from geranylgeranyl diphosphate (GGPP) is one of the most extensively studied pathways in tomato. All the carotenoid biosynthetic enzymes are located in the plastid, even though the genes are encoded by the nuclear genome (Figure 1; Davies, 2009). The bottlenecks in carotenoid biosynthesis have been explored by analyzing the transcript-level alterations in carotenoid biosynthetic genes and their potential correlation with changes in carotenoid content. During tomato fruit ripening, the transcripts of genes encoding 1-deoxy-D-xylulose 5-phosphate synthase (SlDXS), geranylgeranyl pyrophosphate synthase (SlGGPPS), phytoene synthase (SlPSY), phytoene desaturase (SlPDS), ζ-carotene desaturase

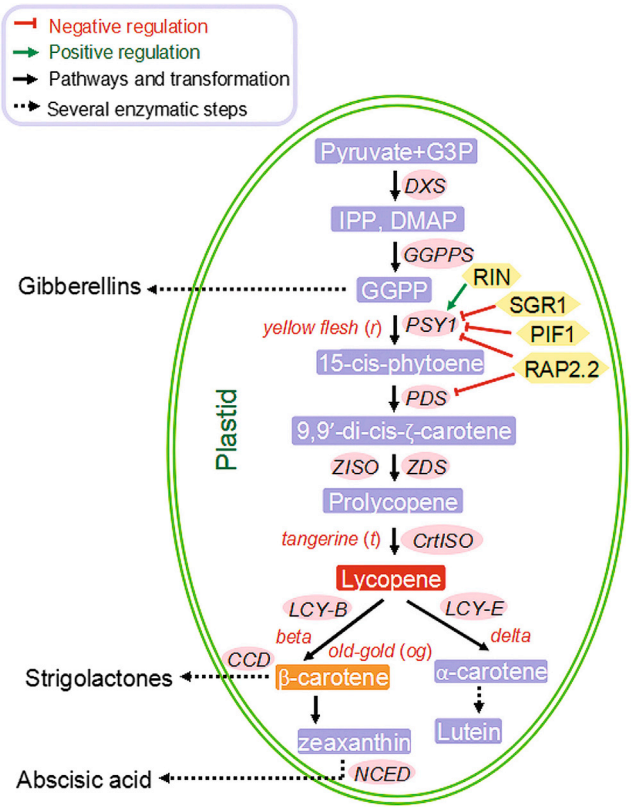

Figure 1. Engineering of Carotenoid Biosynthesis and Catabolism.

Mutants related to carotenoid biosynthesis are indicated in italic red type. The rounded rectangles represent the substrates involved in carotenoid biosynthesis. The pink ellipses represent carotenoid pathway genes localized in plastids. The hexagons represent transcription factors regulating carotenoid biosynthesis. *CCD*, *carotenoid cleavage dioxygenases*; *CrtISO*, *carotene isomerase*; DMAPP, dimethylallyl diphosphate; *DXS*, *1-deoxy-d-xylulose 5-phosphate synthase*; G3P, glyceraldehyde 3-phosphate; GGPP, geranylgeranyl diphosphate; *GGPPS*, *geranylgeranyl pyrophosphate synthase*; IPP, isopentenyl diphosphate; *LCY-B*, *lycopene β-cyclase*; *LCY-E*, *lycopene ε-cyclase*; *NCED*, *9-cis-expoxycarotenoid dioxygenases*; *PDS*, *phytoene desaturase*; PIF1, phytochrome interacting factor 1; *PSY1*, *phytoene synthase*; RAP2.2, related to ap2; RIN, ripening inhibitor; SGR1, stay-green; *ZDS*, *ζ-carotene desaturase*; *ZISO*, *ζ-carotene isomerase*.

(SlZDS), and carotene isomerase (SlCrtISO) are up-regulated and contribute to the formation of lycopene (Giuliano et al., 1993; Fraser et al., 1994; Corona et al., 1996; Pecker et al., 1996). On the contrary, expression of lycopene ε-cyclase (SlLCY-E/CRTL-E) gene and lycopene β-cyclase (SlLCY-B/CRTL-B) gene is dramatically decreased at the breaker stage of ripening (Pecker et al., 1996; Ronen et al., 1999).

The putative initial regulatory step of carotenoid biosynthesis during early fruit ripening is catalyzed by SlDXS. Studies have revealed organ-specific and developmental regulation of tomato *SlDXS* gene expression and a strong correlation with carotenoid accumulation during fruit development (Lois et al., 2000). Two structurally distinct, differentially regulated *SlDXS* isogenes (*SlDXS1*, *SlDXS2*) exist in plants (Walter et al., 2002). Strong

lycopene formation during tomato fruit ripening turned out to be strictly correlated with the expression of *SlDXS1* but not *SlDXS2*. However, *SlDXS2* transcripts are abundant in young tomato leaves, petals, and isolated trichomes (Paetzold et al., 2010). Elevated levels of carotenoids have been found in tomato overexpressing *Escherichia coli DXS* driven by the *FIBRILLIN* (*FIB*) promoter (Enfissi et al., 2005). Two divergent *SlGGPPS* isoforms (*SlGGPPS1*, *SlGGPPS2*) are also found in tomato. The *SlGGPPS1* gene is predominantly expressed in leaves, whereas *SlGGPPS2* is specific for chromoplasts in flowers and/or fruits (Ament et al., 2006).

