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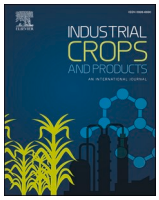

Engineering the production of crocins and picrocrocin in heterologous plant systems

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ABSTRACT

Crocins and picrocrocin are high-value apocarotenoids rarely found in plants. Both are derived from zeaxanthin cleavage in saffron and Buddleja by a 7,8:7′,8′ reaction catalyzed by carotenoid cleavage dioxygenases (CCDs). Although crocins are generated as well from other carotenoid substrates as lycopene and β-carotene, in the case of gardenia. In recent years, there has been growing interest in the therapeutic properties and the potential applications of these apocarotenoids for a wide spectrum of diseases. Saffron is the main source of crocins and picrocrocin but its high price limits further exploitation by different industrial sectors. Competitive and sustainable production of these metabolites can be obtained by engineered plants with the 7,8:7′,8′ CCD enzymes. This review aims to bring together all the available strategies developed to produce these compounds in different plants systems, revealing challenges and limitations for further optimization of their yield and nutritional/industrial exploitation.

Saffron spice is one of the oldest and most well-known spice (Cardone et al., 2020a; Winterhalter and Straubinger, 2000). The color, taste and aroma are provided by crocins, picrocrocin and safranal. Though these compounds play important roles in food, nutraceutical, cosmetics, and pharmaceutical sectors, they also face important actual challenges. Up to date, saffron is the exclusive source of crocins and picrocrocin from commercial exploitation. However, the obtention of these compounds from saffron have a high production cost and is influenced by many environmental parameters (Cardone et al., 2020b), which has resulted in a drastic drop in saffron production. Despite this, the demand for these compounds continues to increase in different economic sectors, especially the medical sector is estimated to witness the fastest growth. In view of this imbalance, the expected consequence is undoubtedly a rise in the prices of these metabolites. Given the rising need and market potential for crocins and picrocrocin, alternative options including biotechnological processes for fast and low-cost production in different plant platforms, and biofortification of crops must be developed. Thus, in this review we present the plants naturally producing these compounds in which the genes encoding for the enzymes responsible for their biosynthesis have been identified. We compare the current state of the different strategies developed to metabolically engineer crocins and picrocrocin in different plants, making use of the identified genes.

1. Carotenoids and apocarotenoids

Carotenoids are important natural pigments with C40-polyenic structures derived from eight isoprene units (C5), which are biogenetically generated from a central head-to-head connection of C20-geranylgeranyl pyrophosphate precursors, with varying degrees of double bond conjugation (Rodriguez-Concepcion et al., 2018). In nature, carotenoids are synthesized by all photosynthetic organisms (i.e., cyanobacteria, algae, higher plants) as well as some non-photosynthetic

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organisms such as fungi, bacteria, and some arthropods (Misawa et al., 2021; Paniagua-Michel et al., 2012; Sandmann, 2022). Carotenoid pigments account for the natural yellow, orange, and red colors, and are the basis for many aromas and scents in fruits and flowers, contributing to the attraction of pollinators and seed-dispersing organisms. Non-enzymatic and enzymatic oxidative cleavage of carotenoids results in the formation of biologically important carotenoid derivatives, known as apocarotenoids. Non-enzymatic apocarotenoid formation can occur primarily on β-carotene via singlet oxygen attack (Havaux, 2014). The enzymatic cleavage at specific double bonds is catalyzed by the Carotenoid Cleavage Dioxygenase enzymes (CCD) (Ahrazem et al., 2016a), which recognized a broad range of carotenoid and apocarotenoid substrates. The CCD enzymes are widely distributed in nature, which is consistent with their biological importance (Beekwilder et al., 2008; Eroglu and Harrison, 2013; Harrison and Quadro, 2018; Meléndez-Martínez, 2019; Zheng et al., 2021). CCDs are nonheme enzymes in which the Fe^{2+} prosthetic group is coordinated by four highly conserved His residues (Sui et al., 2013). The CCDs fold in a characteristic structure consisting of a seven-bladed β-propeller topped by a cluster of α-helical and loop segments forming a dome that covers the bound Fe^{2+} within the active site. The first CCD cloned and functionally characterized was from the mutant maize viviparous-14 (VP14) (Schwartz et al., 1997). This enzyme was catalyzing the cleavage of 9-cis-epoxy-carotenoids to form C25 apo-aldehydes and xanthoxin, the precursor of abscisic acid (ABA). Therefore, this enzyme was renamed as NCED (9-cis-epoxy-carotenoid-dioxygenase), while the name CCD has been used to name the rest of the enzymes involved in the biosynthesis of apocarotenoids other than ABA. While vertebrate genomes have been shown to typically encode only two different CCDs (BCO1 and BCO2) (Harrison and Quadro, 2018), one catalyzing the cleavage of carotenoids and apocarotenoids at the 15:15′ position, and the other catalyzing the asymmetrically cleavage at the 9,10 (and/or 9′,10′) double bond, the plant genomes contain a much higher number of different CCDs (1, 2, 4, 7, 8 and 10), catalyzing the formation of volatile and non-volatile apocarotenoids (Liang et al., 2021; Shi et al., 2020). Among the apocarotenoid compounds synthesized by plants, which showed a strong coloration in addition to several medicinal properties, and with a high economic value are bixin and crocin (Ahrazem et al., 2015b; Rivera-Madrid et al., 2016).

