Prospects and progress on crocin biosynthetic pathway and metabolic engineering

Taoli Liu a,b,1, Suize Yu a,b,1, Zhichao Xu c, Jiantao Tan a,b, Bin Wang a,b, Yao-Guang Liu a,b,⇑, Qinlong Zhu a,b,⇑

a State Key Laboratory for Conservation and Utilization of Subtropical Agro-Bioresources, College of Life Sciences, South China Agricultural University, Guangzhou 510642, China
b Guangdong Laboratory for Lingnan Modern Agriculture, Guangzhou 510642, China
c Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing 100193, China

Abstract

Crocins are a group of highly valuable apocarotenoid-derived pigments mainly produced in Crocus sativus stigmas and Gardenia jasminoides fruits, which display great pharmacological activities for human health, such as anticancer, reducing the risk of atherosclerosis, and preventing Alzheimer’s disease. However, traditional sources of crocins are no longer sufficient to meet current demands. The recent clarification of the crocin biosynthetic pathway opens up the possibility of large-scale production of crocins by synthetic metabolic engineering methods. In this review, we mainly introduce the crocin biosynthetic pathway, subcellular route, related key enzymes, and its synthetic metabolic engineering, as well as its challenges and prospects, with a view to providing useful references for further studies on the synthetic metabolic engineering of crocins.

1. Introduction

Crocus sativus L., a perennial herb belonging to the Iridaceae family, is well known for its dried red stigmas, called saffron [1]. Due to its complicated harvesting process, low yield, and expensive labor costs, saffron, known as “red gold”, is one of the most expensive spices on Earth [2,3]. Saffron not only has powerful...
pharmacological activities but is also the source of some unique apocarotenoids, including crocins (crocetin glycosyl esters), picrocrocin, and safranal, responsible for its red color, bitter taste, and pungent aroma, respectively [4,5]. *Gardinia jasminoides* has been cultivated in China for nearly 1000 years with great ornamental and medicinal value. The dried ripe fruit of *G. jasminoides*, a natural yellow dye, is also a popular traditional Chinese medicine because of its various biological activities [6,7].

Crocins, belonging to apocarotenoid glycosides, are the main bioactive ingredients and colorants in *C. sativus* stigmas and *G. jasminoides* fruits [8,9]. Glycosylation reactions are catalyzed by glycosyltransferases acting on the carboxyl- and glycosyl- groups of crocetins and crocins, and form five kinds of crocins: crocin-Ⅰ, crocin-Ⅱ, crocin-Ⅲ, crocin-Ⅳ, and crocin-Ⅴ [10]. Among these, crocin-Ⅰ is the main constituent [4,11]. Different from most of the lipid-soluble carotenoids (including lycopene, β-carotene, zeaxanthin, and lutein), crocins are water-soluble, which can be attributed to these glycosylations/their glycosylation [12].

Modern pharmacological studies show that crocins have antioxidant [12,13], anti-inflammatory [14,15], anti-hyperlipidemic [16], anticancer [17–19], and antithrombotic [20] properties, as well as potential properties for treatment of Alzheimer’s disease [12]. In recent years, crocins have drawn increasing attention in the medical, food, and cosmetics fields for their excellent pharmacological and coloring functions. Traditionally, crocins have been used for their medical, food, and cosmetics fields for their excellent pharmacological and coloring functions.

2. Crocin biosynthesis

Carotenoids are a group of important pigments found in plants, algae, bacteria and fungi. Carotenoids contribute to the bright colors of vegetables, fruits, and flowers, and play a crucial role in human health [23]. Their cleavage products, called apocarotenoids, are formed after oxidative cleavage catalyzed by a family of double bond-specific carotenoid cleavage dioxygenases (CCDs) [24]. There are some unique apocarotenoids, including crocetins, crocins, picrocrocins, and safranins, which accumulate at high levels only in saffron and gardenia. Among these, crocins, the glycosylated forms of apocarotenoids, are the most valuable and stable constituents in *C. sativus* stigmas [25,26]. Crocins have two glycosyls, including β-D-glucosyl and β-D-gentiobiosyl, and according to the position and numbers of these two glycosyls, crocins could be divided into five forms, crocin-Ⅰ, crocin-Ⅱ, crocin-Ⅲ, crocin-Ⅳ, and crocin-Ⅴ. To date, the crocin biosynthetic pathway in plants has been elucidated by transcriptome and genome sequencing of *C. sativus* and *G. jasminoides* (Fig. 1) [3,24,27–30].