Identification of mutants altered in carotenoid biosynthesis provides new insights into the regulation of carotenoid metabolism. Two tomato mutants related to lycopene biosynthesis are available: *yellow flesh* (locus *r*), a loss-of-function mutant of the *SlPSY1* gene (Fray and Grierson, 1993), and *tangerine* (locus *t*), a mutation in the *SlCrtISO* gene (Isaacson et al., 2002). The yellow-flesh phenotype of the mutant fruits is associated with a lemon-yellow flower corolla, pale-yellow fruit flesh, and more intensely yellow-colored fruit skin. The recessive mutation *yellow flesh* causes a complete lack of carotenoids in the ripe fruit, and overexpression of *SlPSY1* restores carotenoid synthesis (Fray and Grierson, 1993). SlPSY, a key control point during carotenoid biosynthesis in tomato fruits, catalyzes the condensation of two GGPP molecules to produce 15-*cis* phytoene, which is regarded as the major bottleneck in carbon flux to carotenoids (Fraser et al., 2002). SlPSY is the best-studied enzyme and gene family within the plant carotenoid metabolism pathway (Figure 1). Moreover, the SlPSY-catalyzed step has often been the target of attempts to increase carotenoid accumulation in tomato fruits. There are three *SlPSY* genes in tomato: *SlPSY1*, present in very low abundance in leaves, moderate in petals, and extremely high in fruits at the pink and mature-red stages; *SlPSY2*, reported to be expressed in all tissues with the highest level in yellow petals; and *SlPSY3*, found in the tomato genome and predicted to regulate root carotenogenesis induced by abiotic stress, which is consistent with carotenoid-derived ABA and strigolactones production upon abiotic stress (Bartley and Scolnik, 1993; Fraser et al., 1999; Li et al., 2008). Transgenic tomatoes repressing *SlPSY1* by expressing its antisense gene result in only 3% of the normal carotenoid content in the ripe fruit and yellow coloration at maturity (Ray et al., 1992). A *SlPSY1* knockout mutant was identified through Targeting Induced Local Lesions IN Genomes (Gady et al., 2012). The mutant fruit shows yellow flesh on account of the absence of carotenoids, confirming that SlPSY1 is the only candidate enzyme involved in introducing substrate into the carotenoid biosynthesis pathway during fruit ripening. Trials with constitutive overexpression of *SlPSY*1 in tomato have led to detrimental phenotypes, showing dwarfism, altered pigmentation of leaves and flowers, and yellow-colored fruit devoid of carotenoids. It is caused by redirecting GGPP pools for the gibberellin biosynthetic pathway to unscheduled carotenoid formation or by cosuppression effects with SlPSY activity being down-regulated instead of increased (Fray and Grierson, 1993). Interestingly, a bacterial phytoene synthase (*crt*B) was expressed in a fruit-specific manner, which overcame the detrimental pleiotropic effects and generated a high carotenoid content in tomato fruit (Fraser et al., 2002). Furthermore, the construction of transgenic lines overexpressing *SlPSY1* gene significantly increased the carotenoid content in tomato fruit (Fraser et al., 2007). In other tissues, an APETALA2/Ethylene Response Factor–type transcription factor, and phytochrome interacting factor 1, a basic helix–loop–helix transcription factor, have been shown to reduce carotenoid accumulation by binding specifically to the *AtPSY* and *AtPDS* promoter (Welsch et al., 2007; Toledo-Ortiz et al., 2010). In fruit tissues, the MADS box transcription factor, ripening inhibitor (RIN), has been confirmed to regulate carotenoid accumulation by interacting with the *SlPSY1* promoter (Martel et al., 2011). The *STAY-GREEN* (*SGR*) gene is a positively regulated target of RIN, and its product plays a critical role in regulating chlorophyll degradation in tomato leaves and fruits (Hortensteiner, 2009; Fujisawa et al., 2013). Interestingly, SlSGR1 was found to interact directly with SlPSY1 to inhibit its activity during fruit maturation, and therefore, repression of *SlSGR1* significantly increases lycopene and β-carotene accumulation (Luo et al., 2013). Tomato *tangerine* mutant showed orange fruits, yellowish young leaves, and pale flowers due to the accumulation of prolycopene instead of all-*trans*-lycopene, and map-based cloning of the *tangerine* locus of tomato identified *CrtISO*, which encodes an authentic carotenoid isomerase that functions during carotenoid desaturation (Isaacson et al., 2002). Fantini et al. (2013) investigated the functions of ζ-carotene isomerase (*ZISO*), *CrtISO-Like1*, and *CrtISO-Like2* by virus-induced gene silencing. Three metabolic units were identified in the tomato fruit, which are composed of PSY1, PDS/ZISO, and ZDS/CrtISO, catalyzing the synthesis of 15-*cis*-phytoene, 9,9′-di-*cis*-ζ-carotene, and all-*trans*-lycopene, respectively. The disappearance of all-*trans*-ζ-carotene and increased content of lycopene isomers were observed in *CrtISO-Like1*/*-Like2*–silenced fruits, demonstrating that CrtISO-Like1 and CrtISO-Like2 play active roles in a metabolic branch producing all-*trans*-ζ-carotene.

