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The carotenoids metabolism during tomato fruit ripening: insights into multi-level regulatory network

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Abstract

Carotenoids play an indispensable positive role in human nutrition and health. Understanding the biosynthesis and catabolism pathways of carotenoids in tomato fruits has practical value and fundamental importance. Carotenoid biosynthesis and catabolism are developmental program regulated at multiple levels. In recent years, numerous studies have explored the dynamic interactions between phytohormones, transcription factors, and epigenetic modifications to establish a regulatory network for carotenoid accumulation. However, understanding the regulation of the diversity and complexity of carotenoid accumulation remains incomplete. In this review, we summarize the molecular mechanisms of plastid storage capacity, phytohormones, epigenetic modifications, and transcription factors in regulating carotenoid biosynthesis and metabolism in tomato fruit. These insights offer foundational research and considerations for metabolic engineering aimed at altering carotenoid composition and content in tomatoes. We have also identified several unresolved issues for future research, which will help to better understand the factors regulating carotenoid accumulation in tomato fruits.

Keywords Tomato, Carotenoids metabolism, Transcription factors, Epigenetic modifications, Regulatory network

Background

Tomato (*Solanum lycopersicum*) is a global commercial crop that is highly praised by consumers all over the world for its abundance of nutritional components, unique aroma, and diverse consumption methods [1]. The quality of tomato fruit is mainly determined by its intrinsic non visual attributes (including flavor and nutritional value) and extrinsic visual attributes (including size and color) [2]. Appearance quality, including fruit color and uniformity, determines consumers' purchasing preferences and desires. In recent years, consumer demands for the appearance quality of tomatoes have continuously increased [3]. The formation process of tomato fruit quality is often accompanied by fruit ripening [4]. Ripening is a highly intricate process encompassing comprehensive changes in fruit quality, such as aroma, flavor, and color [5]. Among them, the color changes caused by carotenoids biosynthesis/metabolism is one of the most representative features, and strongly influences the nutritional and sensory quality of the fruits [6].

Carotenoids are natural C_{40} lipophilic isoprenoids compounds synthesized in photosynthetic organisms (such as plants, algae, and bacteria) as well as non-photosynthetic fungi and bacteria [7]. In photosynthetic green tissues, carotenoids act as accessory pigments in light-harvesting

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complexes (LHCs), performing essential functions in photosystem assembly, light harvesting, and photoprotection [8]. In non-photosynthetic tissues (such as fruits and flowers), these compounds impart distinctive colors, thereby enhancing their economic value [9]. In addition to their role as pigments, carotenoids are also precursors to some apocarotenoids (β-ionone and geranylacetone). In tomatoes, an increase in the levels of β-ionone and geranylacetone contributes to the enhancement of fruit flavor quality [10, 11]. In *Arabidopsis*, increased emission of β-ionone has been shown to effectively prevent damage caused by insect attacks [12]. Zeaxanthin-derived apocarotenoids (such as crocin, cocetin glycosides, picocrocin, and saffaranal) contribute to the red color of saffron [13, 14]. Carotenoids are also precursors to two important phytohormones, abscisic acid (ABA) and strigolactones [15]. Furthermore, carotenoids play a crucial role in regulating plant growth and development, enhancing resistance to stresses, and other physiological processes [16]. It is noteworthy that carotenoids are also essential components of human diets and have potential health benefits. Since the human body cannot synthesize carotenoids, consuming astaxanthin, cryptoxanthin, lutein, zeaxanthin, and lycopene from diet can help reduce the risk of various chronic diseases, including age-related macular degeneration, cardiovascular disease, and cancer [17–19]. Moreover, apocarotenoids also can play important roles in nutrition and in the prevention of several diseases. For example, vitamin A (also known as retinol, an apocarotenoid substance) contributes to the enhancement of the central nervous system and the improvement of vision [20].

The positive role of carotenoids in nature and human diet combined with high market demands have triggered numerous research [21]. In recent decades, there has been a detailed understanding of the biosynthetic pathways of carotenoids in higher plants. Carotenoid biosynthesis and metabolism depend on several factors beyond the modulation of pathway gene expression. In recent years, there has been rapid advancement in understanding how to regulate the accumulation of carotenoids during tomato fruit ripening [1, 22–36]. Therefore, a thorough understanding of the regulatory network of carotenoids will enable in the rational genetic engineering modifications of their biosynthetic pathways, thereby providing new strategies for improving the quality of tomato fruits. Here, we arranged and summarized the research on carotenoid regulation, emphasizing the latest insights into the comprehensive regulatory network of carotenoid biosynthesis and metabolism through plastid development, phytohormone signaling, transcriptional factors, and epigenetic modifications.

Biogenesis and stable storage of carotenoids

Biosynthesis and catabolism: regulation of carotenoid biogenesis linear pathways

The five carbon isoprenoid building blocks derived from the methylerythritol 4-phosphate (MEP) pathway——specifically isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP)——serve as donors for carotenoid biosynthesis in higher plants [37] (Fig. 1). The glyceraldehyde 3-phosphate (G3P) and pyruvate are utilized as initial substrates to produce 1-deoxy-d-xylulose 5-phosphate (DXP) and MEP, catalyzed by DXP synthase (DXS) [7]. In the subsequent reaction, isomerization reaction occurs through isopentenyl diphosphate isomerase (IPI), forming IPP and DMAPP [38]. Head-to-tail joining of IPP and DMAPP generates the C_{20} intermediate geranylgeranyl pyrophosphate (GGPP), which is the direct substrate for the biosynthesis of carotenoids, a step catalyzed by GGPP synthase (GGPPS) [39].

Phytoene synthase (PSY) catalyzes the condensation of two GGPP molecules to produce 15-*cis*-phytoene, the initial committed intermediate of the carotenoid pathway. This step is recognized as the major rate-determining process that regulates metabolic flux [40]. Three PSY-encoding genes control carotenoid biosynthesis in different tissues. The expression of *SlPSY1* is enhanced during ripening to facilitate the production of carotenoids responsible for fruit pigmentation [41]. Interestingly, a bacterial phytoene synthase (crtB) was expressed in a fruit-specific manner, the CRTB protein was targeted to chromoplast by the PSY1 transit sequence, which overcame the detrimental pleiotropic effects and generated a high carotenoid content in tomato fruit [40]. *PSY2* is mainly responsible for carotenoid synthesis in photosynthetic tissues for photosynthesis and photoprotection, while *PSY3* is mainly expressed in roots [42, 43]. In various crops, the transcription of *PSY* is induced by salt, light, temperature, drought, photoperiod, and development cues. For example, salt and drought induced *PSY3* transcript abundance and this correlated with increased carotenoid flux and ABA in maize (*Zea mays*) and rice (*Oryza sativa*) roots [44, 45]. The different expression profiles of the rice *PSY1* and *PSY2* correlate strongly with the presence of promoter regulatory cis-elements that mediate light [45]. However, stress-induced changes in *PSY* mRNA do not always result in changes in carotenoid flux. For example, elevated temperatures decreased *PSY1* mRNA abundance in maize leaves and unexpectedly there was a concurrent increase in carotenoids [46]. Spontaneous mutants of key genes in the carotenoid biogenesis pathway provide a new perspective for a deeper understanding of the carotenoid regulatory network. A loss-of-function mutant of the *SlPSY1*, known as *yellow flesh* (locus *r*), results in an intense yellow pigmentation in the pericarp [47]. The spontaneous mutation r^y is an

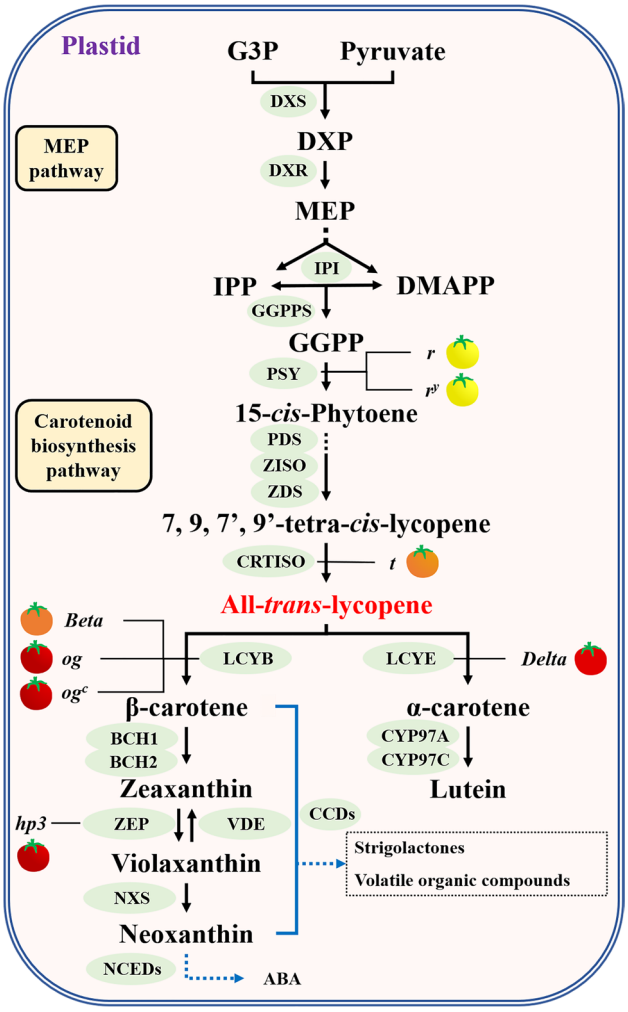

Fig. 1 Schematic carotenoid biosynthesis and catabolism linear pathway. Mutants related to carotenoid biosynthesis are indicated in italic black type. The green ellipses represent carotenoid pathway genes. G3P, glyceraldehyde 3-phosphate; DXP, 1-deoxy-d-xylulose 5-phosphate; MEP, methylerythritol 4-phosphate; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GGPP, geranylgeranyl pyrophosphate; *DXS*, 1-deoxy-d-xylulose 5-phosphate synthase; DXR, *1-deoxy-d-xylulose 5-phosphate reducto-isomerase*; *IPI*, *isopentenyl diphosphate isomerase*; *GGPPS*, *geranylgeranyl pyrophosphate synthase*; *PSY*, *phytoene synthase*; *PDS*, *phytoene desaturase*; *ZISO*, *ζ-carotene isomerase*; *ZDS*, *ζ-carotene desaturase*; *CRTISO*, *carotene cis-trans isomerase*; *LCYE*, *lycopene ε-cyclase*; *LCYB*, *lycopene β-cyclase*; *BCH*, β-carotene hydrolase; CYP97A, *cytochrome P450 carotene β-hydroxylase*; *CYP97C*, *cytochrome P450 carotene ε-hydroxylase*; *ZEP*, *zeaxanthin epoxidase*; *VDE*, *violaxanthin de-epoxidase*; *NXS*, *neoxanthin synthase*; *CCD*, *carotenoid cleavage dioxygenase*; *NCED*, *9-cis-epoxycarotenoid dioxygenase*