2. Crocetin, crocin, safranal and picrocrocin as pharmacological agents

Crocetin ($C_{20}H_{24}O_4$), is a lipophilic yellow apocarotenoid consisting in a polyunsaturated conjugated acid structure, with four side-chain methyl groups, and seven conjugated double bonds, and present in nature as *cis*- and *trans*-forms (Fig. 1A). Crocetin is mainly found in nature esterified with sugars and re-named as crocins. The oligosaccharide moieties present in crocins are highly variable, from mono- to bis-ester derivatives with mono-ester up to trisaccharide substituents, with a complex pattern of substitution and a total number of up to five or eight monosaccharides per crocin derivative (Ahrazem et al., 2015a; Carmona et al., 2006; Rubio Moraga et al., 2013), with glucose the main sugar found (Fig. 1B). The presence of sugars in crocins confer to them high solubility in water. In addition, crocetin and crocins are powerful free radical quenchers, displaying a broad range of health benefits (Bastani et al., 2022). Interest in the therapeutical properties of crocins is increasing due to their sedative and analgesic properties, neurological protection, anti-hypertensive, anti-atherosclerotic, and their anticancer activities (Hashemzaei et al., 2020; Hong and Yang, 2013). Further, clinical assays indicate that crocins have positive effects in the treatment of depressions and dementia (Siddiqui et al., 2022). Crocins have anti-HSV-1 and anti-HIV-1 potential preventing virus entry and replication (Soleymani et al., 2018). More recently, in *silico* analyses have

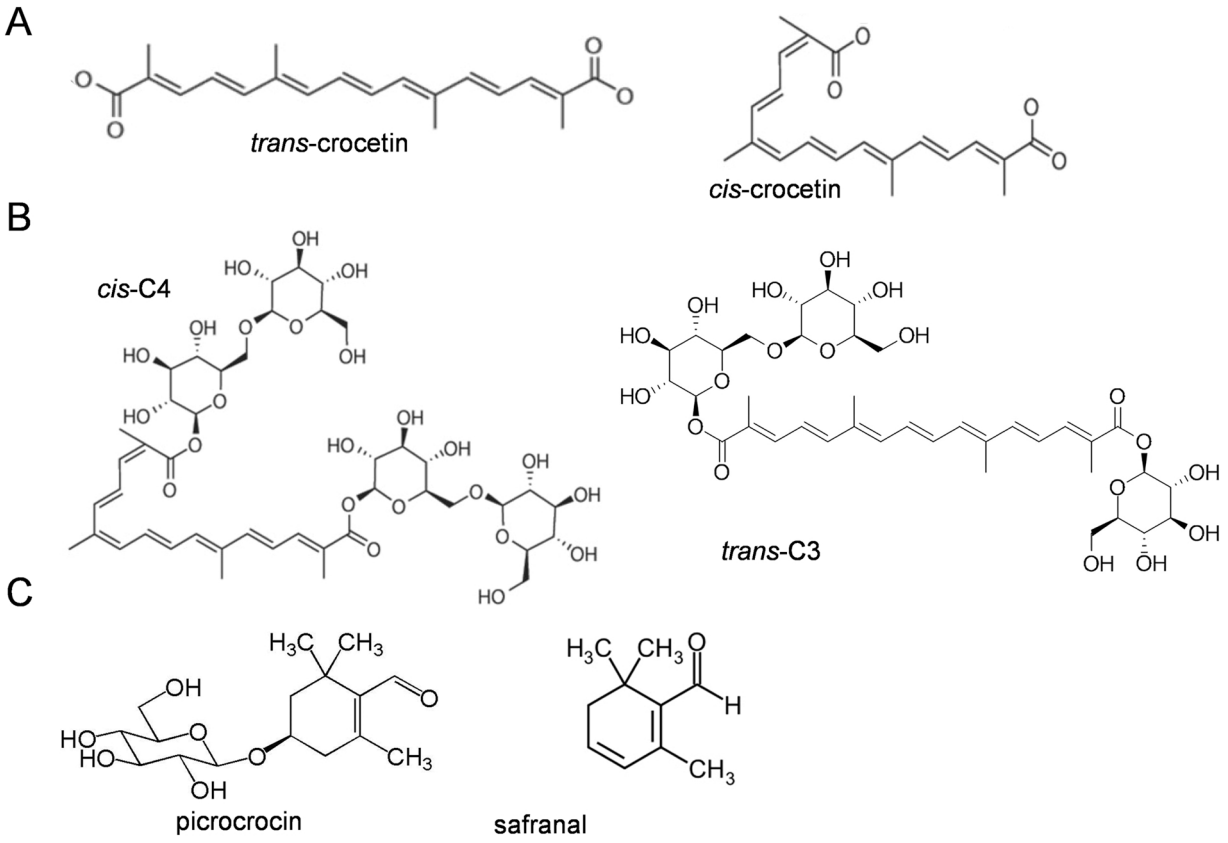

Fig. 1. Structures of crocetin, crocins, picrocrocin and safranal. (A) structures of *trans*-crocin and *cis*-crocin. (B) structures of c-C4 and t-C3. (C) structures of picrocrocin and safranal.