The cyclization of lycopene is a central branch point in the carotenoid biosynthetic pathway (Figure 1). One route leads to β-carotene, zeaxanthin, violaxanthin, and neoxanthin, providing precursors for the synthesis of ABA and strigolactones. The alternative pathway leads to α-carotene and lutein. Thus, the goal of controlling carotenoid composition can be achieved by regulating these two types of lycopene cyclizations. The bifurcation point of these two types of lycopene cyclizations is governed by SlLCY-B and SlLCY-E. There are two *SlLCY-B* genes in tomato: *SlLCY-B1* (*SlCRTL-B*) is active in green tissues and flowers and *SlLCY-B2* (*SlCYC-B*) is chromoplast-specific (Ronen et al., 2000). A high level of β-carotene accumulates in the fruit of *beta*, which is caused by the up-regulation of *SlLCY-B2* (Ronen et al., 2000). Conversely, null mutation of *SlLCY-B2* leads to the abolishment of β-carotene and an increase in lycopene content, and therefore the related mutant *old-gold* (*og*) has deep-red colored fruits (Ronen et al., 2000). The *delta* mutant with orange-colored fruits contains elevated levels of monocyclic δ-carotene, and the transcript level of *SlLCY-E* in *delta* increases by 30-fold during fruit ripening (Ronen et al., 1999).

Carotenoid accumulation is an outcome of its synthesis and degradation. A metabolic equilibrium between biosynthesis and catabolism of carotenoids is essential for maintaining their content and composition in photosynthetic tissues (Beisel et al.,

2010). Apocarotenoids are either non-enzymatically formed by reactions initiated by reactive oxygen species or produced by enzymatic degradation of carotenoids (Yanishlieva et al., 1998). Enzymatic steps have recently attracted increased interest with the discovery of a new class of apocarotenoid phytohormones, strigolactones (Morris et al., 2001). The carotenoid cleavage enzymes include four carotenoid cleavage dioxygenases (CCDs) and five 9-*cis*-epoxycarotenoid dioxygenases (NCEDs) in *Arabidopsis* (Auldridge et al., 2006a; Vogel et al., 2008; Huang et al., 2009). *CCD1* expression level was reported to be correlated to ripening with a concomitant decrease in lutein content in strawberry (Garcia-Limones et al., 2008). Investigation on the activity of SlCCD1B and its homolog, SlCCD1A, provides direct evidence for the 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 the vast majority of tomato isoprenoid volatiles (Ilg et al., 2014). CCD7 and CCD8 are associated with strigolactone biosynthesis, while NCED2, NCED3, NCED5, NCED6, and NCED9, which catalyze the formation of xanthoxin and apocarotenal from 9-*cis*-violaxanthin or 9′-*cis*-neoxanthin, are associated with ABA biosynthesis (Auldridge et al., 2006b; Walter et al., 2010). Fruit-specific RNAi-mediated suppression of *SlNCED1* produces deep-red fruit with reduced *SlNCED1* transcription and ABA biosynthesis, as well as increased accumulation of lycopene and β-carotene (Sun et al., 2012).

Beyond their functions as colorants and nutrients, carotenoids also produce important volatile organic compounds (VOCs) as essential positive contributors to fruit ripening (Vogel et al., 2010). Sixteen volatile compounds of more than 400 detectable VOCs positively contribute to the flavor of ripe tomato fruits (Tieman et al., 2012). Tomato fruit volatiles can be derived from carotenoids, fatty acids, terpenoids, and amino acids (Vogel et al., 2010; Klee and Giovannoni, 2011). Those carotenoid-derived VOCs produced by the cleavage activity of carotenoid dioxygenase or non-enzymatically formed by oxidative cleavage of linear and cyclic carotenoids (Schwartz et al., 2001; Vogel et al., 2008) affect the perception of sweetness in tomato fruits (Baldwin et al., 2008; Vogel et al., 2010). The *tangerine* mutant with a deletion in the *SlCrtISO* gene exhibits lower levels of carotenoid-derived VOCs (Isaacson et al., 2002). The *old-gold* mutant with repressed *SlLCY-B* expression also showed reduced levels of VOCs derived from carotenoids (Vogel et al., 2010).