allele of the *r* locus and its fruit color is similar to the normal flower corolla color [47]. These mutant fruits exhibit yellow flesh due to carotenoid deficiency, affirming that *SlPSY1* is an extremely important candidate gene responsible for initiating substrates into the carotenoid biogenesis pathway.

Uncolored 15 cis-phytoene undergoes four desaturation reactions catalyzed by phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS). Additionally, two intermediary isomeric conversion steps facilitated by ζ-carotene isomerase (ZISO) and carotene *cis-trans* isomerase (CRTISO) transform it into the red-colored all-trans lycopene [48–51]. The accumulation of phytoene has been postulated to involve negative feedback regulation. For example, in the *pds3* mutant, genes encoding upstream enzymes such as IPI, GGPPS, and PSY, were downregulated [50]. Although the *PDS* gene is often used as a marker for gene silencing in tomatoes, little is known about the role of *PDS* in fruit ripening [52, 53]. The use of virus-induced gene silencing (VIGS) in tomatoes was first achieved by silencing *PDS* in leaves [54], followed by many more successful *PDS*-silencing experiments on tomato fruit, the silencing of *PDS* gene in tomato fruit reduced the accumulation of carotenoids and led to yellow-color fruit [53, 55]. In overexpression-*SlZDS* fruits, lycopene level was significantly increased, and the ZDS substrate 9, 9' *di-cis*-ζ-carotene was completely depleted. This indicates that the steps catalyzed by ZDS represent an additional bottleneck in carotenoid biosynthesis in tomatoes [56]. *Y9* in maize or *ZIC* in *Arabidopsis* encodes the enzyme ZISO, which is essential for the *cis*-to-trans conversion of the 15-*cis*-bond in 9, 15, 9'-tri-*cis*-ζ-carotene (the product of PDS) to 9, 9'-di-*cis*-ζ-carotene, the substrate of ZDS [51, 57]. In tomato fruits, VIGS of *ZISO* did not significantly alter total carotenoid content, yet did alter carotenoid composition [58]. The 'Pinalate' sweet orange mutant, caused by a single nucleotide insertion in the *ZISO* gene, has reduced content of β, β-xanthophylls and derived apocarotenoids, resulting in yellow pigmentation in the fruit [59]. Analysis of *ZISO* promoter and 5'-UTR sequences revealed more than 130 *cis*-regulatory elements involved in responses to light, stress and hormones, as well as in the binding of transcription factors. Different tomato cultivars, such as green-fruited (*Solanum chilense*, *Solanum habrochaites*, and *Solanum pennellii*) and red-fruited (*Solanum pimpinellifolium*), differed in the number and position of *cis*-elements, suggesting that transcriptional regulation of *ZISO* expression has changed during tomato evolution, which may contribute to differences in fruit color [60]. The *cis-trans* reactions catalyzed by CRTISO has emerged as a crucial regulatory node in the carotenoid synthesis pathway, which capable of isomerizing *cis* bonds at 7, 9, 7', 9' positions, thereby converting tetra-*cis*-lycopene into all-*trans*-lycopene [7]. The isomerase activity of CRTISO depends upon the flavin adenine dinucleotide (FAD) binding motif, which bonds to the reduced form of cofactor (FAD_{red}) to catalyze a reaction without net redox changes [61]. Two distantly related *CRTISO-like* single copy genes (*CRTISO-like1* and *CRTISO-like2*) have been discovered in tomato [58]. The disappearance of all-*trans*-ζ-carotene and increased

content of lycopene isomers were observed in *CRTISO-like1*-/*CRTISO-like2*-silenced fruits, demonstrating that *CRTISO-like1* and *CRTISO-like*2 play active roles in a metabolic branch producing all-*trans*-ζ-carotene [58]. *CRTISO* mutants, *tangerine* (locus *t*), showed orange fruits and yellowish young leaves due to the accumulation of 7, 9, 7′, 9′-tetra-*cis*-lycopene in the etioplasts (dark-grown plastids) of seedlings and chromoplasts of fruit [48]. Epistatic interaction of *crtiso* mutant (*tangerine*) on *psy1* mutant (*yellow flesh*), where the transcription of *PSY1* is partially restored (involving *cis*-carotenoid metabolites) for sufficient production of phytoene and downstream carotenoids in *tangerine* background [41]. This indicated towards the complex regulation of carotenogenesis in tomato fruits through interaction between different mutant alleles.

The branch point for carotenoid synthesis and metabolism is carried out using all-*trans*-lycopene as the substrate (Fig. 1). This cyclization reaction, which commences at the terminal end of the linear lycopene synthesis chain, and is essential for the production of a variety of carotenoids [10]. The orange-colored α- and β-carotene are produced in the α- and β-branches catalyzed by lycopene ε-cyclase (LCYE) and lycopene β-cyclase (LCYB), respectively [62]. Subsequently, the β-branch flows toward zeaxanthin, violaxanthin, and neoxanthin to provide precursors for the synthesis of ABA and strigolactones, while the α-branch moves towards the lutein [16]. The genes encoding LCYB and LCYE share significant sequence identity, suggesting that they may have originated from a common ancestor [63]. There are two *SlLCYB* genes in tomatoes, among which *SlLCYB1* (also known as *SlCRTLB*) is mainly expressed in flowers and green tissue, while *SlLCYB2* (also known as *SlCYCB*) plays a specific role in chromoplast [9]. In *Beta* mutant, the up-regulation of the *SlLCYB2* resulted in a large accumulation of β-carotene. Conversely, in *old-gold* (*og*) and *old-gold crimson* (og^c) mutants (frame-shift mutations in *SlLCYB2*, leading to the production of non-functional protein), where the lack of lycopene β cyclase activity causes a complete absence of β-carotene and a significant increase in lycopene content resulting in a deep-red colored of ripe tomato fruits [9]. The expression of *SlLCYE* is decreased during fruit ripening, but in the *Delta* mutant with orange-colored fruits, the transcript level of *SlLCYE* increases by 30-fold [9]. Subsequently, α- and β-carotene undergo hydroxylation catalyzed by two heme-containing cytochrome P450 type hydroxylases (CYP97A/C) and two non-heme β-carotene hydrolases (BCH1/2) to produce lutein and zeaxanthin, respectively [10, 22]. Zeaxanthin is epoxidized by zeaxanthin epoxidase (ZEP) to yield violaxanthin. The mutation in *high pigment 3* (*hp3*) had occurred in the gene for ZEP. This mutant accumulates 30% more carotenoids in ripe fruits, and higher concentrations of carotenoids and chlorophyll were also measured in leaves and the pericarp of green fruit. Furthermore, the plastid compartment size in fruit cells is at least 2-fold larger in *hp3* plants compared with the wild-type [32]. Subsequently, the de-epoxidation steps catalyzed by violaxanthin de-epoxidase (VDE) reverses this process. The interconversion between this epoxidation and de-epoxidation reaction forms the xanthophyll cycle, which is an important mechanism for plants to protect against photo-damage [64]. The conversion of violaxanthin into neoxanthin by neoxanthin synthase (NXS) [65]. The steady-state concentration of carotenoids in tomato fruit is regulated by the balance between biosynthetic pathway flux and degradation processes catalyzed by carotenoid cleavage enzymes [66]. The carotenoid cleavage enzymes include 5 9-*cis*-epoxycarotenoid dioxygenases (NCEDs) and 4 carotenoid cleavage dioxygenases (CCDs) [67]. According to reports, CCDs strongly controls the content of carotenoids, while also influencing the formation of downstream phytohormones and contributing to the colors or aromas of fruits [68, 69]. Multiple homologs have been found in the CCD subfamily, such as *CCD1a* and *CCD1b* in tomato [70] and *CCD4a* and *CCD4b* in citrus [71]. *SlCCD1a* and *SlCCD1b* have high specificity for substrates and cleavage sites, which can mediate the oxidative cleavage of *cis*- and all-*trans*-carotenoids and different apocarotenoids to produce volatile substances such as isoprene as well as mono-apocarotenoids and dialdehyde products (including *cis*-pseudoionone, neral, geranial, and farnesylacetone) [70]. β-Citraurin gives citrus peel its distinctive red color, which is caused by the cleavage of β-cryptoxanthin and zeaxanthin by CCD4 [71]. Furthermore, downstream in the carotenoid pathway, CCD7 and CCD8 sequentially cleave 9-*cis*-β-carotene to form carlactone, resulting in strigolactones [68, 72]. NCED are associated with abscisic acid (ABA) biosynthesis. In tomato, *SlNCED1*-RNAi resulted in reduced ABA biosynthesis in fruit, increased β-carotene and lycopene accumulation, and ultimately the production of deep red fruit [73]. The tomato mutants *notabilis* (*not*, a null mutation in the gene *SlNCED1*), *flacca*, and *sitiens* are deficient in ABA and are phenotypically wilty, stunted and epinastic [74]. ABA biosynthesis begins with the epoxidation of zeaxanthin by the enzyme ABA deficient 1 (ABA1) to form epoxycarotenoids. The oxidative cleavage of 9-*cis*-epoxycarotenoids is a key regulatory step catalyzed by NCED to form xanthoxin, which are converted to ABA in further reactions mediated by ABA deficient 2 (ABA2), ABA deficient 3 (ABA3), and abscisic aldehyde oxidase 3 (AAO3) [75]. Recently, an ABA1-independent ABA biosynthesis pathway was discovered in *Arabidopsis*, starting from upstream of zeaxanthin. β-apo-11-carotenoid was identified as an intermediate of ABA1-independent ABA

biosynthesis pathway. This result provides new insights into the plant apocarotenoid metabolic network [75].