shown that crocins may have the potential to limit the progression and severity of the SARS-CoV2 infection via its anti-inflammatory, immune-modulatory, anti-oxidative, and pulmonal-protective effects (Gholami et al., 2021; Mentis et al., 2021).

Picrocrocin, a monoterpene colorless glycoside, is the β-D-glucoside of hydroxysafranal (4-hydroxy-2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde) (Fig. 1C) and mainly responsible for the bitter taste of saffron (Winterhalter and Straubinger, 2000). In addition to the taste, picrocrocin is related to the aroma of saffron, because it is the safranal (2, 6, 6-trimethyl-1, 3-cyclohexadien-1-carboxaldehyde or $C_{10}H_{14}O$) (Fig. 1C) precursor (Tarantilis et al., 1995), the major volatile oil component (60–70%) responsible for the aroma of saffron spice generated during the dehydration of the spice (Winterhalter and Rouseff, 2001). Several studies have shown that picrocrocin inhibits the proliferation of several types of cancers. For instance, picrocrocin was reported to inhibit the cell viability of melanoma and leukemia cells (Bukhari et al., 2018; Yu et al., 2018). In addition, safranal is also known as a cytotoxic substance effective against specific cancer cells (Abdalla et al., 2021; Malaekeh-Nikouei et al., 2013).

3. Crocin-source species

3.1. Saffron

Crocus plants are perennial herbs that belong to the *Iridaceae* family. The genus *Crocus* comprises more than a hundred species, which are primarily located in the Mediterranean-Europe region and Western Asia. *Crocus* is divided into two subgenera, based on morphological and cytological characters, as well as on genetic analysis (Harpke et al., 2013; Nemati et al., 2019), with viz. subgenus *Crocus* including all the characterized species except one of them, viz. *C. banaticus*, which is the sole member of the subgenus *Crociris*. *Crocus sativus*, known as saffron, belongs to the subgenus *Crocus* and derives by hybridization events of *C. cartwrightianus* cytotypes (Schmidt et al., 2019). Saffron is known world-wide for its red stigmas, which dehydrated constitute the saffron spice, one of the most expensive species in the world, mainly due to the method of the cultivation, collection, and further processing (Cardone et al., 2020a). Saffron spice is characterized for its water solubility and yellow coloration, conferred by crocins, and a bitter taste and a characteristic fragrance, which are caused by picrocrocin and safranal, respectively (Winterhalter and Straubinger, 2000). Saffron has undergone an extensive phytochemical and biochemical characterization, and many studies have shown a wide range of pharmacological effects, including anticonvulsant, antidepressant, anti-inflammatory, antigenotoxic, antihypertensive, antioxidant, antitussive, anxiolytic, cytotoxic, and relaxant properties (Abu-Izneid et al., 2022; Hatziagapiou et al., 2022; Mykhailenko et al., 2019). Saffron extracts also help to improve learning skills and memory, and to increase blood flow in the retina and choroid (Srivastava et al., 2010). In addition, many anti-tumor activities have been reported (Patel et al., 2017).

Interestingly, practically all *Crocus* species are widely characterized by the biosynthesis and accumulation of picrocrocin, crocetin and crocins (Castillo et al., 2005; Rubio Moraga et al., 2013). In *C. sativus* and *C. ancyrensis*, the accumulation of these apocarotenoids is developmentally regulated(Ahrazem et al., 2015a; Gomez-Gomez et al., 2017; Moraga et al., 2009; Rubio-Moraga et al., 2010), and also the biosynthesis of zeaxanthin, the precursor of these metabolites (Ahrazem et al., 2010; Castillo et al., 2005). Notably, it has been also shown that saffron has four phytoene synthase isoforms, and only one (*CsPSY2*) is mostly expressed in the stigmas and is responsible for apocarotenoid biosynthesis (Ahrazem et al., 2019).

Cleavage of zeaxanthin at the 7,8 and 7′,8′ double bonds by the plastidial enzyme CCD2, allows the formation of crocetin and HTCC (Ahrazem et al., 2016b; Frusciante et al., 2014; Pfander and Schurtenberger, 1982) (Fig. 2). Further oxidation and glucosylation reactions, mediated by specific glucosyltransferases (Gomez-Gomez et al., 2018; López-Jimenez et al., 2021; Moraga et al., 2004), result in the formation of crocins and picrocrocin (Diretto et al., 2019) (Fig. 2).