THE BIOSYNTHETIC CAPACITIES OF THE PLASTID

Carotenoids are formed and sequestered at very high levels in plastids, mainly chloroplasts and chromoplasts (Egea et al., 2010). In green chloroplast-containing tissues, carotenoid biosynthesis mostly takes place in the envelope and the thylakoid membrane, and xanthophylls are predominantly produced to perform photosynthetic functions (Deruere et al., 1994; Vishnevetsky et al., 1999; Joyard et al., 2009). However, specific carotenoid in the form of lycopene crystalloid is accumulated at a high level in membrane-shaped structures in chromoplast-containing tissues during tomato fruit ripening accompanied by breakdown of the thylakoid membrane, the appearance of carotenoid-containing crystalloids, the synthesis of new membranes of sites of formation of carotenoid crystals, as well as an increase in the number and size of plastoglobules (Harris and Spurr, 1969; Simkin et al., 2007). ZDS, LCY-B, and two β-carotene β-hydroxylase proteins were detected in the proteome of pepper (*Capsicum annuum*) plastoglobules, while ZDS was identified in the proteome of tomato chromoplasts (Barsan et al., 2010). There is evidence that tomato fruits with more active chloroplasts at the green stage can develop ripe tomatoes with more active chromoplasts, producing higher amounts of carotenoids (Isaacson et al., 2002; Galpaz et al., 2008; Nashilevitz et al., 2010). Therefore, the biosynthetic capacity of carotenoids in a given cell also determines the level of carotenoid accumulation as well as the biosynthesis rate. Carotenoid accumulation in extremely high concentrations is accompanied by changes in the plastid's size and anatomical structure, as well as plastid division, differentiation, and cell enlargement (Figure 2).

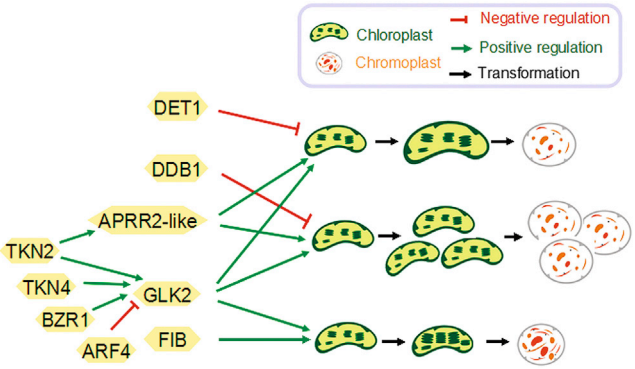

Figure 2. Engineering of the Biosynthetic Capacities of the Plastid to Improve Plastid Size, Number, and Thylakoid Stack.
The hexagons represent transcriptional factors regulating plastid development in tomato. APRR2-like, *Arabidopsis* pseudo response regulator2-like; ARF4, auxin response factor 4; BZR1, brassinazole resistant1; DDB1, UV-damaged DNA-binding protein 1; DET1, de-etiolated1; FIB, fibrillin; GLK2, golden2-like; TKN2, knotted1-like homeobox2; TKN4, knotted1-like homeobox4.

The enhanced chromoplast number and size are responsible for the significant enrichment of carotenoids in tomato high-pigment mutants. The tomato mutant *high pigment 2* (*hp2*) possesses relatively larger plastids and a higher level of pigment with the mutation in *DE-ETIOLATED1* (*DET1*), whose product is a negative regulator of light signal transduction (Mustilli et al., 1999; Kolotilin et al., 2007). The plastid number increases in fruits of the *hp1* mutant, resulting in elevated carotenoid pigmentation (Liu et al., 2004). Interestingly, *hp1* has been identified as a mutation in a tomato UV-damaged DNA-binding protein 1 (*DDB1*) homolog, and its counterpart in *Arabidopsis* interacts with DET1 (Bernhardt et al., 2006). The tomato *hp3* mutant possesses an enlarged plastid compartment, producing mature fruits with more carotenoids (Galpaz et al., 2008). Recently, a transcription factor related to the *ARABIDOPSIS PSEUDO RESPONSE REGULATOR2*-like (*APRR2-like*) gene was identified, and increases in the plastid number, area, and carotenoid

content were observed in transgenic tomato fruit overexpressing tomato *SlAPRR2-Like* gene driven by the *35S* promoter (Pan et al., 2013). The fibrillin protein is implicated in the formation of plastoglobules and fibrils in certain chromoplasts, and an *FIB* gene was expressed in tomato fruit to examine its effect on differentiating chromoplasts. The delayed loss of thylakoids in differentiating chromoplasts, increased carotenoid content (particularly lycopene and β-carotene), and carotenoid-derived flavor volatiles were observed in transgenic fruits (Simkin et al., 2007).