Stable storage of carotenoids in tomato fruits: the role of plastids

In addition to carotenoid biosynthesis and degradation, the stable storage of carotenoids in plastids significantly influences the final carotenoid levels in tomato fruits [38]. During tomato fruit development, there is a transition from partially photosynthetic to fully heterotrophic metabolism, accompanied by the parallel transformation of chloroplasts into chromoplasts to generate red or orange colored plastid types dominated by carotenoids [76, 77]. During this interconversion process, massive carotenoid accumulation following the plastid's internal membranes undergo remodeling and the formation of carotenoid-sequestering structures within chromoplasts [78]. These structures include three types: globular chromoplasts, crystalline chromoplasts, and membranous chromoplasts [79]. In tomato fruits, globular chloroplasts are characterized by the accumulation

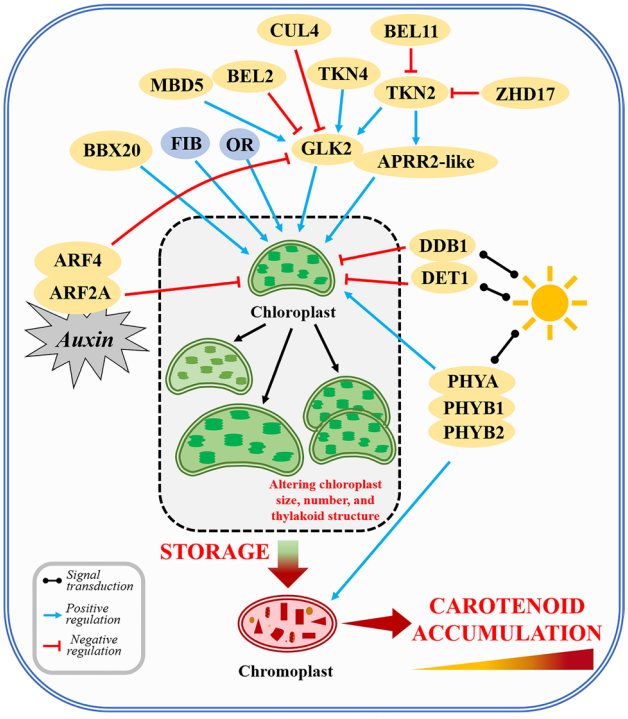

Fig. 2 Plastid structures and carotenoid stable storage. The yellow ellipses represent transcriptional factors regulating plastid development in tomato. The blue ellipses represent plastid proteins. DDB1, UV-damaged DNA binding protein 1; DET1, deetiolated1; FIB, fibrillin; OR, orange; GLK2, golden 2-like; APRR2-like, *Arabidopsis* pseudo response regulator2-like; TKN2, knotted1-like homeobox 2; TKN4, knotted1-like homeobox 4; MBD5, methyl-CpG-binding domain 5; BEL2, BEL1-like homeodomain 2; BEL11, BEL1-like homeodomain 11; ZHD17, Zinc-finger homeodomain17; CUL4, Cullin4; BBX20, B-box 20; ARF4, AUXIN response factor 4; ARF2A, AUXIN response factor 2 A; PHYA, phytochrome A; PHYB1, phytochrome B1; PHYB2, phytochrome B2

of pigment-containing plastid globules [79, 80], while crystalline chloroplasts are red or orange due to the over-accumulate lycopene or β-carotene [80]. During the transition from chloroplasts to chromoplasts, there is a general reduction in proteins related to photosynthesis, while many non-photosynthetic proteins accumulate [81, 82]. As an example, genes responsible for carotenoid biosynthesis are upregulated during the formation of chromoplasts [83], while those encoding essential photosynthetic proteins are downregulated like major light-harvesting chlorophyll-binding proteins and the small subunit of Rubisco [84].

In plant cells, carotenoids are mainly formed in plastids and stored at high levels [78]. Plastids originate from undifferentiated and colorless proplastids. As cells differentiate, proplastids transform into various types of plastids, such as amyloplasts, chloroplasts, and chromoplasts [85]. Studies have shown that high level of carotenoid accumulation are accompanied by changes in plastid differentiation, structure, and size [78]. Therefore, the plastids type, number, and size significantly influence stable accumulation of carotenoids [76, 86]. For instance, tomato *high pigment* (*hp1*, *hp2*, and *hp3*) mutants accumulate significant levels of carotenoid in mature fruits due to an increase in the chromoplast number or plastid compartment volume [32, 87, 88]. In addition, the influence of different plastid ultrastructures (including globular, crystalline, and membranous substructures) on carotenoid biosynthesis and storage capacity varies [89]. Crystalline chromoplasts, for instance, typically amass lycopene and β-carotenoids as red/orange crystals in tomato fruits [80]. During chromoplast formation, the accumulation of plastoglobules and the increase in size are essential for carotenoid production [90, 91]. Perhaps the absence of suitable lipoprotein sequestering substructures in amyloplasts limits their ability to synthesize and store carotenoids effectively [78].

Indeed, numerous known plastidial proteins can also regulate carotenoid production by manipulating the plastid sink strength (Fig. 2). In gene overexpression of FIBRILLINS (FIB, it is related to the formation of plastid beads and fibril in chromoplasts) fruits, the degradation of thylakoids in chromoplasts was delayed, and the contents of lycopene and β-carotene was increased [92]. ORANGE (OR) encodes a plastidial DnaJ cysteine-rich domain-containing protein and serves as a key regulator of chromoplast biogenesis and carotenoid accumulation in melon fruit [93]. Overexpression of *AtOR* in tomato led to the conversion of chloroplasts into chromoplasts at the early stage of fruit ripening [94].

Carotenoid synthesis and metabolism in chromoplasts is regulated by phytohormones as well as environmental cues [7, 16] (Fig. 2). Auxin response factor 2 A (SlARF2A) directly binds to the *chloroplast-targeted*

metalloendopeptidase (*RCM1*) promoter to regulate the number and membrane structure of thylakoids, thereby affecting chloroplast development distinctly [95]. *SlARF4* functions as a negative regulator of chloroplast development by suppressing *GOLDEN 2-like* (*SlGLK2*) activity. And suppression of *SlARF4* expression results in a dark green phenotype in tomato fruits [96, 97]. In abscisic acid (ABA) deficient mutants such as *hp3*, *flacca* and *sitiens*, the number of plastids and lycopene content were increased, which inversely correlates with ABA levels [32], indicating that ABA regulation of carotenoid accumulation may be related to its control of plastid development [16]. *HP1* and *HP2* encode homologs of tomato UV-damaged DNA-binding protein 1 (DDB1) and deetiolated1 (DET1), respectively, involved in light signal transduction. Mutations in both genes lead to a significant enlargement of chloroplast size in mature green stage fruits [87, 98]. Loss of *DET1* function increased plastid number, plastid size, and cell index, and elevated levels of isoprenoids were associated with increased plastid volume in cells [99]. Altered plastid biogenesis leading to an increased plastid compartment per cell is also believed to be a contributing factor to elevated chlorophyll and carotenoid levels in *hp* mutants [99]. In tomato fruits, plant phytochromes (PHYs) play a crucial role in regulating chlorophyll and carotenoid synthesis as well as plastid development and expansion [100, 101], and *phyaphyb1phyb2* triple mutant exhibits a significant decrease in chlorophyll content [102]. Furthermore, silencing of *SlPHYA* in fruit results in reduced number of chloroplast and decreased content of chlorophyll [103]. Given that carotenoid accumulation and storage reflects the coordinated regulation of multiple programs, inducing chromoplast biogenesis can further promote biosynthesis of carotenoid and enhances storage stability [104]. Therefore, deeper exploration of the molecular mechanisms governing chromoplast biogenesis could offer substantial potential for biofortification aimed at enhancing carotenoid accumulation and stability.