2.1. Crocin biosynthetic pathway in plants

Crocins are accumulated in stigmas of *C. sativus* and fruits of *G. jasminoides* in a tissue-specific manner, and their biosynthesis requires a high degree of coordination of several pathways, including the upstream methylethylthiol phosphate (MEP) pathway, the midstream carotenoid biosynthetic pathway, and the downstream crocin biosynthetic pathway [3,31].

In the MEP pathway, deoxyxylulose-5-phosphate (DXP) synthase utilizes pyruvate and glyceraldehyde-3-phosphate (GAP) as initial substrates to form isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). Carotenoid biosynthesis occurs in plastid and begins with geranylgeranyl diphosphate (GGPP), which is produced by the condensation of IPP and DMAPP [27]. Although IPP and GPP can be synthesized by the cytoplasm-localized mevalonate pathway (MEV) pathway and the plastid-localized MEP pathway, only the latter provides precursors for plant carotenoid biosynthesis in plastid. Firstly, phytoene synthase (PSY), the first rate-limiting enzyme of the carotenoid biosynthetic pathway, catalyzes the condensation of two molecules of GGPP to form the first carotenoid, 15-cis-phytoene [32]. Then, 15-cis-phytoene is converted into all-trans-lycopene by desaturation and isomerization, which is a multi-step process requiring two desaturases, phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS) [33], as well as two isomerases, ζ-carotene isomerase (Z-ISO) [34] and carotenoid isomerase (CRTISO) [35,36]. Lycopene, the branching point in carotenogenesis, acts as the substrate for lycopene ζ-cyclase (ζ-CLY) and lycopene β-cyclase (β-CLY) leading to formation of α-carotene, which is further hydroxylated to lutein, or for β-CLY alone leading to formation of β-carotene, which is further hydroxylated to zeaxanthin by β-carotene hydroxylase (BCH) [37]. The key enzymes involved in general carotenoid biosynthesis, including GGPP, GGPPS, PSY, PDS, BCH, ZDS, Z-ISO, CRTISO, β-CLY, and α-CLY, have so far been identified from *C. sativus* [24,27,30,38], and further functional characterization of enzyme-coding genes indicates that CsBCHI [38], CsLcyB2a [39], and CsPsy2 [40] are responsible for carotenoid accumulation in stigmas of *C. sativus*. Recently, Ji et al. [3] performed the first transcriptome sequencing of fruits in *G. jasminoides* and identified candidate genes encoding the key enzymes involved in the MEP and carotenoid biosynthesis pathways.

Both β-carotene and zeaxanthin are important precursors for apocarotenoid biosynthesis and are converted by different CCDs leading to different apocarotenoid compounds [38,41]. For example, CsCCD1 and CsCCD4 cleave β-carotene at the 9, 10 and 9,10' double bonds into β-ionone and β-cyclocitral [24,41], while *Arabidopsis thaliana* CCD7 (AtCCD7) and *Arabidopsis thaliana* CCD8 (AtCCD8) are responsible for strigolactone production by cleavage of β-carotene [42]. In the crocin biosynthetic pathway, CCD2, a recently identified CCD family member from *C. sativus*, cleaves the 7, 8 and 7', 8' double bonds of zeaxanthin to produce crocetin dialdehyd and 3-OH-β-cyclocitral, which is the first step of saffron crocin biosynthesis [22]. Aldehyde dehydrogenase (ALDH) catalyzes the conversion of crocetin dialdehyde to crocetin. UDP-glucosyl transferases (UGT) transform crocetin and β-cyclocitral into crocins (crocin-Ⅰ to crocin-Ⅴ) and picrocrocin, respectively. Finally, picrocrocin is converted into a safranal under the combined action of heating and β-glucosidase (β-GS) [27]. The reported transcriptome data and functional assays show that *CsCCD2* [2], CsALDH31 [43], and CsUGT74A1 [43] as well as *GjCCDA4* [29, GjALDH2C3 [29], GjUGT74FB [29,44], and GjUGT94E13 [29,44], have been identified to be involved in the complete crocin biosynthetic pathway in stigmas of *C. sativus* and fruits of *G. jasminoides*, respectively.
2.2. Subcellular routes for crocin biosynthesis