The intensity and pattern of plastid and chlorophyll distribution in tomato fruit are controlled by the dominant *Uniform ripening* (*U*) locus, which has recently been identified by positional cloning (Powell et al., 2012). *U* encodes a GOLDEN2-LIKE (SlGLK2) transcription factor, a member of the glutamic acid-rich protein (GARP) family belonging to the MYB transcription factor superfamily in plants (Waters et al., 2008; Waters et al., 2009). The green-shoulder phenotype of green *U*/*U* tomato fruit seems to result from a latitudinal gradient of *GLK2* expression independent of the ripening gradient (Nguyen et al., 2014). *SlGLK* is the closest global relative of *SlAPPR2-like* in tomato (Fitter et al., 2002). Tomato contains two *SlGLKs* (*SlGLK1* and *SlGLK2*), which function redundantly to regulate the expression of chloroplast genes and promote chloroplast development (Fitter et al., 2002; Waters et al., 2008; Waters et al., 2009). The introgression of the loss-of-function *u* mutation (*u*/*u*) into tomato fruits converts tomato dark-green–shouldered immature fruits into uniform pale fruits with less pigment accumulation. Interestingly, in the fruit of the *u* mutant, the rest of the fruit's needs might be satisfied by recruiting the *SlGLK1* gene, which plays a role in vegetative tissues. The *u* fruit is attractive to both merchants and consumers since the uniform phenotype facilitates the determination of optimal timing for harvest and provides better exterior quality, making it a widely selected tomato variety. However, tomato carrying the *u* mutation produces fruit with defective chloroplasts, lower sugar and lycopene levels. Adding *SlGLK2*, either by crossing or by genetic engineering under a different promoter, results in tomato fruit with more chloroplasts containing more grana/thylakoids, a higher level of chlorophyll and starch at the mature green stage, and more sugars and lycopene at the red ripe stage (Powell et al., 2012). It was found recently that TKN2 and TKN4, the class I KNOTTED1-like homeobox transcription factors, act upstream of *SlGLK2* and/or *SlAPRR2*-like to establish a gradient of chloroplast development (Nadakuduti et al., 2014). Transcript profiling analysis shows that GLK2 acts independently of DDB1, while GLK and DDB1 play additive roles in the regulation of fruit quality (Nguyen et al., 2014). Furthermore, the general ripening systems controlled by RIN, colorless non-ripening (CNR; SQUAMOSA promoter-binding protein-like family transcription factor), tomato agamous-like (SlTAGL1), and ethylene (ET) seem not to be involved in the GLK2-regulated gradient (Nguyen et al., 2014).

The regulation of chromoplast biogenesis plays a crucial role in providing a site not only for active carotenoid biosynthesis but also for carotenoid storage (reviewed by Li and Yuan, 2013). Therefore, regulation of chromoplast differentiation opens up a way to enhance carotenoid accumulation and to improve other quality traits of tomato fruit (Figure 2).

ENVIRONMENTAL REGULATION OF CAROTENOID METABOLISM

The carotenoid metabolism in tomato fruit can be influenced by both the genetic potential and environmental factors, such as radiation intensity, CO_2 concentration, and temperature (Figure 3). Phytochromes are light receptors involved in response to red light and far-red light, but only red light can activate the protein to induce a physiological response (Quail, 2002). Phytochromes have been implicated in regulating the extent of lycopene accumulation in tomato fruit (Alba et al., 2000). When pericarp discs from tomato fruit at breaker stage were incubated in darkness, darkness interrupted by red light, or red light followed by far-red light, darkness/red light-treated discs with enhanced activity of SlPSY accumulated higher levels of carotenoids than darkness- and red light/far-red light-treated discs (Schofield and Paliyath, 2005). Cryptochromes are blue-light photoreceptors found in plants as well as bacteria and animals. Transgenic tomato plants overexpressing *SlCRY2* show a phenotype with higher levels of pigment, including the overproduced anthocyanin and chlorophyll in leaves, as well as flavonoids and lycopene in fruits accompanied by decreased expression of *SlLCY-B* (Giliberto et al., 2005). RNAi-mediated repression of the negative regulator genes of light signaling, *SlDDB1*, *SlDET1*, and *CONSTITUTIVELY PHOTOMORPHOGENIC 1 like* (*SlCOP1-like*), increased lycopene content in tomato fruits, whereas silencing of the positive regulator *LONG HYPOCOTYL 5* (*SlHY5*) gene showed the opposite effect (Davuluri et al., 2004; Liu et al., 2004; Davuluri et al., 2005).

Among the environmental factors that influence the metabolism of carotenoid, moderate stress is especially effective without inducing senescence or necrosis (Poiroux-Gonord et al., 2010). The idea that stress affects the metabolism of carotenoids in tomato fruit makes sense because many carotenoids are antioxidants, and some carotenoids can dissipate excess absorbed energy in the xanthophyll cycle (Mozzo et al., 2010; Garcia-Plazaola et al., 2012). Environmental factors might offer excellent prospects for enhancing carotenoid accumulation to alleviate the negative attitude toward genetically modified food.

Photo-oxidative stress occurs when the absorbed energy exceeds the capacity of the plant photosynthetic apparatus (Jarvis and Lopez-Juez, 2013). When tomato fruit is exposed to high irradiance and high temperature, oxidative stress occurs, which may result in fruit plastid bleaching and impaired shoulders of the ripening fruit (Cocaliadis et al., 2014). The phenotype of *u* fruit might be due to the oxidative stress induced in very active fruit chloroplasts as they become overheated to cope with high irradiation. Oxidative stress in tomato fruit increases coordinately with fruit ripening and reaches a peak at the final stages, which facilitate metabolic changes and fruit softening (Jimenez et al., 2002). High amounts of liposoluble antioxidant compounds, such as lycopene and β-carotene, accumulate to protect the photosystems and fruit cells (Dall'Osto et al., 2013). It should be possible to improve the fruit nutrients by enhancing fruit plastids (chloroplasts and chromoplasts) at different stages of fruit development, as well as balancing light absorption and redox protection (Cocaliadis et al., 2014).