Regulation of plastid development by light signaling-related transcription factors

The intensity and pattern of chloroplast distribution in tomato fruit are governed by the dominant *Uniform ripening* (*U*) locus [105]. *U* encodes a GLK transcription factor that controls the chloroplast-related genes expression and promotes chloroplast development [106]. Overexpression of *SlGLK2* increases chlorophyll levels by increasing plastid number and size [105]. Similarly, overexpression of the tomato *ARABIDOPSIS PSEUDO RESPONSE REGULATOR2-like* (*SlAPRR2-like*) gene, which is related to *SlGLK2*, causes enhanced chloroplasts development, increased plastid number, and elevated pigment levels in fruits [107]. TKN2 and TKN4, which are KNOTTED1-like HOMEOBOX transcription factors, function upstream of *SlGLK2* and *SlAPRR2-like* in tomato. Specifically, TKN2 promotes development of chloroplast by regulating the both *SlGLK2* and *SlAPRR2-like* expression, whereas TKN4 regulates only *SlGLK2* expression to establish chloroplast development gradients [108]. Furthermore, previous study showed that GLKs activate the transcription of *PSY* in a G-box binding factor (GBF)-dependent manner in *Arabidopsis*, thereby regulating carotenoid biosynthesis [109]. Overexpression of *SlMBD5* (a methyl-CpG-binding domain protein) increases plastid levels and pigmentation by enhancing the expression of *SlGLK2* [110]. SlBEL2 (BEL1-LIKE HOMEODOMAIN 2) directly binds to the *SlGLK2* promoter region and represses its transcription level, thereby affecting the chloroplasts development [111]. Additionally, SlBEL11 and SlZHD17 (Zinc-finger homeodomain17) binds to the *SlTKN2* and *SlPOR* promoter, respectively, leading to the downregulation of their transcription levels to negatively regulate development of chloroplast and synthesis of chlorophyll in tomato fruits [112, 113]. Furthermore, inhibition of CUL4 (Cullin4) increases plastid number, resulting in enhanced lycopene accumulation in tomato fruit [114]. *SlBBX20*, a B-box zinc-finger transcription factor family member, regulates development of chloroplast by modulating the *SlCHL27*, *SlCAB6A*, and *SlCAB1B* expression [115].

Regulation of carotenoid in tomato fruit: molecular network of phytohormones signals

During the tomato fruits ripening, the synthesis and metabolism of carotenoids undergoes a highly intricate biological process, governed by phytohormones including ethylene, ABA, brassinosteroids (BR), and auxin, gibberellin (GA), and strigolactones [16, 116, 117].

The burst of ethylene is a trigger for tomato fruit ripening. At the same time, ethylene induces the expression of *SlPSY1* and *SlPDS*, which in turn cause accumulation of lycopene and its downstream β-carotene [16]. This pivotal role in tomato fruit ripening underscores the specificity of ethylene's role in regulating carotenoid accumulation (Fig. 3). The synthesis of ethylene is catalyzed by 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS), and ACC oxidase (ACO). 14 *ACS* and 6 *ACO* genes were identified in tomatoes. Among them, the expression levels of *SlACS2*, *SlACS4*, and *SlACO1* showed significant induction during fruit ripening, and their high expression in tomatoes significantly accelerated the transition from green to red in the fruit [24, 118]. This means that manipulating ethylene biosynthesis is a key strategy for regulating carotenoid accumulation [22]. Apart from ethylene biosynthesis, ethylene signaling also plays a crucial role in carotenoid accumulation and fruit ripening [119]. The different reactions to ethylene are

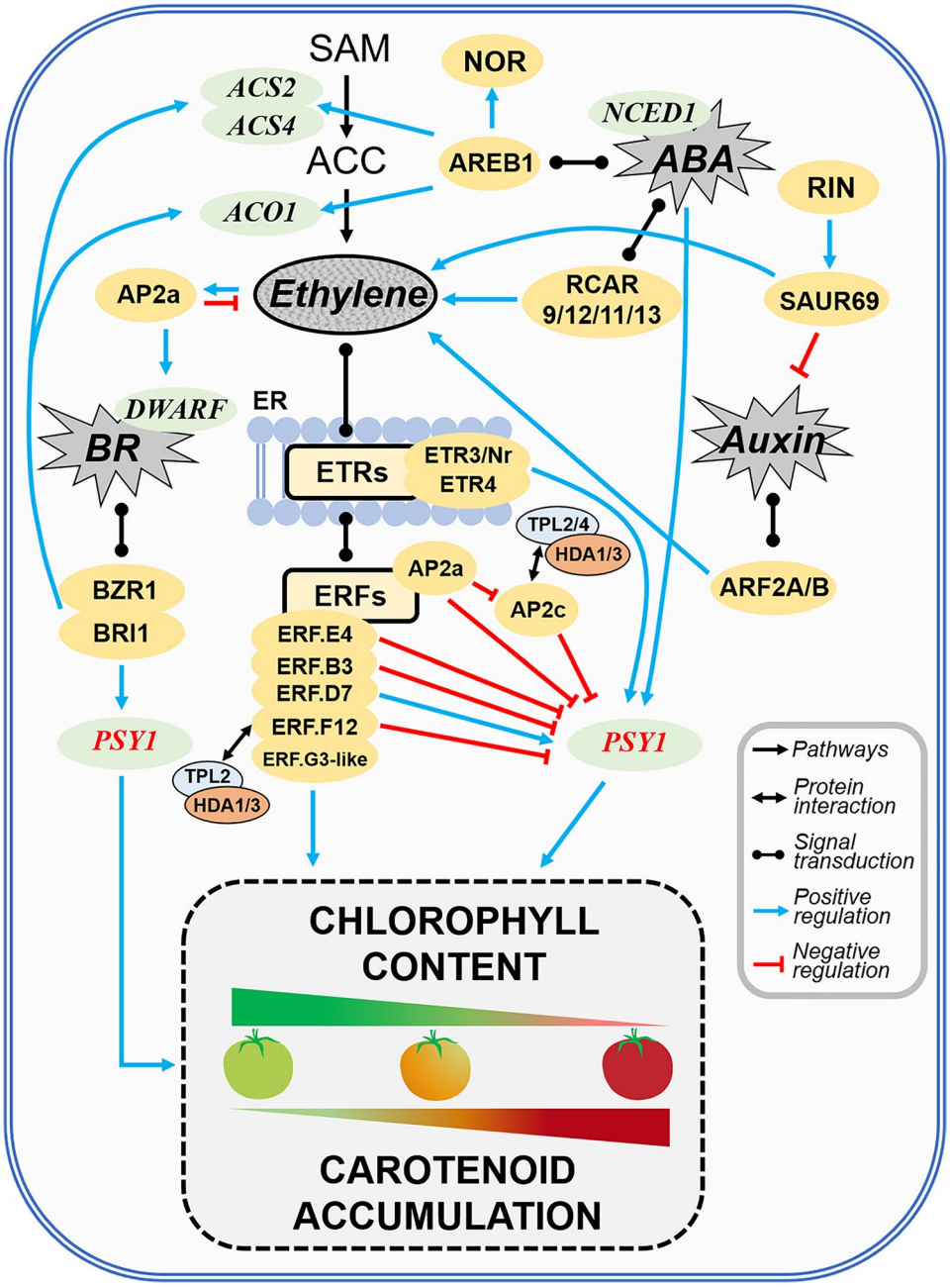

Fig. 3 The regulatory network of phytohormones signals on carotenoids in tomato fruits. The yellow ellipses represent transcriptional factors signaling components. The green ellipses represent genes involved in the synthesis of carotenoids, ethylene, and other phytohormones. ER, endoplasmic reticulum; ABA, abscisic acid; BR, brassinosteroids; RIN, ripening inhibitor; NOR, non-ripening; ETRs, ethylene receptors; ERFs, ethylene response factors; AP2a/c, APETALA2/ERF transcription factor; ARF2A/B, AUXIN response factor 2 A/B; SAUR69, Small Auxin-Up RNA 69; AREB1, ABA response element binding factor 1; RCAR, regulatory components of ABA receptors; BZR1, brassinazole resistant 1; BRI1, brassinosteroid insensitive 1; TPL2/4, co-repressor TOPLESS 2/4; HDA1/3, histone deacetylase1/3; *ACS2/4, 1-aminocyclopropane-1-carboxylic acid synthase 2/4*; *ACO1, 1-aminocyclopropane-1-carboxylic acid oxidase 1*; *NCED1, 9-cis-epoxycarotenoid dioxygenase 1*; *PSY1, phytoene synthase 1*

mainly mediated by the ethylene receptors (ETRs), the initial component of the ethylene signaling cascade and is necessary for tomato fruit ripening [120]. *SlETR3* (also referred to as *Never ripe* (*Nr*)), *SlETR4*, and *SlETR7* are recognized as the three main receptor genes expressed early in fruit ripening [121]. The ripening-defective fruit phenotype and inability to accumulate lycopene exhibited by tomato *etr3*/*Nr* mutant further demonstrate the role of ETRs in carotenoid formation [122]. Ethylene response factors (ERFs, belong to the APETALA2/ethylene response factors family) located at the end of the linear pathway of ethylene signaling have a typical role