It has been reported that zeaxanthin and crocin are localized in plastids and vacuoles, respectively, suggesting that the crocin biosynthetic pathway might involve multiple subcellular compartments [43,45,46]. Initially, CsCCD2 that cleaves zeaxanthin to produce apocarotenoid was reported to be localized in the cytoplasm [2]. However, Ahrazem et al. [47] identified a longer CsCCD2 version, CsCCD2L, and the transient expression experiment in tobacco leaves indicated that CCD2 is a plastid-localized enzyme rather than a cytoplasm-localized enzyme, which was further confirmed by immunogold electron microscopy on C. sativus stigmas and confocal fluorescence microscopy in N. benthamiana leaves [43]. At present, there are two models regarding the subcellular route of crocin biosynthesis in saffron. In 2017, Gómez-Gómez et al. [46] proposed the first model through a proteome analysis and microscopy studies: crocins are synthesized in the chromoplast and then accumulated in chromoplast-localized vesicles to form crocino-plasts, which are further transported from the end of the chromoplast to the vacuole for storage. In 2018, Demurtas et al. [43,48] analyzed the localization of CsCCD2, CsALDH3I, and CsUGT74AD1, which have been characterized as responsible for crocin biosynthesis, and proposed a different model: crocetin dialdehyde, crocetin, and crocins are synthesized in the chromoplast, the endoplasmic reticulum (ER), and the cytoplasm, respectively. Finally, crocins are transported from the cytosol to the vacuole for storage through unidentified tonoplast transporters. Recently, Demurtas et al. [49] developed a transportomic approach to identify saffron tonoplast
transporters mediating crocin accumulation in the vacuole, and CsABCC4a, a tonoplast transporter belonging to the ATP-binding cassette C (ABCC) transporter family, was characterized to be involved in crocins transport in saffron. Since plastid-localized ALDH and UGT have not been identified and the subcellular route of stevioside biosynthesis in *Stevia rebaudiana* leaves is similar to the second model described above [50], we prefer to consider the second model as the true subcellular route of crocin biosynthesis in saffron (Fig. 2).

### 2.3. Key enzymes involved in crocin biosynthesis

In recent years, with the increasing number of reports on genomic and transcriptomic analyses of *C. sativus* and *G. jasminoides* [3,24,27–30], the functional characterization and localization of three key enzymes (CCD, ALDH, and UGT) involved in crocin biosynthesis have been described [2,23,29,43,44,47,51]. From these studies, we have a better understanding of the crocin biosynthetic pathway and provide guidance for using metabolic engineering to enhance crocins production. The key enzymes for crocin biosynthesis that have been identified are summarized in Table 1.

#### 2.3.1. Enzyme catalyzing the cleavage reaction: carotenoid cleavage dioxygenase (CCD)

The first step in crocin biosynthesis is the cleavage of carotenoids by CCD to produce crocetin dialdehyde. CCDs, a class of double bond-specific enzymes involved in apocarotenoid biosynthesis, have been grouped into five clusters in plants according to their substrate preference and/or the cleavage position, named CCD1, CCD4, CCD7, CCD8, and nine-cis-epoxy-carotenoid cleavage dioxygenases (NCEDs) [24,52,53]. Structurally, all CCDs contain a conserved seven-bladed β-propeller structure, a less-conserved dome region formed by a-helix elements, strands and loops, and a Fe²⁺ ion essential for the catalytic activity [54,55]. Zeaxanthin cleavage dioxygenase (ZCD) was first reported to mediate crocetin production by cleavage of zeaxanthin at the 7, 8 and 7', 8' double bonds [45]. However, later studies including sequence and structure
analyses, in vivo and in vitro assays, showed that ZCD is an N-truncated CCD4 form with a lack of activity to convert zeaxanthin into crocetin dialdehyde [2,41].

Through deep transcriptome sequencing, Fruscianti et al. [2] identified a new di-oxygenase named CCD2 from C. sativus, which belongs to a novel CCD clade closely related to the CCD1 subfamily, and found that the expression pattern of CCD2 is consistent with crocins accumulation during stigma development. Further in vivo and in vitro functional analyses revealed that CsCCD2 is able to catalyze the conversion of zeaxanthin into the crocins precursor crocetin dialdehyde [2]. The second member of the CCD2 subfamily, CaCCD2, was identified from C. sativus by Ahrazem et al. [47], and like CsCCD2, it is functionally characterized as involved in crocin formation. The identification and functional characterization of CsCCD2 and CaCCD2 confirm that the cleavage of zeaxanthin by the CCD2 enzymes can give rise to crocetin dialdehyde. Lately, according to full-length transcriptome data of C. sativus, Yue et al. [30] proposed that CCD2 might have evolved from the CCD1 subfamily via the whole-genome duplication event.