Among crocuses, *C. sativus* accumulates crocins at the highest levels in the stigma (Castillo et al., 2005; Moraga et al., 2009; Rubio Moraga et al., 2013). The main crocins that have been identified in saffron are *trans*-crocin-2 (t-C2), *trans*-crocin-2′ (t-C2'), *trans*-crocin-3 (t-C3), *trans*-crocin-4 (t-C4), *cis*-crocin-2 (c-C2), *cis*-crocin-3 (c-C3), *cis*-crocin-4 (c-C4), and *trans*-crocin-5 (t-C5). Among all these different crocins, t-C4

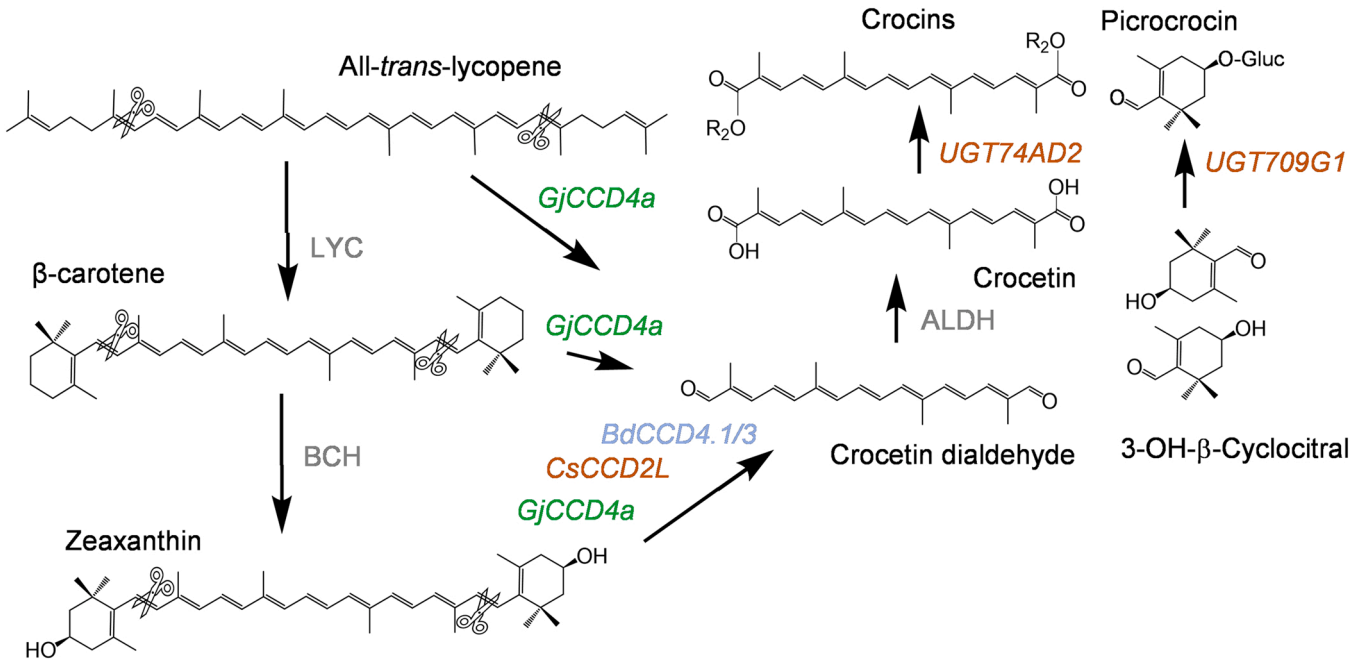

Fig. 2. Carotenoid substrates for crocins production in *Crocus sativus*, *Gardenia jasminoides* and *Buddleja davidii*. In color are shown the enzymes used for the transformation experiments in different plant species. The enzymes from saffron used for transformation are shown in orange. The enzymes from *B. davidii* are shown in blue, and the enzyme from gardenia is shown in green. LYC-B: lycopene β-cyclase, BCH: β-carotene hydroxylase.

is found to be the most abundant crocin in most of the investigated saffron samples followed by t-C3 (Amin and Hosseinzadeh, 2012; Chen et al., 2020a; D'Archivio et al., 2016; Gonda et al., 2012; Koulakiotis et al., 2015; Li et al., 2018a; Masi et al., 2016; Si et al., 2022). However, in other Crocus species crocins are not exclusively restricted to the stigma; rather, they also accumulate in tepals (Ahrazem et al., 2018; Rubio Moraga et al., 2013), conferring a characteristic yellow color to the tepals of these species.

3.2. Gardenia

Gardenia jasminoides Ellis is a shrub that belongs to the *Rubiaceae* family. The genus is native to the regions of Africa, Asia, Madagascar, Pacific Islands, and Australia.