in controlling the ethylene reactions and carotenoid pathway flux in tomato fruits [123]. The ERF subfamily regulates its expression by binding to the GCC-box (GCCGCC) or DRE (CCGAC) motifs of downstream target gene promoters [123]. *SlERF6* (named *SlERF.E4* by Liu et al.) [124] inhibits carotenoid biosynthesis and additional ripening phenotypes by reducing lycopene and β-carotene accumulation [124, 125]. Overexpression of the dominant suppressor version of *SlERF.B3* (*SlERF.B3-SRDX*) altered the carotenoid pathway flux by downregulating *SlPSY1* and *SlPDS* expression, allowing ripen fruits to remain orange [126]. Recently, it has been reported that *SlERF.D7*, *SlERF.F12*, and *SlERF.G3-Like* controlled tomato fruit color development and ripening by participating in the ethylene signal transduction pathway [127–129]. The expression level of *SlPSY1* and lycopene content were significantly increased in *SlERF.F12*-RNAi fruits. In addition, SlERF.F12 interacts with the corepressor TPL2 through the C-terminal EAR motif and recruits histone deacetylases SlHDA1 and SlHDA3 to form a tripartite complex in vivo, which negatively regulates tomato fruit ripening by repressing the transcription of *SlACS2* and *SlACS4* by reducing the levels of histone acetylation marks H3K9Ac and H3K27Ac in their promoter regions [128]. Dual-Luc assay confirmed that SlERF.G3-like did not bind to the promoters of *SlPSY1* and *SlPDS*, but the CRISPR-*slerf.g3-like* line fruit showed an increase in lycopene content and a decrease in flavonoid biosynthesis, indicating that these two pathways may compete for substrates [129]. These results suggested that the influence on carotenoids might be caused by cascade changes induced by SlERF.G3-like induced redistribution of flavonoid pathway metabolic flux [129, 130]. *SlAP2a*, a member of the APETALA2/ERF family, is considered a negative regulatory factor involved in ethylene production, carotenoid accumulation, and chromoplast differentiation [131]. Another member of the AP2/ERF family, SlAP2c, represses the expression of genes involved in lycopene biosynthesis (*SlPSY1*, *SlZISO*, and *SlCRTISO*) by directly binding to cis-elements in the promoter. In addition, SlAP2c relies on its EAR motif to recruit the corepressor TPL2/4 and interact with HDA1/3 to form a complex, thereby reducing the histone acetylation level of lycopene biosynthesis genes [132]. Meanwhile, SlAP2a acts upstream of SlAP2c and inhibits its transcription during fruit ripening, thereby alleviating the inhibition of lycopene biosynthesis by SlAP2c [132]. Other ethylene related transcription factors located upstream of ERF, such as EIN2, EIN3/EIL, EBF1/2, EBF2 like, and EBF3, activate or inhibit tomato carotenoid accumulation by coordinating ethylene dependent programs or combining with other proteins into transcription modules to coordinate multiple positive or negative feedback loops [133–136].

BRs are considered as positive regulatory factors for tomato fruit carotenoid accumulation [22]. Exogenous BR treatment of tomato fruits accelerated fruit ripening and increased lycopene content (Fig. 3). On the contrary, the application of BR synthesis inhibitor brassinazole (BRZ) inhibited the carotenoids accumulation [137]. BR synthesis and signaling play crucial roles in carotenoids synthesis in tomato fruit. For instance, overexpression of the BR synthesis gene *SlCYP90B3* and *SlDWARF* in tomato fruits increased lycopene content. In contrast, *SlCYP90B3*-RNAi and CRISPR-*sldwarf* fruits showed significantly reduced lycopene content [22, 138]. Overexpression the core genes of BR signaling, including *BRASSINAZOLE RESISTANT 1* (*SlBZR1*) and *BRASSINOSTEROID INSENSITIVE 1* (*SlBRI1*), significantly upregulated the expression levels of *SlACS2*, *SlACS4*, *SlACO1*, and *SlPSY1*, while promoting ethylene release and carotenoid accumulation. However, ethylene release and carotenoid content were significantly reduced in the the CRISPR-*slbzr1* line fruits [24, 139]. Dual knocking out *SlBZR1* and *BRI1-EMSSUPPRESSOR 1* (*SlBES1*) significantly reduced the carotenoids accumulation in tomatoes [24]. SlBZR1 directly binds to and induces expression of the *SlPSY1* promoter, which initiates chromoplasts differentiation and carotenoid accumulation [24]. Furthermore, it was found that AP2a binds to the promoter region of *SlDWARF* and directly activate its expression [29]. In the *slap2a* mutant, *SlPSY1* expression level and lycopene accumulation were significantly reduced, whereas exogenous 28-homobrassinolide (HBR, a bioactive BR) treatment significantly increased *SlPSY1* expression and lycopene content, suggesting that the increased BR level partially compensated for the loss of function of AP2a [29].

Previous studies have confirmed that auxin response factors (ARFs) participate in nuclear auxin signaling and regulate physiological processes such as pigment accumulation in tomato fruits [140, 141]. Double silencing of the central components of auxin signaling, *SlARF2A* and *SlARF2B*, results in tomato fruits exhibiting a phenotype that fails to turn red normally [142]. In addition, the role of crosstalk between ethylene and auxin in regulating tomato fruit ripening has long been reported [143]. In tomatoes, ARF2 and Aux/IAA27 (IAA27), play crucial roles in the crosstalk between ethylene and auxin [144]. Silencing of *SlARF2* has been shown to significantly affect the expression of *ETRs* and *ERFs* genes, as well as ethylene biosynthesis. Additionally, it down-regulates key ripening regulators such as *SlRIN*, *SlCNR*, and *SlNOR* [142]. *SlIAA27* expression was significantly decreased in the *ERF.B3-SRDX* lines, and ERF.B3 could directly bind to the *SlIAA27* promoter and regulate its expression. Consequently, ERF.B3 integrates ethylene and auxin signaling pathways through the regulation of the auxin signaling

component *SlIAA27* [145]. RIN activates its transcription by binding to the promoter region of *Small Auxin-Up RNA 69* (*SlSAUR69*), and *SlSAUR69* reduces free auxin level or activity, which enhances the sensitivity of fruit tissues to ethylene, leading to the transition of autocatalytic ethylene production from system Ⅰ to system Ⅱ [146]. Thus, the interactions mediated by RIN and SlSAUR69 between auxin and ethylene are essential for initiating and normalizing the tomato fruit ripening program [146]. These findings underscore the critical role of auxin-ethylene crosstalk in the transition of tomato fruit from unripe to ripe states (Fig. 3).

In tomatoes, ABA is a derivative of the carotenoid pathway and its content shows an increasing trend with carotenoid synthesis [144]. Meanwhile, exogenous application of ABA can increase the accumulation of carotenoids in tomato fruits [147], whereas silencing of the key gene *9-cis-epoxycarotenoid dioxygenase 1* (*SlNCED1*) related to ABA biosynthesis seriously inhibits coloring and softening [148]. Exogenous ABA treatment significantly induced ethylene production in tomatoes, and SlACS2, SlACS4 and SlACO1 expression levels was significantly up-regulated [149]. However, the interplay between ABA- and ethylene- signaling pathways during tomato fruit ripening has garnered significant attention, particularly regarding their upstream-downstream relationship. After ABA treatment, *SlNCED1* was expressed prior to *SlACS2*, *SlACS4* and *SlACO1* in tomato fruit, while ACC treatment had no effect on the concentration of ABA in fruit [150]. In addition, the transcription of *ABA response element binding factor 1* (*SlAREB1*) and *NOR* is significantly induced by ABA, and SlAREB1 has the ability to bind to the promoter region of *NOR*,thereby activating its transcription [151]. Co-silencing of four regulatory components of ABA receptor genes (including *SlRCAR9*/*12*/*11*/*13*) weakens ethylene synthesis and signaling during the early stages of tomato fruit ripening, resulting in delayed fruit pigmentation and ripening [149] (Fig. 3). Actually, the real role of ABA in fruit ripening is still under debate. Some studies have highlighted the possibility that ABA delays fruit ripening and thus extends shelf life [152, 153]. ABA delays peach fruit ripening by downregulating the transcriptional levels of ethylene biosynthesis and signaling, cell wall softening-related, and auxin-related genes [152]. In addition, Fruit-specific overexpression of *SlLCYB* can increase ABA content, reduce ethylene production, delay fruit softening, and extend shelf life. The results of the experiment using the ABA biosynthesis inhibitor abamine to treat *SlLCYB*-overexpressing fruits showed that ABA content was reduced, ethylene production was increased, and the long shelf life phenotype was reversed [153].

In recent years, some other phytohormones have emerged as new actors in regulating tomato fruit ripening, such as GA and strigolactones [116, 117, 154]. Exogenous applications of GA delay tomato ripening [154]. Yeast one-hybrid and dual-luciferase reporter gene assays have confirmed that SlNAP1 directly binds to the promoters of GA degradation genes *SlGA2ox1* and *SlGA2ox5* and activates their expression, thereby positively regulating fruit ripening [116]. Strigolactones treatment promoted the accumulation of fructose and glucose and increased the total sugar content of the fruit by upregulating the expression of *SlAI*, *SlNI*, *SlSPS*, and *SlSS* genes in the sucrose metabolic pathway [117].

Regulation of carotenoid in tomato fruit: from transcription factors to epigenetic modifications

In recent decades, advances in high-throughput sequencing technology and a deeper mastery of "Large-scale data" assembly technology, the transcriptional and epigenetic regulatory underlying carotenoid synthesis and metabolism in tomato fruits have become an emerging research topics [119]. So far, among the identified transcription factors (TFs) controlling the fruit ripening and carotenoids synthesis in tomato, spontaneous mutations or gene editing mutations of some TFs have revealed their specific involvement in the biogenesis of carotenoids in tomatoes [155, 156]. Some TFs promote or inhibit the biosynthesis of carotenoids in tomato fruits by directly targeting structural genes related to carotenoids or fruit ripening or interacting with other proteins [157, 158]. Here, we reviewed recent findings about the functional mechanisms of the transcription factors and epigenetic landscape in controlling carotenoids biosynthesis in tomatoes.

Transcriptional regulation of carotenoid in tomato fruit

The spontaneous mutations of TFs genes known as the "master regulators" for tomato fruit ripening, include *RIPENING-INHIBITOR* (*rin*), *NON-RIPENING* (*nor*), and *COLORLESS NONRIPENING* (*Cnr*). These mutations hinder the transition from ethylene production system Ⅰ to system Ⅱ before the maturation program begins, resulting in a failure to undergo the characteristic color change from green to deep red. These typical phenomena greatly contribute to our understanding of the transcriptional regulatory mechanisms underlying tomato fruit ripening and carotenoid biogenesis.