To date, the CCD2 enzymes have only been identified from Crocus species [2,47], but crocetin has also been found in other plant organs, such as fruits of G. jasminoides [56] and flowers of Buddleja davidii [57]. Recently, BdCCD4.1 and BdCCD4.3 were identified from Buddleja davidii and exhibited cleavage activity over zeaxanthin in vivo and in vitro, leading to production of crocetin dialdehyde [23]. It is reported that BdCCD4.1 and BdCCD4.3 are located in plastids, which is consistent with CCD2 localization [23]. However, it is worth noting that GjCCD4a identified from G. jasminoides not only has the same catalytic activity as CsCCD2, CaCCD2, BdCCD4.1, and BdCCD4.3 to cleave zeaxanthin, but also catalyzes the production of crocetin dialdehyde from lycopene and \( \beta \)-carotene [29].

Therefore, the first dedicated step in crocin biosynthesis can be catalyzed by the CCD2 enzymes from Crocus [2,47] and by the CCD4 enzymes from Buddleja [23] and Gardenia [29], indicating that these CCD enzymes acquire the ability to cleave zeaxanthin at the 7, 8 and 7', 8' double bonds through convergent evolution in different species [29].

### 2.3.2. Enzyme catalyzing the dehydrogenation reaction: ALDH

The second step in crocin biosynthesis is the dehydrogenation of crocetin dialdehyde to produce crocetin. The ALDH superfamily is generally characterized by the use of NAD(P)\(^+\) as a cofactor to catalyze the conversion of aldehyde groups to carboxyl groups [58]. The plant ALDHs have been grouped into 13 subfamilies [58]. In 2018, Demurts et al. [43] identified six candidate genes encoding ALDHs from C. sativus stigmas, and only one of their corresponding proteins, CsALDH3I1, was characterized as an enzyme capable of converting crocetin dialdehyde into crocin by using an Escherichia coli expression system and an in vitro enzyme activity assay. Like those ER-localized proteins containing a C-terminal KXXX signal, CsALDH3I1 harboring a C-terminal KKPK signal was also confirmed to be localized in the ER [43,59]. Recently, GjALDH2C3 from G. jasminoides, whose expression accompanied the accumulation of crocins in fruits of G. jasminoides, was reported to be involved in the second step in crocin biosynthesis by Xu et al., and they also identified crocetin semialdehyde as an intermediate product in the catalytic process from crocetin dialdehyde to crocin [29]. In addition, some candidate genes that might encode ALDHs responsible for crocin production have also been identified from plants by transcriptome analyses, such as ADH2946, ADH11367, and ADH54788 derived from C. sativus [46], and ALDH12 and ALDH14 derived from G. jasminoides [3]. However, further in vivo and in vitro studies are needed to determine their functions.

### 2.3.3. Enzyme catalyzing the glycosylation reaction: uridine diphosphate glycosyltransferase (UGT)

The final step in crocin biosynthesis is the glycosylation of crocin by UGTs to produce crocins. UGTs catalyze glucose molecules and specific receptors to be linked together by glycosidic bonds. In