The desiccated fruits of gardenia have been traditionally used for medicinal purposes (Chen et al., 2020b), and it is also widely used in dyestuff and food industries (Giménez et al., 2015). An increasing number of scientific investigations have focused on the pharmacological activities of its chemical constituents. Iridoids are the characteristic phytoconstituents of the fruits of gardenia and are present in high contents (Fu et al., 2008). Besides iridoids, crocins are considered the second major active constituents in gardenia (Hong and Yang, 2013; Ozaki et al., 2002; Tian et al., 2022). In comparison with saffron, picrocrocin and safranal are not present, suggesting lycopene and/or β-carotene as substrates for crocins biosynthesis in gardenia, being β-carotene the main carotenoid present in non polar fruit extracts from gardenia, where zeaxanthin is not detected. In fact, the enzyme responsible for crocins biosynthesis in gardenia, GjCCD4a (Xu et al., 2020) recognized as substrates lycopene and β-carotene, as well as zeaxanthin in in vitro and *bacterio* experiments (Xu et al., 2020) (Fig. 2). All the other components of the biosynthetic pathway of crocins in gardenia have been identified, including ALDH and UGTs enzymes, through the sequencing and analysis of the gardenia genome (Xu et al., 2020). Recently, the presence of crocins have been also detected in full mature flowers (Sommano et al., 2020). Four crocins have also been mainly identified in gardenia: t-C4, c-C4, t-C3 and t-C2; with t-4 C as the main crocin detected, followed by t-C2.

3.3. Buddleja sp

Buddleja davidii is an ornamental multi-stemmed shrub that belongs to the *Scrophulariaceae* family (Tallent-Halsell and Watt, 2009), which comprise c. 108 species displaying wide morphological diversity, especially in the flower and inflorescence, and are one of the only two tribes that have a major radiation in the Southern and Northern Hemispheres (Tank et al., 2006). Several of these species are known for their horticultural value and for their use in traditional medicine (Houghton, 1984). The dried flower buds and inflorescences of buddleja are used as a traditional herbal medicine to treat neurological disorders, strokes, eye diseases, diabetes and vascular diseases (Backhouse et al., 2008; Bukhari et al., 2016; Cheng and Si-Ying, 1991). The plant is also cultivated as a natural food dye and aqueous extracts of the flowers have been used since ancient times as a yellow rice colorant in local festivals in southwest China (Cheng and Si-Ying, 1991). Crocins are responsible for the yellow pigmentation (Aoki et al., 2001; Xie et al., 2019), as well as for some of the pharmacological properties of buddleja (Liao et al., 1999). Besides crocin, picrocrocin and safranal were also detected in the flowers of *B. davidii* (Diretto et al., 2021). The pathway for the biosynthesis of these apocarotenoids in *B. davidii* has been fully elucidated (Ahrazem et al., 2017; Diretto et al., 2021; Gomez-Gomez et al., 2018). Two plastidic CCD enzymes, BdCCD4.1 and BdCCD4.3 are involved in the cleavage of zeaxanthin at the 7,8;7′,8′ double bonds for the biosynthesis of crocins (Fig. 2). Crocins with two molecules of glucose are the main crocins that accumulate during the development of the flower, achieving the highest levels at anthesis (Diretto et al., 2021).

4. Crocins and Picrocrocin metabolic engineering

4.1. Crocins production in Nicotiana species

Nicotiana species grow naturally in many places around the world. Plants in this genus have been exploited by humans for their ornamental characteristics and for their psychoactive and toxic activities (Jassbi et al., 2017). In addition, these species have been used as factories for the production of a broad range of metabolites in high concentrations. *N. tabacum* was the first plant to be genetically modified (Horsch et al., 1985), being followed by other species including *N. benthamiana* and *N. glauca*. Furthermore, genome data from all the main species have been obtained. The first draft genome of *N. benthamiana* was obtained in 2012 (Bombarely et al., 2012), and in 2014 the genomes of three tobacco varieties were published, representing the three main classes of commercially grown tobacco (Sierro et al., 2014), while in 2018, a 3.5 Gbp draft genome of *N. glauca* was published (Usadel et al., 2018).