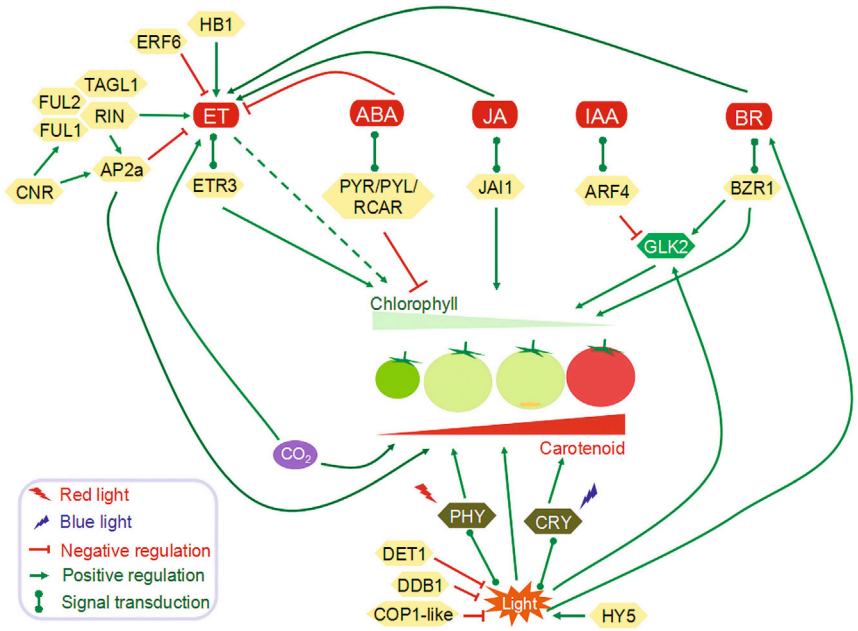

Figure 3. Roles of Plant Hormones, CO_2, Light, and Their Interplay in Carotenoid Accumulation During Tomato Fruit Ripening.

The hexagons represent transcriptional factors and signaling components. ABA, abscisic acid; AP2a, apetala2; ARF4, auxin response factor 4; BR, brassinosteroids; BZR1, brassinazole resistant1; CNR, colorless non-ripening; COP1, constitutively photomorphogenic 1; CRY, cryptochrome; DDB1, UV-damaged DNA-binding protein 1; DET1, de-etiolated1; ERF6, ethylene response factor 6; ET, ethylene; ETR3, ethylene receptor 3; FUL1, fruitfull1; FUL2, fruitfull2; GLK2, golden2-like; HB1, an HD Zip homeobox protein; HY5, hypocotyl 5; IAA, indole-3-acetic acid; JA, jasmonic acid; JAI1, jasmonic acid-insensitive-1 (Li et al., 2003b); PHY, phytochrome; PYR/PYL/RCAR, pyrabactin resistance1/pyr1-like/regulatory components of ABA receptors; RIN, ripening inhibitor; TAGL1, tomato agamous-like.

Temperature has a significant influence on the growth and development of tomato fruits. It has been reported that a high temperature (35°C) can specifically inhibit the accumulation of lycopene by stimulating the conversion of lycopene into β-carotene (Hamauzu et al., 1998). Temperatures below 12°C and above 32°C strongly inhibit and completely block lycopene biosynthesis, respectively (Dumas et al., 2003).

Supply of CO_2, the primary substrate of photosynthesis, is expected to have human benefit by improving the yield. Interestingly, CO_2 enrichment also plays a role in regulating tomato fruit quality (Zhang et al., 2014). The contents of health-promoting compounds, including lycopene, β-carotene, and ascorbic acid, as well as the flavor, indicated by glucose, titratable acidity, and the sugar/acid ratio, are significantly increased in fruits enriched in CO_2. Furthermore, CO_2 enrichment also enhances ET production and organoleptic characteristics, including color, firmness, aroma, and sensory attributes in tomato fruits, indicating that CO_2 enrichment has potential in promoting the nutritional value and organoleptic characteristics of tomato fruits (Figure 3).

HORMONAL REGULATORY NETWORK OF CAROTENOID METABOLISM

The elegant modulation of carotenoid metabolism by both environmental and endogenous signals is tightly coordinated with the developmental changes in tomato fruits. Classic phytohormones, including ET, auxin, and ABA, are involved in the regulation of ripening and carotenoid accumulation in tomato fruits (Figure 3).