RIN, also known as *MADS-RIN*, which regulates fruit ripening and carotenoid synthesis [155]. In the *rin* mutant, the loss of RIN function results in a loss of autocatalytic system Ⅱ ethylene synthesis, and prevents the accumulation of lycopene in fruits [159, 160]. And even with an appropriate amount of exogenous ethylene supply, the fruits still cannot recover their normal mature phenotype [161]. Based on these findings, *rin* was widely recognized as a loss-of-function mutant [162]. Under

normal conditions, MADS-RIN primarily regulates fruit ripening, while MACROCALYX (MADS-MC) is involved in sepal development and inflorescence determinacy. In the *rin* mutant, a 2.6 kb genomic deletion results in the fusion of the *MADS-RIN* and *MADS-MC* genes, forming the RIN-MC fusion protein. This fusion prevents the RIN-MC protein in the *rin* mutant from performing the normal function of MADS-RIN in promoting fruit ripening, thereby affecting both tomato fruit ripening and floral organ development [161]. However, CRISPR-induced MADS-RIN knockout mutation had a milder influence on fruit ripening and carotenoids synthesis, and exhibiting a partial ripening phenotype and low levels of lycopene, which is in sharp contrast to the phenotypes of the classical *rin* mutant [163]. Knockout of chimeric gene *rin-mc* in the *rin* mutant background resulted in partially restored ripening, suggesting that *rin* may be a gain-of-function mutation, which led to a re-evaluate of its function [163, 164]. After overexpression of the *RIN-MC* gene, tomato fruit exhibit a yellow phenotype and show inhibited expression of ripening-related genes, indicating that the *RIN-MC* chimeric gene may also possess repressor characteristics [163, 164]. It has been reported that RIN controlled tomato fruit ripening and the accumulation of carotenoid by targeting several ethylene- and carotenoid-related genes, including *SlACS2*, *SlACS4*, *ETR3*/*NR*, *E4*, and *E8*, as well as *SlPSY1* and *SlZDS* [165–168] (Fig. 4A). Futhermore, some TF-genes can also interact with RIN to regulate carotenoid accumulation in tomatoes (Fig. 4A). For example, RIN can directly bind to the CArG-box motif in the promoter regions of *SlWRKY35*, *SlERF.G3-like* and *SlbHLH95*,

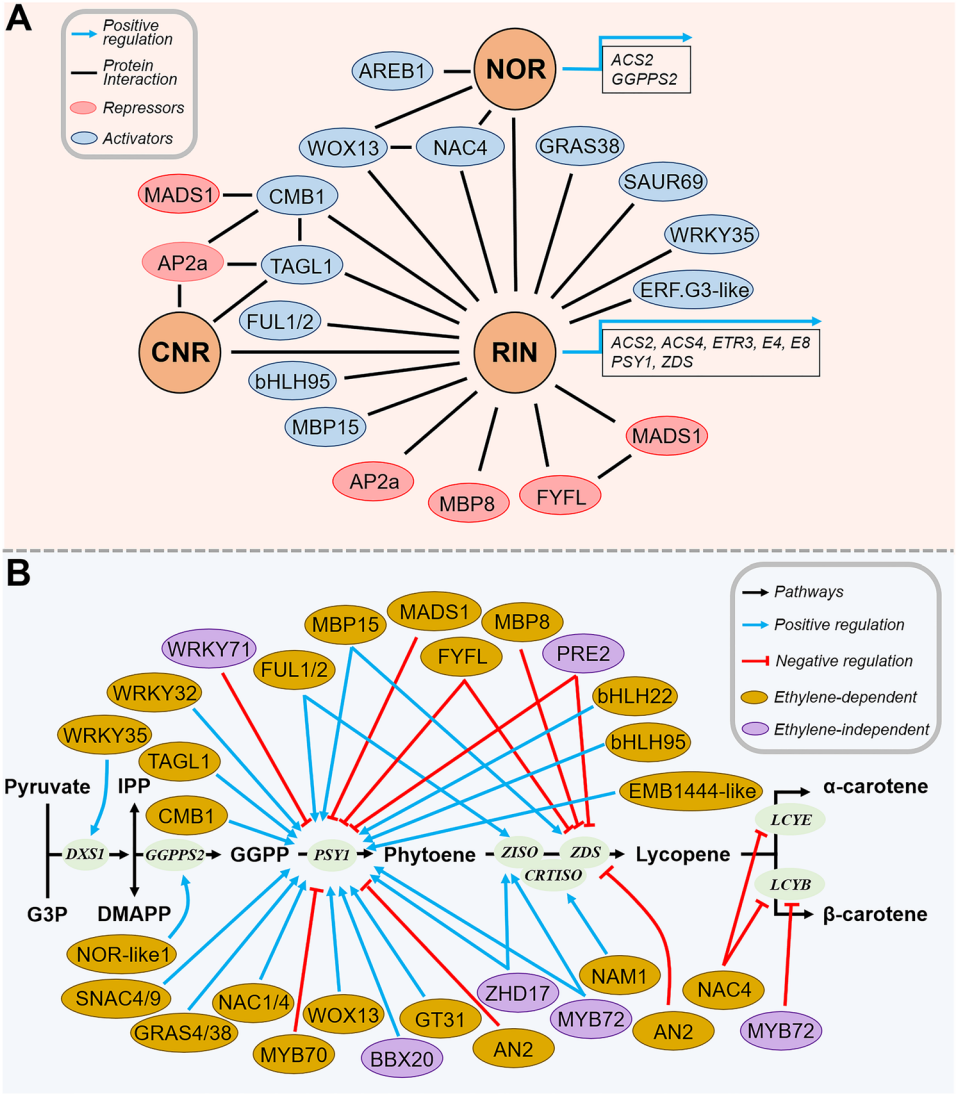

Fig. 4 Schematic diagram of transcriptional regulatory network involved in tomato fruits carotenoid synthesis. **A** Interaction between "master regulators" and other proteins in tomato fruit. **B** Transcriptional regulation of carotenoid synthesis by other TFs in tomato fruits

and activate their expression [1, 129, 169]. SlWOX13 can directly bind to the promoter region of *RIN*, and induce its expression [170]. *SlSAUR69*, *SlGRAS38*, and *SlAP2a* are target genes of RIN, and their expression can be activated by RIN [146, 166, 171]. Although there is no evidence that CNR directly interacts with RIN, CNR can influence the binding activity of RIN to its target genes [168]. Additionally, yeast two-hybrid assays have shown that RIN can bind to *SlNAC4*, *SlMADS1*, *SlFYFL*, *SlMBP8*, *SlMBP15*, and *SlCMB1* [31, 172–176]. Electrophoretic mobility shift assays have demonstrated that RIN can directly bind to *FRUITFULL1* (*SlFUL1*), *SlFUL2*, and *TAMOTO AGAMOUS-like 1* (*SlTAGL1*) through the CArG motif [177]. In addition to RIN, several other MADS-box family TFs have been demonstrated to participate in the regulation of carotenoids during fruit ripening (Fig. 4B). SlMADS1, which belongs to the SEPALLATA genesubfamily, acts as a negative regulator of tomato fruit ripening. The expression of *SlPSY1* was up-regulated in *SlMADS1*-RNAi fruits, and the accumulation of carotenoids was increased compared with that in wild-type fruits [172]. Silencing tomato *SlFUL1* or *SlFUL2* alone only results in a slight decrease in lycopene, but double mutant of these two genes leads to a significant reduction in carotenoid accumulation or even inhibited ripening by blocking ethylene synthesis [177, 178]. Overexpression of the *SlTAGL1* (a member of AGAMOUS clade of the MADS-box TFs family) showed a quite higher concentrations of lycopene, whereas *SlTAGL1* co-suppression fruit was not different from the wild type [179]. Silencing of *SlMBP8* (FLC/MAF group member of the MADS-box TFs family) resulted in up-regulation of *SlPDS* and *SlZDS* and increased carotenoid accumulation [174]. *SlFYFL* (*SlMBP18*) belonging to the SOC1 group of the MADS-box TFs family, its overexpression revealed reduction of carotenoid concentration [173]. In the *SlCMB1*-suppressed lines, the expression of carotenoid synthesis genes such as *SlPSY1* and *SlPDS* is significantly down-regulated, while catabolism genes like *SlLCYB* and *SlLCYE* are up-regulated [176].

The *nor* spontaneous mutant is formed by the deletion of two adenines at the C-terminus of the *NAC-NOR* gene [180]. Spontaneous *nor* mutant retains the capability to bind to downstream gene promoters but fails to activate expression of ethylene- and carotenoid-related target genes. This deficiency prevents tomato fruit ripening and the accumulation of carotenoids, indicating that the classical *nor* mutant exhibits dominant negative regulatory activity [178, 181]. The fruit ripening was partially induced in *NOR* knockout mutant generated using CRISPR, showing an orange phenotype, even though ethylene emission and carotenoid accumulation were significantly suppressed, the impact was far less severe than *nor* spontaneous mutant [178, 181]. Interestingly, these results are similar to those of the CRISPR-*rin* mutant [157]. These "master regulators" may play redundant roles in a complex network integrating multiple signals [19, 155, 182]. Indeed, their influences on ethylene production, lycopene level have been shown to have additive effects [183]. In addition to *NOR*, several other members of the NAC-TFs family have also been identified as candidates for regulating the accumulation of carotenoids during tomato fruit ripening (Fig. 4B). For instance, overexpression of *SlNAC1* leads to a yellow or orange phenotype in mature fruits by reducing carotenoid pathway flux [184]. *SlNAC4*-RNAi reduces carotenoids by inhibiting the expression of system Ⅱ ethylene related genes and carotenoid pathway flux [31]. NOR-like1 controls carotenoid and chlorophyll metabolism by binding to the promoter regions of *SlGGPPS2* and *SlSGR1*. And the CRISPR-mediated *slnor-like1* loss-of-function mutant showed reduced lycopene levels and delayed chlorophyll loss [185]. *SNAC4* (*SlNAC48*) and *SNAC9* (*SlNAC19*) positively regulate fruit ripening and carotenoid accumulation by influencing ABA- and ethylene-related genes [186]. The lycopene content decreased significantly in CRISPR-*snac9* mutants, and there was a notable deceleration in the transformation of chloroplasts into chromoplasts [28]. Although there is no difference in the color of the red ripening stage fruit between the CRISPR-*slnam1* or OE-*SlNAM1* lines and the WT, the time to reach the breaker stage is prolonged in the CRISPR-*slnam1* lings was delayed [187]. Current evidence suggests that NAC-TFs can affect fruit color formation by mediating ethylene or ABA signals or directly targeting key genes in the carotenoid pathway. However, more NAC-TFs involved in regulating fruit ripening and carotenoid accumulation remain poorly understood. Moving forward, it will be crucial to integrate interactions between NAC proteins and other specific ripening-related genes, TFs, and hormonal signals. This approach will enhance our understanding of the transcriptional regulatory network governing tomato fruit ripening and carotenoid synthesis metabolism.