In previous studies of transient expression in *N. benthamiana*, and carried out for the characterization of the different components of the crocin and picrocrocin biosynthesis pathway in saffron, it was observed that the introduction of only *CsCCD2L* allows for the accumulation of crocins in the infiltrated leaves, without the inclusion of the other biosynthetic enzymes, proving that the carotenoid cleavage is the only essential step and thus allowing the accumulation of crocins up to 0.03 mg/g DW (Diretto et al., 2019; López-Jimenez et al., 2021) (Fig. 3A). The first attempt to produce and accumulate crocins in plants non naturally producing these compounds was achieved using a virus-driven system, in particular a viral vector derived from Tobacco etch virus (TEV, genus Potyvirus,family Potyviridae), more specifically TEVANIb, engineered to transiently express the CCD enzymes from saffron and Buddleja, CsCCD2L and BdCCD4.1, alone or in combination with other carotenoid biosynthetic enzymes in *N. benthamiana* plants (Martí et al., 2020). More in detail, transformed *N. benthamiana* stably expressing TEV nuclear infusion b (NIb), were agro-inoculated with the different TEV recombinant clones, and crocetin, picrocrocin and crocins accumulation were analyzed at different time points post-inoculation (0, 4, 8, 11, 13, 15, 18 and 20 dpi), coming to the conclusion that the accumulation of these apocarotenoids increased from approximately 8 to 13 dpi, and reaching a plateau up to the end of the analysis (20 dpi). In this virus-driven experiment, CsCCD2L was most efficient in the production of crocetin dialdehyde, crocins and picrocrocin followed by BdCCD4.1, allowing the production of 2.18 mg/g DW of crocins and 8.24 mg/g DW of picrocrocin at 13 dpi (Fig. 3A). On the contrary, the introduction of BdCCD4.1 only produced 0.76 mg/g DW of crocins (Fig. 3B), suggesting that these differences were related with the stability of the introduced sequences in the virus vector. In fact, *BdCCD4.3* was extremely unstable in the viral vector and its activity could not be evaluated (Martí et al., 2020). The levels of crocins and picrocrocin were further improved by adding the phytoene synthase (*PSY*) gene, encoding for the first committed enzyme for carotenoid biosynthesis, in the construct containing the *CsCCD2L* gene. The inclusion of a *PSY* gene from *Pantoea ananatis* (PaCrtB), indeed, increased the levels of crocins up to 3.493 mg/g DW (Fig. 3A). It should be mentioned that the profiles of crocins obtained were different with respect to those obtained in saffron stigma or Buddleja flowers, which can be due to the activities of the endogenous glucosyltransferase and/or glucosidase enzymes and/or to the intrinsic tissue properties.

Constitutive expression of *CsCCD2L* in stable transformed *Nicotiana* species, *N. tabacum* and *N. benthamiana*, resulted in the accumulation of crocins in leaves at 0.036 mg/g and 0.4 mg/g DW, respectively (Ahrazem et al., 2022b) (Fig. 3A). Additionally, the total amount of crocins was increased 3.5-fold in *N. tabacum* when the gene encoding for β-carotene hydroxylase, *BCH*, which could enhance the levels of zeaxanthin, used as substrate by CsCCD2L (Frusciante et al., 2014), along with the mutated Orange gene from Arabidopsis (*AtOrMut*), were introduced together with *CsCCD2L* (Ahrazem et al., 2022b) (Fig. 3A).