Among them, ET plays a central role in fruit ripening, and the effect of ET in regulating carotenoid accumulation during fruit development in tomato has been increasingly reviewed. In tomato fruits, the onset of ripening is triggered by a dramatic increase in ET production, correlating with the rapid accumulation of β-carotene and lycopene, and the expression of *SlPSY1* and *SlPDS* is dependent on ET (Marty et al., 2005). Several transcription factors involved in regulating ET-dependent carotenoid accumulation during tomato fruit ripening have been identified (Figure 3). As a master regulator of ET-regulated tomato fruit ripening, RIN interacts directly with the promoters of ET biosynthetic genes, *ACC SYNTHASE2* (*ACS2*) and *ACS4*, as well as the ET perception gene, *ETHYLENE RECEPTOR 3* (*ETR3*/*NR*) (Martel et al., 2011; Fujisawa et al., 2012, 2013). The corresponding mutant of RIN, *rin*, fails to synthesize climacteric ET and accumulates lycopene in its fruits (Giovannoni et al., 1995; Vrebalov et al., 2002). Additional MADS-domain ripening regulators, TAGL1 and FRUITFULL 1 and 2 (FUL1/TDR4 and FUL2/MBP7) have been found to positively regulate the ET pathway and carotenoid accumulation during ripening (Vrebalov et al., 2009). Inhibition of ET and carotenoid biosynthesis was observed in their corresponding mutants (Itkin et al., 2009; Vrebalov et al., 2009; Bemer et al., 2012; Fujisawa et al., 2014), the *TAGL1* knockdown lines (Itkin et al., 2009; Vrebalov et al., 2009), and the *FUL1*/*FUL2*-suppressed fruits (Bemer et al., 2012; Shima et al., 2014; Wang et al., 2014). TAGL1, FUL1, and FUL2 interact with RIN in a yeast two-hybrid assay, which supports the speculation that MADS box genes function in a combinatorial manner to control fruit ripening (Leseberg et al., 2008; Martel et al., 2011; Bemer et al., 2012; Shima et al., 2013). More data demonstrate the possible tetramers of RIN–TAGL1 with RIN–FUL1/RIN–FUL2. RIN was unable to bind to its targets in the absence of a functional *CNR* allele because of the possibility that the reduced expression of *FUL1* in *cnr* mutant fruits may affect tetramer formation (Eriksson et al., 2004; Martel et al., 2011). SlHB1, an HD-ZIP homeobox protein, directly regulates *1-AMINOCYCLOPROPANE-1-CARBOXYLIC OXIDASE1* (*ACO1*) expression to foster increased ET release and ripening (Lin et al., 2008). Surprisingly,

RNAi-mediated repression of *SlAP2a* resulted in orange-colored ripe fruits with higher levels of ET production and a reduced level of carotenoids, indicating that *SlAP2a* plays a positive role in carotenoid accumulation but a negative role in ET biosynthesis (Chung et al., 2010). These results do not seem to be consistent with the effect of ET on qualitative and quantitative carotenoid constituents. *SlAP2a* may exert an intricate influence on tomato fruit ripening via ET or possibly other pathways independent of ET. Furthermore, RIN and CNR were shown to function upstream of SlAP2, and CNR positively regulates the expression of *SlAP2* by directly binding to its promoter (Chung et al., 2010; Karlova et al., 2011; Fujisawa et al., 2013). ETHYLENE RESPONSE FACTOR 6 (SlERF6), a tomato ET response factor, is also found to be a negative carotenoid modulator. Reduced expression of *SlERF6* by RNAi enhanced both ET release and carotenoid accumulation during tomato fruit ripening (Lee et al., 2012). Six tomato ET receptors, ETHYLENE RECEPTOR 1 (ETR1), ETR2, ETR3 (NR), ETR4, ETR5, and ETR6, have been characterized and isolated, among which, *ETR4*, *ETR6*, and *NR* are the most highly expressed genes in ripening fruits (Klee and Tieman, 2002; Klee, 2004; Klee and Giovannoni, 2011). The role of ET in carotenoid formation is further demonstrated by the tomato ET-insensitive mutant, *Never ripe* (*Nr*), which exhibits the ripening-defective fruit phenotype and fails to accumulate lycopene (Lanahan et al., 1994).

The levels of free indole-3-acetic acid (IAA; the most abundant auxin) decline prior to the onset of tomato fruit ripening, whereas the levels of IAA–Asp (the IAA–amino acid conjugate) increase with its biosynthetic gene, *GH3*, being up-regulated (Bottcher et al., 2010). The above evidence supports a role for IAA in controlling the onset of fruit ripening by modulating the level of its amino acid conjugates (Bottcher et al., 2010). Down-regulation of the tomato *AUXIN RESPONSE FACTOR* 4 (*ARF4*) resulted in a dark-green fruit with increased chloroplasts (Jones et al., 2002). The effect of *ARF4* on fruit plastid accumulation seems to be mediated through the transcriptional up-regulation of *SlGLKs* in tomato fruit, which is consistent with the presence of a perfectly conserved canonical ARF-binding motif (TGTCTC box) found in the promoter region of the *SlGLK1* gene (Sagar et al., 2013).

Additionally, ABA is implicated in tomato fruit development, but knowledge of its role in regulating carotenoid accumulation during tomato fruit ripening is limited. A pyrabactin resistance1/PYR1-like/regulatory components of ABA receptors (PYR/PYL/RCAR)–mediated ABA signaling has been described to regulate the content and composition of carotenoid during tomato ripening (Ma et al., 2009; Park et al., 2009; Sun et al., 2011). Suppression of the key ABA biosynthetic gene, *SlNCED1*, results in increased ET production, down-regulation of *SlLCY-B* expression, and up-regulation of *SlPSY1* expression with increased levels of lycopene and β-carotene (Sun et al., 2012). The possible reason for the enhanced carotenoid accumulation in transgenic tomato fruits could be an enhanced carbon flow to carotenoid production or inhibition of carbon flux to free ABA and its metabolites. On the other hand, 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 the build-up of the storage capacity of plastids.