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**Table 1**

| Key enzymes | Name       | Species          | GenBank accession no. | Identification methods                                         | Refs   |
|-------------|------------|------------------|-----------------------|---------------------------------------------------------------|--------|
| PSY         | CcPSY2     | Crocus sativus   | MH112439              | bioinformatics analysis, subcellular localization, expression analysis, functional complementation | [40]   |
| β-LCY       | CsLcyB2a   | Crocus sativus   | GQ202141              | bioinformatics analysis, expression analysis, in vivo functional analysis | [39]   |
| BCH         | CbBCH1     | Crocus sativus   | CAC95130              | various PCR-based methods, bioinformatics analysis, expression analysis | [38]   |
| CDD         | CsCCD2     | Crocus sativus   | KPS41748              | subcellular localization, bioinformatics analysis, in vivo functional analysis | [47]   |
|             | CsCCD2L    | Crocus sativus   | KPS87110              | bioinformatics analysis, in silico analysis                   | [47]   |
|             | CaCCD2     | Crocus ancyrensis| KP792756              | bioinformatics analysis, in silico localization, expression analysis, in vivo functional analysis | [47]   |
| BdCCD4.1    | Buddleja davidii | KX374547       | bioinformatics analysis, in silico localization, expression analysis, in vivo and in vitro assays | [23]   |
| BdCCD4.3    | Buddleja davidii | KX374549       | bioinformatics analysis, in silico localization, expression analysis, in vivo and in vitro assays | [23]   |
| ALDH        | CsALDH3I1  | Crocus sativus   | MF596165              | subcellular localization, transcripts analysis, expression analysis, in vivo and in vitro assays | [43]   |
|             | GjALDH2C3  | Gardenia jasminoides | KYS31926       | Genome-wide analysis, bioinformatics analysis, expression analysis, in vivo and in vitro assays, LC/LC-MS analyses | [29]   |
| UGT         | GjUGT756   | Gardenia jasminoides | AY262037       | Genomic analysis, expression analysis, in vitro functional assays | [25]   |
|             | GjUGT74E5  | Gardenia jasminoides | FBW38K05       | expression analysis, bioinformatics analysis, in vitro functional assays | [51]   |
|             | GjUGT74F8  | Gardenia jasminoides | MN944054       | Genomic analysis, expression analysis, in vitro functional assays | [29,44]|
|             | GjUGT94E13 | Gardenia jasminoides | MN944055       | Genomic analysis, bioinformatics analysis, expression analysis, in vivo and in vitro assays, LC/LC-MS analyses | [29,44]|
| Bc-GT       | Bacillus subtilis | WP_003220110.1     | bioinformatics analysis, in vitro functional assays | [10]   |
saffron, the glycosylation of crocetin results in the transformation of lipid-soluble carotenoid into water-soluble and stable apocarotenoid glycosides, crocins. Moraga et al. [25] cloned two UGTase-encoding genes (UGTs2 and UGTS3) containing the plant secondary product GTase (PSPG) box from C. sativus using degenerate primers, and found that UGTs2 and UGTS3 were expressed at high levels in stigmas and stamens, respectively. Expression of UGTS2 in E. coli and an in vitro enzyme activity assay show that UGTs2 exhibits strong glucosylation activity toward crocetin, β-D-glucosyl ester, and crocetin β-D-gentibiosyl ester [25]. To further verify the function of UGTS2, Demurtas et al. [43] searched for the corresponding transcript in the transcriptome derived from their own and others' studies [27], and found a new gene encoding a different version of UGTS2, CsUGT74AD1, whose corresponding protein CsUGT74AD1 was identified to be responsible for the primary glycosylation of crocetin [43]. It is reported that CsUGT74AD1 displays a cytoplasmic localization [43]. Recently, UGT709G1, a novel UGT involved in picrocrocin biosynthesis, was identified and characterized from saffron, which contributes to the industrial production of safranal [60].

Besides saffron, crocins are also highly enriched in gardenia. In 2012, Nagatoshi et al. [51] identified and functionally characterized two glucosyltransferases, GjUGT75L6 and GjUGT94E5, which mediate crocetin production in G. jasminoides. An in vitro enzyme activity assay demonstrate that the carboxyl group of crocetin is first glycosylated by UGT75L6 forming crocetin glucosyl esters, which is further glycosylated on the 6-position hydroxyl group of the glucose moiety by UGT94E5, yielding other forms of crocins [50]. However, the expression profiles of the genes encoding these two UGTs are not correlated with the distribution of crocins in gardenia, suggesting that there may be other UGTs involved in crocin synthesis in gardenia [51]. Recently, GjUGT74F8 and GjUGT94E13 from G. jasminoides identified by Xu et al. showed an expression pattern consistent with crocin accumulation, and in vitro functional analysis confirmed that their corresponding proteins were responsible for the primary and secondary glycosylation of crocetin [29]. Furthermore, using E. coli as an expression system, GjUGT74F8 and GjUGT94E13 can lead to efficient conversion of crocin to five types of crocins [44], indicating that GjUGT74F8 and GjUGT94E13 are involved in crocin biosynthesis in gardenia.