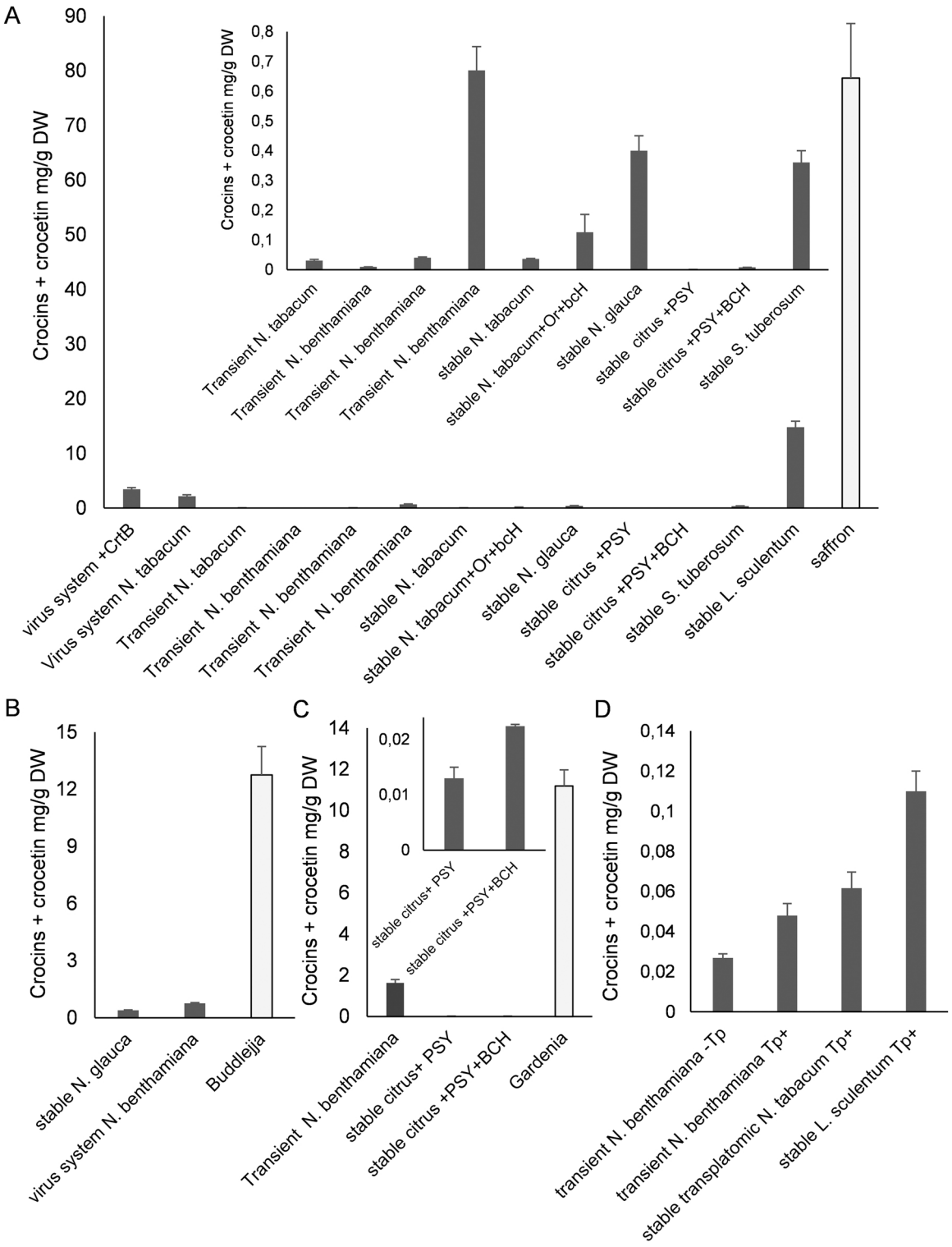

Fig. 3. Levels of crocins + crocetin in transformed plant tissues with CCDs of different plant origin (dark-grey bars), and comparison with the accumulation of crocins in their natural sources (light-grey bars). (A) levels obtained in different systems using the saffron enzyme CsCCD2L, and in stigmas of *Crocus sativus*. (B) Contents obtained in different systems using the BdCCD4.1 enzyme, and in flowers of *Buddleja davidii*. (C) levels obtained in different systems using GJCCD4a and levels in *Gardenia jasminoides* fruits.

Additional efforts using crocins non-saffron enzymes have been carried out. More specifically, the enzyme from *B. davidii*, BdCCD4.1, was constitutively expressed in *N. Glauca* plants, and the levels of crocins were determined in leaves and flowers (Huang et al., 2022) (Fig. 3B). Differences in the quantity and the profile of crocins between both tissues were observed in the analyzed lines, and the highest levels obtained were over 0.300 mg/g DW, with this level being lower than the one reached using the saffron enzyme, CsCCD2L (Ahrazem et al., 2022b), but higher compared the ones obtained using the virus-driven system (Martí et al., 2020).

Finally, the *CCD* gene from *G. jasminoides*, *GjCCD4*, was also transiently expressed in *N. benthamiana* plants and the levels of picrocrocin

and crocins were compared with the ones obtained using the same conditions with the *CsCCD2L* gene from saffron (Zheng et al., 2022). Overall, the transient expression of *GjCCD4a* allowed for the accumulation of 1.630 mg/g DW, while 0.67 mg/g DW were detected in agro-infiltrated leaves with the *CsCCD2L* gene (Zheng et al., 2022) (Fig. 3A and C). These results were above the values previously obtained in two other independent works (0.039 mg/g DW (Frusciante et al., 2022) and 0.030 mg/g DW (Diretto et al., 2019)), despite having used the same promoter in all the studies. The observed differences between the crocin levels obtained using GjCCD4a or CsCCD2 were attributed to the greater availability of substrates for the gardenia enzyme in tobacco leaves, since this enzyme can act on lycopene, β-carotene and zeaxanthin, compared to CsCCD2L that only cleaves zeaxanthin. However, the profile of crocins obtained was the same in both cases, with t-C3 as the predominant crocin (Zheng et al., 2022).

Interestingly, in a search for the CCDs enzymes involved in bixin production, Frusciante et al. in 2022 (Frusciante et al., 2022) tested six possible CCD candidates. Unexpectedly, they discovered that the plastidial BoCCD4–3 can produce crocetin dialdehyde from lycopene, rather than bixin as previously published (Carballo-Uicab et al., 2019). In addition, β-carotene and zeaxanthin were also used as substrates by BoCCD4–3 for crocin production. The transient expression of BoCCD4–3 in *N. benthamiana* leaves allowed the accumulation of 0.026 mg/g DW of crocins (Fig. 3D), with t-C2 as the main crocin present in the extract (Frusciante et al., 2022). The addition to BoCCD4–3 of the CsCCD2L transit peptide for plastidial location allowed for further increase in the crocin content of 0.048 mg/g DW (Fig. 3D), suggesting that the addition of this peptide allowed a better location, and thus an enhanced content of this enzyme in the plastids (Frusciante et al., 2022). However, *BoCCD4–3* expression did not produce picrocrocin, which was also absent from leaves obtained from stable expression of *BoCCD4–3* in plastids, by generating stable chloroplast-transformed *N. tabacum*, but allowing the accumulation of 0.061 mg/g DW of crocins (Frusciante et al., 2022) (Fig. 3D).