Another *Cnr* spontaneous mutant arises from alterations in the cytosine methylation levels within the promoter region of *SPL-CNR*, a member of the SQUAMOSA Promoter Binding Protein-like (SPL) family [188]. Although *Cnr* spontaneous mutant plants can grow and develop normally, the fruits cannot ripen and remain light-yellow [189]. The *CNR* knockout mutation generated using CRISPR only delayed the ripening time compared with the spontaneous mutant, and the fruit can completely turn red [180]. Epigenetic changes in *Cnr* mutants lead to reduced expression of fruit ripening-related genes and significantly affect fruit ripening and carotenoid accumulation [188]. Silencing of *chromomethylase 3* (*SlCMT3*), a key gene for DNA methylation,

resulted in reversal of ripening in *Cnr* fruits [190]. These results suggest that DNA methylation could be responsible for the colorless non-ripening phenotype observed in *Cnr* spontaneous mutants, or alternatively, unknown gain-of-function mechanisms may also be operative in *Cnr* mutant lines.

Other TFs that regulate the biosynthesis of carotenoids in tomato fruits have also been characterized over the past decade, such as bHLH-, MYB-, WRKY-, GRAS-, and B-box zinc finger-TFs, mainly acting in an ethylene-dependent or ethylene-independent manner (Fig. 4B). SlPRE2, an atypical bHLH-TFs gene, overexpression of the *SlPRE2* gene resulted in yellowed ripe fruit phenotype, and the accumulation of chlorophyll and carotenoids in the fruit pericarp was reduced [191]. OE-*SlbHLH22* changes fruit color from green to orange by regulating ethylene-mediated fruit ripening and carotenoid accumulation [192]. Repression of *SlbHLH95* reduced fruit sensitivity to ethylene and decreased carotenoids accumulation. In addition, the promoter activity of *SlbHLH95* is under the regulation of RIN and affects the expression of RIN-targeted ripening-related genes, including *SlFUL1* and *SlFUL2* [169]. Y1H and EMSA analysis showed that another bHLH-TF, SlEMB1444-like, binds to the E-box motif in the promoter region of *SlYFT1* (encoding the SlEIN2 protein, a core component of ethylene signaling) and regulates chromoplast differentiation and carotenoid accumulation [193]. SlMYB70 dependent on the EAR repression motif to directly bind to the ethylene synthesis genes *SlACS2* and *SlACO3* promoters and inhibit their expression, thereby reducing ethylene production. Meanwhile, *SlPSY1* was down-regulated and the accumulation of carotenoids was reduced in the fruit of OE-*SlMYB70* lines [194]. The down-regulation of *SlMYB72*, a member of the R2R3-MYB subfamily, reduced lycopene content, promoted the production of β-carotene and chromoplast development, resulting in uneven color phenotype. SlMYB72 protein interacts with the SlARF4, directly targeting the *SlPOR* and *SlCHLH* genes and regulated chlorophyll biosynthesis and chloroplast development [30]. Overexpression of *SlAN2* (a member of R2R3-MYB proteins) reduced lycopen, β-carotene, and lutein levels by inhibiting the transcription of *SlPSY1*, *SlPDS*, and *SlZDS*, resulting in an orange phenotype in the fruit [195]. SlWRKY32 binds to W-box and W-box like motifs in the region of *SlYFT1* promoter and induces its expression. In *SlWRKY32*-RNAi line fruits, ethylene signalling is inhibited, carotenoid accumulation is reduced, chromoplast development is delayed, and exhibit a yellow fruit phenotype [25]. SlWRKY35 can directly activate the transcription of *SlDXS1*, thereby reprogramming the MEP pathway to enhance carotenoid accumulation [1]. In addition, the lutein production in the fruits of hybrid lines co-overexpressing *SlWRKY35* and *SlLCYE* was significantly increased [1]. These results indicate that SlWRKY35 can be used as a potential target for metabolic engineering of carotenoid derivatives to enhance the production of specific carotenoid compounds. Overexpression of *SlWRKY71* significantly inhibited the expression of *SlPSY1* and reduced lycopene content, indicating that SlWRKY71 is a repressor of tomato ripening and carotenoid accumulation [196]. SlWRKY71 binds to the W-box motif in the promoter region of CYANOALANINE SYNTHASE1 (CAS1, an enzyme that controls H_2S synthesis in plants) and inhibits its expression. H_2S signaling induces a decrease in BRG3 (BOI-related E3 ubiquitin-protein ligase 3) ubiquitination activity and reduces interaction with SlWRKY71, thereby enhancing the binding dynamics between SlWRKY71 and SlCAS1, ultimately delaying tomato fruit ripening [196]. SlGRAS4 directly binds to the promoters of the ethylene biosynthesis genes *SlACO1* and *SlACO3*, promoting their expression to increase ethylene production. This upregulation of ethylene stimulates the expression of *SlPSY1* and enhances the accumulation of carotenoids [118]. The results of high-resolution spatiotemporal transcriptome analysis showed that SlGRAS38 serves as a regulatory factor downstream of the RIN, and ethylene release and lycopene accumulation in the fruits of its silenced lines are blocked [171]. SlBBX20, a member of B-box zinc finger protein, modulates carotenoid accumulation in tomatoes by directly activating *SlPSY1* [115]. Another member of zinc-finger protein, SlZHD17, regulates plastid transformation and carotenoid synthesis by directly regulating the expression of *SlPSY1* and *SlZISO*, and inhibiting the expression of *SlPOR-B* and *SlTKN2* [113]. SlGT31 (a Trihelix TF) functions as a fruit ripening activator by increasing ethylene content in tomatoes through binding to the promoters of ethylene synthesis genes *SlACO1* and *SlACS4* [197]. In addition, overexpression of *SlGT31* significantly upregulated the expression of *SlPSY1*, thereby enhancing carotenoid accumulation [197]. SlWOX13, a member of WUSCHEL-related homeobox (WOX) TF family, directly regulates carotenoid accumulation and fruit ripening by positively activating *SlPSY1*, *SlPDS*, and *SlACO1* in tomato fruit [170]. Additionally, SlWOX13 directly induces the expression of *RIN*, *NOR*, and *SlNAC4*, which subsequently modulate the expression of ripening-related genes, thereby promoting fruit ripening [170]. In tomatoes, 2026 genes are annotated as TFs, and among them, 516 are expressed in the ripening fruit [180]. This suggests that regulatory network of TFs involved in modulating the ripening process is more intricate and robust than anticipated. Nevertheless, the future challenge is that the molecular mechanisms of fruit ripening and carotenoid accumulation mediated by

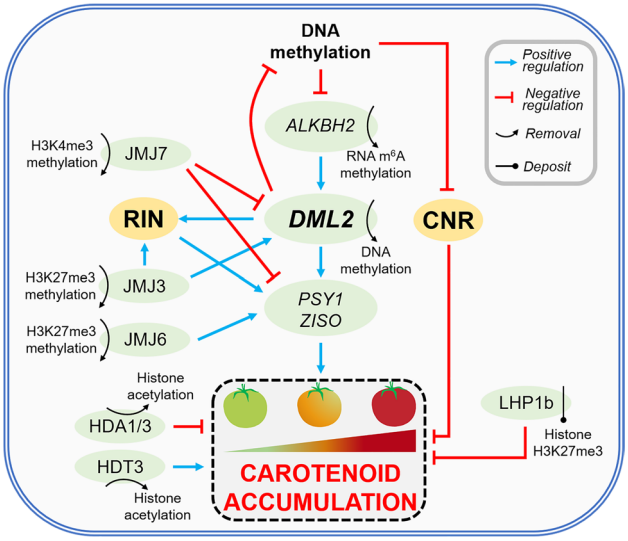

Fig. 5 Roles of epigenetic modifications in tomato fruit carotenoid accumulation. DNA methylation, RNA m^6A methylation, histone methylation, and histone acetylation are the main epigenetic modifications that affect the accumulation of carotenoids in tomato fruits. In tomatoes, *ALKBH2* regulates the stability of *DML2* through RNA m^6A demethylation modification, thereby affecting the expression of carotenoid related synthesis genes and carotenoid accumulation. The histone demethylase JMJ3/6 erases the H3K27me3 marker from genes related to *RIN*, *DML2*, and carotenoid synthesis, thereby positively regulating carotenoid accumulation; The histone demethylase JMJ7 eliminates the H3K4me3 marker from these genes and inhibits carotenoid accumulation. Histone deacetylases HDA1/3 and HDT3 negatively and positively regulate carotenoid accumulation, respectively. PRC1 protein LHP1b can bind to H3K27me3 markers in the chromatin regions of *ACS* and *RIN*, inhibiting the accumulation of carotenoids in tomato fruits

more unknown TFs, or how to balance the interactions between them, still need to be further explore.