Based on the substrate and position of glycosylation, the above UGTs can be divided into three types: UGT-I type (UGTCS2/CSUGT74AD1/UGT75L6/UGT74F8/BS-GT) for the primary glycosylation of crocetin to produce crocetin monoglucosyl and diglucosyl esters; UGT-II type (GjUGT94E5) for the secondary glycosylation of Glc groups to form one or two gentiobiose groups; UGT-III type (GjUGT94E13) for the primary and secondary glycosylations. Since the functions of most of the reported UGTs are determined by in vitro enzyme activity assays, it is necessary to further verify their functions in plants.

3. Crocin metabolic engineering

As a high-value carotenoid, crocins have great potential in pharmacology. Currently, the main production method of crocins is extraction and purification from C. sativus. However, complicated production methods and expensive raw materials have been the main factors limiting the mass production and broad application [44]. To improve the manufacturing technology, the research of crocin metabolic engineering has attracted increasing attention, and the development of engineered microorganisms and biofortified plants to produce crocin in vivo is the main research direction.

The biosynthesis of apocarotenoids in C. sativus was studied and analyzed by transcriptomics and dynamic metabolomics [28]. The synthetic pathway of crocins has been largely revealed, and many key enzymes have also been found and applied in metabolic engineering. The biosynthetic pathway of carotenoids in C. sativus includes: starting with geranylgeranyl diphasophate, zeaxanthin was gradually synthesized, and the specific synthesis of crocetin and crocins by cleavage of zeaxanthin. Enzymes of the upstream crocin synthesis pathway, such as PSY, PDS, and CRT, have been used to synthesize carotenoids such as lycopene, β-carotene, and zeaxanthin in various crops. The combined overexpression strategy of multiple biosynthetic genes co-transformation can effectively improve the accumulation of target carotenoids. Zeaxanthin is the key intermediate product in the synthetic pathway, an important direct precursor for the synthesis of crocins [8]. In the published studies, researchers either integrated the β-carotene hydroxylase gene to synthesize zeaxanthin, or directly optimized a microbial strain already producing zeaxanthin. According to the existing reports (Table 2), many microbes have been successfully transformed to synthesize crocin or crocins, including E. coli [39,44], Chlorella vulgaris [61], and yeast [62]. Recently, the transient transformation of Nicotiana benthamiana to synthesize crocins were reported [63].

E. coli has a simple and clear genetic background, and its related genetic engineering technologies are mature and widely used, which make it an excellent host bacterium for the production of crocin by metabolic engineering. Based on the terpenoid producing strains, Wang et al. used pyruvic acid and glyceraldehyde-3-phosphate as substrates, and introduced a series of genes, cte, cteB, cteT, cteV, and cteZ, to gradually synthesize lycopene to β-carotene from upstream prenyl diphasophate (IPP) and dimethylallyl diphasophate (DMAPP), then finally obtained the zeaxanthin-producing strains YL4 and YL5. Also, by integrating and optimizing the expression of heterologous genes, CsCCD2 from C. sativus and ALD8 from Neurospora crassa in YL4 and YL5, crocetin was produced. Finally, the glycosyltransferase genes YjiC, YdhE, and YojK from Bacillus subtilis were introduced, and five crocins were produced [26].