ET, IAA, and ABA are considered as essential modulators in tomato fruit development. In recent years, the roles of new phytohormones, jasmonic acid (JA) and brassinosteroids (BR), in the regulation of tomato fruit ripening were also explored (Figure 3). JA and its volatile methyl ester, MeJA, are well-studied plant growth regulators, participating in the regulation of fruit ripening, pollen viability, and plant resistance, as well as the metabolism of many secondary metabolites (Chen et al., 2006). MeJA in lanolin paste was used to treat tomato mature green fruits in early studies, and it was observed that lycopene accumulation was almost totally inhibited during treatment (Saniewski and Czapski, 1983). On the contrary, lycopene content did not change in tomato fruits during MeJA exposure but increased after the treatment (Tzortzakis and Economakis, 2007). We investigated the role of JA in regulating carotenoid accumulation during tomato fruit ripening (Liu et al., 2012) by using the JA-deficient mutants *def1* (with a defective octadecanoid synthesis pathway), *spr2* [a suppressor of (pro) systemin-mediated responses 2 mutation with a reduced level of trienoic fatty acids] (Howe et al., 1996; Li et al., 2003a), and a *35S::prosystemin* transgenic line (*35S::prosys*; produce increased JA levels and constitutive JA signaling). The lycopene content was significantly decreased in fruits of *spr2* and *def1*, but increased in *35S::prosys* fruits. Moreover, the expression of lycopene biosynthetic genes followed a similar trend. ET production is depressed in *spr2* and *def1* and increased in *35S::prosys*. The exogenous application of MeJA to *Nr* significantly enhanced lycopene accumulation, as well as the expression of lycopene biosynthetic genes. It is proposed that JA might function independently of ET to promote lycopene biosynthesis in tomato fruits (Liu et al., 2012).

2,4-Epibrassinolide (EBR) has been previously shown to regulate carotenoid accumulation (Vidya Vardhini and Rao, 2002). Furthermore, EBR-treated pericarp discs of *Nr* accumulated more carotenoids than those of the control, suggesting the existence of a BR-induced carotenoid accumulation pathway independent of NR-mediated ET signal transduction (Liu et al., 2014). The transcription factor BRASSINAZOLE RESISTANT1 (BZR1) is a key component of BR signaling. Tomato fruit overexpressing the *Arabidopsis BZR1-1D* gene exhibited enhanced carotenoid accumulation and increased soluble solid, soluble sugar, and ascorbic acid contents during fruit ripening (Liu et al., 2014). In addition, the fruits of two transgenic lines show a dark-green shoulder at the mature green stage, in accordance with the up-regulated expression level of *SlGLK2*. This study indicates the importance of BZR1-centered BR signaling in regulating carotenoid accumulation and quality attributes, as well as the potential application of the BZR1-like(s) for improvement of nutritional quality and flavor of tomato fruit. Carotenoid biosynthesis is a dynamic process in which the complexes are formed in a dynamic fashion and recruited as needed (Shumskaya et al., 2012). ET-independent induction of carotenoid may have other effects besides their classic roles in light-harvesting complexes and photosynthetic reaction centers.

CONCLUSIONS AND FUTURE PERSPECTIVES

As important as yield improvement and stress resistance, enhancement of tomato fruit quality has gained extensive attention. Carotenoid content is one of the most important quality traits of tomato fruit, with industrial, health, and nutritional attributes. The amounts of fruit volatiles are correlated to carotenoid levels; thus, the more carotenoids in a fruit, the more of the essential flavor volatiles are generated, making a more nutritious and tastier tomato (Klee and Giovannoni, 2011). The high demand for natural carotenoids to serve as healthy antioxidants requires plants to be green factories for the economical production of high-value carotenoids and this has triggered increasing interest in exploring the regulation of carotenoid metabolism.

Plant carotenoid metabolism is regulated at multiple levels by developmental programs, environmental factors, and metabolic signals. All these events indicate that carotenoids are integral components of a multilayer dynamic metabolism network. Environmental factors, for example, CO_2 and light, confer prospects for enhancing carotenoid accumulation in fruits to attenuate the negative attitude of consumers toward genetically modified food. Although the application of phytohormones in agriculture to control plant growth and development has been expanded along with the progress in research on signal transduction and physiological mechanism, the function and molecular mechanism of phytohormones in regulating carotenoid metabolism and fruit development are still far from being understood. To fully unveil the regulation of carotenoid metabolism, it is therefore important to understand the complexity of the carotenoid metabolic network together with its associated pathway networks, such as isoprenoid metabolism and primary metabolism, as well as the volatiles contributing to flavor. The transcription factors and their interacting partners involved in the carotenoid metabolic network remain to be further identified. The advanced "omic" technologies (phenomics, genomic, transcriptomics, proteomics, metabolomics, and fluxomics) will extend our knowledge of the carotenoid metabolic network together with its associated pathway networks.

In conclusion, it is evident that better understanding and technological advancement of the regulation of carotenoid metabolism in tomato hold enormous promise for satisfying both agricultural needs and scientific interests. Efforts should be made to facilitate the manipulation and improvement of agronomical and economical quality in tomato crops by elucidating the regulatory mechanisms of the carotenoid metabolism network in the future.

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