Epigenomic regulation of carotenoid in tomato fruit

As mentioned above, these regulatory factors that have been characterized primarily influence fruit ripening and carotenoid accumulation at the transcriptional level. Epigenetic regulatory factors, as a participants in the carotenoid regulatory network, is considered as supplementary transcriptional regulation. And it work together with phytohormones or TFs, playing critical roles in fruit ripening and carotenoids accumulation [5, 6, 198, 199]. Here, we discussed the latest research progress in DNA methylation and demethylation, and histone modifications in carotenoid biosynthesis and accumulation (Fig. 5).

DNA methylation is an epigenetic modification that depends on the maintenance of DNA methyltransferase 1 (MET1), chromomethylase 3 (CMT3), CMT2, and RNA-directed DNA methylation (RdDM) pathway, characterized by the addition of methyl groups to the C5 of the cytosine ring [199–201]. Locus-specific changes in DNA methylation can alter tomato fruit phenotypic traits [199]. In the spontaneous *Cnr* mutant, high levels of cytosine methylation in a 286 bp continuous region 2.4 kb upstream from the first ATG of SPL-CNR, which completely blocks fruit ripening and consequently results in insufficient carotenoid accumulation [188]. To explored whether demethylation could promote early ripening and carotenoid accumulation, researchers treated immature tomato fruit with 5-azacitidine, a MET activity inhibitor. They observed that treatment with 5-azacitidine led to reduced DNA methylation levels and caused an uneven ripening phenotype in fruits. This treatment also resulted in differential expression of carotenoid pathway genes [165]. More importantly, methylation can also be reversed by DNA DEMETHYLASE (DML) enzymes [202], in which DML2 playing a central role in mediating DNA hypomethylation in carotenoid synthesis and chloroplast development [6, 36, 198] (Fig. 5). The level of DNA methylation at *TAGL1* was enhanced in CRISPR mediated *sldml2* knockout lines, resulting in a fruit phenotype with green stripes [198, 203], meanwhile, *PSY1*, *ZDS*, and *CRTISO* were highly methylated, thereby preventing the formation of carotenoids and stable storage in fruit tissues [83, 198]. These results underscore the essential role of *SlDML2* in ripening-induced demethylation and the expression of carotenoid-related genes [198]. This mechanism well explains why DNA methylation levels generally show a downward trend during tomato fruit ripening [36, 165, 198]. In addition, the promoter region of the RIN target genes are hypomethylated during fruit ripening [165], this suggests that TFs may interact with DNA methylation to regulate fruit ripening and development. The mRNA modification mediated by N^6 methyladenosine (m^6A) regulates the phenotype color of ripen tomato fruits by interacting with DNA methylation [204]. The CRISPR mediated *slalkbh2* mutant fruits exhibited a delayed ripen phenotype, and the mRNA level of *SlDML2* decreased. This indicates that SlALKBH2 may be involved in the formation of tomato fruit ripen phenotype color by regulating *SlDML2* mRNA level via m^6A demethylation [204] (Fig. 5). Nevertheless, the modulation of m^6A during fruit ripening is intricate, and there might be additional specific regulatory factors acting as bridges to coordinate the relationship between DNA methylation and mRNA m^6A methylation to regulate fruit ripening.

Histones acetylation and deacetylation, controlled by histone acetyltransferases (HATs) and histone deacetylases (HDACs), respectively, influence carotenoid abundance in tomato fruits by modifying the interaction between histones and DNA chains [119, 205–208]. HDACs appear to play contrasting functions in carotenoid accumulation. Specifically, the knockdown of *SlHDT3* impeded accumulation of carotenoids compared to wild-type fruits [205], while knockdown of *SlHDA1*

and *SlHDA3* leads to a significant increase in carotenoid accumulation [206, 207]. These results suggest that histone acetylation may be involved as a fine-tuning factor in the dynamic balance of carotenoid accumulation in tomato fruit (Fig. 5). The trimethylation of histone H3 at lysine 27 (H3K27me3) is a relatively conserved inhibitory epigenetic marker associated with fruit ripening, which is responsible by the Polycomb Repressive Complex 2 complexes (PRC2s) [209–211]. H3K27me3 was detected on *RIN* and *SlACS2* in immature fruits, indicating that H3K27me3 may participate in fruit ripening regulation by inhibiting system II ethylene production [212]. Several recent studies have further demonstrated that histone H3K27me3 and DNA methylation synergistically control the accumulation of carotenoids in fruits (Fig. 5). For example, a Polycomb Repressive Complex 1 (PRC1)-like protein, such as Like Heterochromatin Protein 1b (SlLHP1b), suppresses tomato fruit ripening and carotenoid content by preserving epigenetic mark H3K27me3 in *SlACS* and *RIN* chromatin [213]. The *SlJMJ6* (a histone lysine demethylase) enhances accumulation of carotenoids by removing H3K27me3 levels on *SlGGPPS2*, *SlPSY1*, and *SlZISO* [214]. SlJMJ3 directly interacts with the chromatin regions of *RIN*, *NOR*, *FUL1*, and *DML2*, which demethylates H3K27me3 of these genes and activates their transcription, further up-regulating the expression of *SlDXS1*, *SlPSY1*, and *SlZISO*, which promotes tomato fruit carotenoid synthesis [215]. Additionally, it has been confirmed that SlJMJ7 upregulates *SlPSY1* expression and increases lycopene content through crosstalk between H3K4me3 and DML2 mediated DNA demethylation [35].

Conclusions and future perspectives

Our understanding of carotenoid biosynthesis and regulation in tomato fruits has significantly advanced due to the in-depth application of high-throughput sequencing and "Large-scale data" assembly technologies. Transcription factors, epigenetic modification, developmental programs, phytohormones and environmental signals constitute a complex regulatory network, and these events work together to maintain a dynamic balance of carotenoid synthesis. In tomatoes, 2026 genes are annotated as TFs, and among them, 516 are expressed in the ripening fruit [180]. This suggests that regulatory network of TFs involved in modulating the ripening process is more intricate and robust than anticipated. Ethylene seems to play the role of a "central system" in the synthesis of carotenoids, current research mainly focuses on TFs controlling ethylene biosynthesis to regulate carotenoid synthesis, and forming a relatively systematic regulatory network; In addition, RIN induces ethylene synthesis by mediating some TFs and epigenetic modifications. Based on current research foundations and advancements, we believe several unresolved issues still require in-depth exploration.

I. Although a sufficient number of transcription factors involved in regulating carotenoid accumulation in tomato fruits have been identified, most studies have focused on specific transcription factors triggered by environmental changes and plant hormones. In the future, it is likely that more transcription factors involved in regulating carotenoid accumulation in response to exogenous stimuli will be discovered, particularly those related to emerging research topics such as gaseous signaling molecules and pathogen infection. For example, both H_2S and NO have been shown to inhibit carotenoid accumulation; when tomato fruits are subjected to pathogen stress, carotenoid accumulation may be affected through enhanced antioxidant capacity. However, the specific transcriptional regulatory mechanisms underlying these processes still require further investigation.
II. Some transcription factors usually do not work alone, but interact through complex transcriptional cascade networks to jointly regulate the synthesis of carotenoids. For example, the transcriptional cascade mediated by AP2a and AP2c regulates the biosynthesis of lycopene during tomato ripening; the transcriptional cascade formed by AP2a/DWARF/BZR1 promotes lycopene synthesis. The analysis of these specific transcription factor modules helps to understand the metabolic regulation of carotenoid accumulation in tomato fruit. Therefore, future studies should perhaps focus more on the systemic regulatory mechanisms of these transcription factor networks.
III. The integration of multi-omics data can provide a panoramic view of the regulation of carotenoids and help reveal the complex regulatory network from genes to metabolites. The expression profile of carotenoid-related genes in tomato fruits can be obtained through high-throughput RNA sequencing technology, and the concentration changes of carotenoids and their derivatives (such as lycopene, β-carotene,) can be accurately determined by combining liquid chromatography-mass spectrometry (LC-MS). In addition, the analysis of metabolic channels and the precise monitoring of metabolic flux are crucial to reveal the dynamics of metabolic reactions in the carotenoid synthesis pathway. Metabolic flux analysis, combined with stable isotope labeling and metabolic network model optimization, can quantitatively analyze the metabolic flux in the carotenoid synthesis pathway. In tomato fruits, researchers can track how these precursors flow to the carotenoid synthesis pathway

by labeling specific precursor substances (such as IPP), thereby finding the key links to improve carotenoid synthesis.

IV. In post-transcriptional regulation, the role of non-coding RNA (such as miRNA, lncRNA) in the stability and translation efficiency of carotenoid synthesis gene is studied. For example, RNA-Seq and CLIP-Seq are used to analyze how specific miRNA targets carotenoid synthesis genes and regulates their stability and translation efficiency. In post-translational regulation, mass spectrometry, phosphorylation and acetylation can be used in the future to deeply analyze the post-translational modification patterns of key transcription factors to study the regulatory effects of specific modifications on transcription factor activity. In addition, by changing the specific sites of transcription factors through CRISPR-mediated site-directed mutagenesis, the specific role of post-translational modifications in regulating carotenoid synthesis can be verified.

Therefore, in the future, it is necessary to establish a comprehensive network based on the identification of multi-level regulatory modules of tomato fruit carotenoids to provide a theoretical basis and technical support for improving tomato fruit quality and enhancing carotenoid biosynthesis.

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Authors' contributions
YW and JY conceived the review. JW wrote the manuscript. YW and JY added valuable comments and improved the paper. All the authors read and approved the finalized manuscript.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
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Competing interests
The authors declare no competing interests.

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