In the research of eukaryotes, Saccharomyces cerevisiae and Chlorella vulgaris have been successfully engineered to produce crocetin. Chai et al. introduced heterologous CroZ, CCD, and ALD into a β-carotene producing strain (S. cerevisiae, SyBE_SC0014CY06), and successfully accomplished the biosynthesis of crocetin in S. cerevisiae [62]. The enzyme source combination PsCrZ (from Pantoea stewartii) CCD2 with the highest yield was found by introducing nine CroZs and four CCDs from various sources. By overexpressing CCD2 and ALD (from Synecochysis sp.) and controlling the reaction temperature, the reported highest titer in shake flask was 1219 μg/L. The titer of crocetin in a 5-L bioreactor reached 6278 μg/L through fed-batch fermentation, which is the highest crocetin titer reported in eukaryotic cells. In C. vulgaris, Lou et al. transformed crtRB and CCD1 into a wild type strain with a similar design as above, and produced crocetin without exogenous ALD by using the rich secondary metabolites and potential aldehyde oxidase activity in C. vulgaris [61]. Recently, Martí et al. [63] constructed transient transformation vectors for Nicotiana tabacum by using the Tobacco etch virus (TEV), and transformed the tobacco plants with a series of CCD genes and several carotenoid synthesis related genes. The accumulation of yellow pigments of crocins and picrocrocin can be seen with naked eyes in leaves infected by the virus. Crocins (0.2%) and picrocrocin (0.8%) were detected in the plants expressing only the CCD gene, which were in relatively low levels. Meanwhile, expression of the phytoene synthase gene (PcCrtB) could increase the crocin content, reaching as much as 0.35% of dry weight [63]. This is a successful case of crocin synthesis in higher plants. However, stably inherited plants cannot be obtained by the transient transformation. To develop valuable bioreactors, it will be necessary to create de novo synthetic genetic engineering plants using stable transformation methods.
The above cases provide solid theoretical support and practical basis for further synthesizing crocins in higher plants, especially in crop plants. There have been many reports on the successful synthesis of carotenoids, such as β-carotene, zeaxanthin, canthaxanthin and astaxanthin, in crops like maize, rice, and tomato [65,66], which prove the feasibility of synthesizing crocins in plants. Many plants have robust carotenoid biosynthesis and accumulation providing potential precursors for crocins. For example, tomato contains a high content of lycopene, and even zeaxanthin, the direct precursor of crocetin, which is the main carotenoid in maize. When these plants are genetically engineered, the carotenoids present in them can be used as the synthetic raw materials. During the transformation, the existing endogenous synthetic pathways can be utilized, and the existing or low-expression genes in them can be complemented, which can simplify the operation. For plants with little carotenoid accumulation and lack of suitable endogenous genes, such as rice endosperm, it is necessary to introduce a complete synthetic pathway to complete the de novo synthesis of crocins. Using multi-gene stacking technology, such as TransGene Stacking II (TGSI) [64], multiple gene expression cassettes can be transferred at the same time, and with high-expression promoters, so that high-level enzymes can be accumulated at specific tissues to synthesize target products [64,65,66].

4. Challenges and prospects

In recent years, with the mining of genomic and transcriptomic data of crocin-producing species (mainly saffron and gardenia) [3,24,29,30], the complete crocin biosynthetic pathway in plants has been elucidated, and some crocin biosynthetic genes have been identified from several plants and microorganisms by in vivo and/or vitro assays. However, there are still some challenges to overcome, such as the lack of studies of the regulatory mechanism of crocin biosynthesis and genome-wide data of C. sativus, as well as the so far missing identification of the enzyme catalyzing secondary glycosylation of crocetin in saffron. In addition, the production of crocins through metabolic engineering to meet current demands remains a challenge, despite relevant studies on crocin or crocetin production in microbial cell factories such as E. coli [10,26,44] and yeast [28,62]. How to improve the productivity and yield of industrial platform strains has become the first problem to be solved in the industrialization of crocin metabolic engineering. Some new technologies and methods may be used to improve metabolic engineering of microbial strain for production of glycosylated crocins, for examples: (1) development of specific CRISPR engineered E. coli strains or yeast to redirect metabolic fluxes for crocins production [67]; (2) using bacterial microcompartments (BMCS) technology to efficiently synthesize crocins without affecting normal metabolic activities of cells [68]; and (3) Using the synthetic microbial consortia method [69], which can maximize the synthesis efficiency.

On the other hand, there are many directions for future research on crocin biosynthesis and metabolic engineering in plants, for examples: (1) genome-wide sequencing of C. sativus to gain an in-depth understanding of the evolution of crocin biosynthesis; (2) identification of the enzyme(s) responsible for secondary glycosylation of crocetin in saffron to understand the complete crocin biosynthetic pathway in C. sativus; (3) identification of more genes encoding transcription factors (TFs) involved in the crocin biosynthetic pathway by comprehensive analysis of genomics, transcriptomics, proteomics, and metabolomics to reveal the regulatory mechanism of crocin biosynthesis; (4) determination of the subcellular localization of more key enzymes to provide novel insights into the subcellular route of crocin biosynthesis; (5) characterization of more key enzymes responsible for crocin biosynthesis to construct suitable cell factories in plants or microorganisms for crocin biosynthesis to address crocins shortages; and (6) introduction of genes involved in the crocin biosynthetic pathway into important agricultural or industrial crops by using a multi-gene stacking system to develop crocin-rich functional foods or raw materials.

In conclusion, further researches on crocin biosynthesis and metabolic engineering will contribute to the industrial production of crocins, which will not only bring huge economic benefits but also have beneficial effect on human health.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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