4.2. Crocin production in tomato

Since tomato (*S. lycopersicum* L.) was imported to Europe in the 16th century, it has become one of the most important crops in the world. In recent years, interest in tomato as a model plant has increased significantly, due to the fact that its genome has been sequenced (2012). Tomato is an excellent model for basic and applied research programs for the following reasons: it possesses a relatively small genome (950 Mb),), a broad genetic resources including an important collection of mutants (http://tgrc.ucdavis.edu), the ability to grow under different growing conditions, a relatively short life cycle, high self-fertility and homozygosity, easy control of pollination and hybridization, seed production capacity the ability of asexual propagation by grafting, and the possibility of transforming and regenerating whole plants from different explants (Gerszberg et al., 2014). Additionally, tomato is globally the second largest vegetable crop with a yearly worldwide production of over 180 million tons (FAO, http://www.fao.org/). Finally, tomato fruit represents an essential component of the Mediterranean diet, due to its high nutritional value, indeed tomato berry synthesizes a series of beneficial compounds as carotenoids, that accumulate in the chromoplasts, the organelles adapted to their storage. As a dietary source of nutrients, it has a complex blend of bioactive components, including a variety of carotenoids like lycopene, β-carotene, and lutein.

Tomato already has a broad range of tools and resources for biotechnological applications (Li et al., 2018b). This crop is an attractive platform to be used for the introduction of different genes involved in the production of carotenoids. Enhancing tomato fruit quality has received substantial attention. One of the most significant qualities of tomato fruit is its carotenoid concentration, which has benefits for industry, health, and nutrition (Meng et al., 2022). The high demand for natural carotenoids to function as beneficial antioxidants requires that plants serve as environmentally friendly factories for the cost-effective production of high-value carotenoids, which has led to an increased in interest in engineering carotenoid metabolism in tomato.

In this context, the fruit of *S. lycopersicum* is an optimal platform for the production of crocins, carotenoid-derived metabolites due to its high potential for carotenoid accumulation. The *BoCCD4–3* and *CsUGT74AD1* genes were constitutively expressed in tomato plants, which generate 0.110 mg/g of crocins DW fruits (Fig. 3D) (Frusciante et al., 2022). Data reported in this study regarding BoCCD4–3 activity raises the question why crocin or crocetin are not detected in *B. Orellana*, which only accumulates bixin (Rivera-Madrid et al., 2016), if it owns an enzyme that is able to cleave lycopene, β-carotene, and zeaxanthin producing crocetin dialdehyde in bacteria.

Using the enzymes from saffron: CsCCD2L, CsUGT2 (CsUGT74AD1) and CsUGT709G1, Ahrazem et al. (2022) (Ahrazem et al., 2022a) introduced the pathway for crocins and picrocrocin biosynthesis into tomato by combining fruit specific promoters pE8 and p2A11, and the constitutive promoter CaMV p35S. The obtained tomato fruits, in which one *CsCCD2L* was under fruit specific promoters, accumulated up to 14.800 mg/g of crocins and 2.920 mg/g of picrocrocin DW, representing the highest level reported for these specific apocarotenoids in heterologous systems up to know, as a result of transient or stable metabolic engineering, without compromising the normal development of the plant (Fig. 3A). However, in those fruits where *CsCCD2L* was expressed under a constitutive promoter, although accumulating up to 12 mg/g DW of crocins, the obtained fruits were devoid of seeds. Notably, the levels of lycopene, β-carotene, and α-carotene were reduced in all the obtained fruits, due to the activity of CsCCD2L that was able to intercept the carotenoid metabolic flux to direct it towards the production of crocins and picrocrocin. In addition, these obtained tomatoes develop a good coloring capacity in aqueous solution and showed high antioxidant activity (Ahrazem et al., 2022a).

4.3. Crocin production in citrus

Citrus crops are high-yielding fruits representing an important economic activity worldwide. Citrus fruits contain a high concentration of carotenoids and are an important part of the human diet (Lado et al., 2016). The genomic origin and evolution of citrus has been recently determined (Wu et al., 2018), revealing complex taxonomic relationships. Different biotechnological tools have developed (Peña and Navarro, 2000), however the in vitro regeneration process is an important bottleneck for citrus genetic transformation (Conti et al., 2021). In addition to *Nicotiana* spp. And tomato, citrus has been used as model system for crocins production. More in detail, white-colored callus of Citrus paradise Macf, which naturally contains a very low content of carotenoids, were engineered to obtain different lines expressing several combinations of carotenoid and apocarotenoid biosynthetic genes: *tpCrtB* (bacterical PSY with transit peptide), *OsBCH* (rice beta-carotenehydroxylase), *GjCCD4a* or *CsCCD2L* (Zheng et al., 2022). The first step of agrobacterium-mediated serial transformations was obtained using the gene encoding for the bacterial PSY fused with the transit peptide, TpCrtB, which resulted in callus showing an orange-red color, mostly due to the high accumulation of beta- and alpha-carotene. The second step implied to remodel the xanthophyll content and,for this purpose, the *OsBCH* gene was used for a second level of transformation. The obtained PSY/BCH yellow callus lines accumulated a high level of b-b xanthophylls and lower content in cyclic carotenoids. Subsequently, these two newly produced transgenic lines were supertransformed with *CsCCD2L* and *GjCCD4a* respectively, to finally achieve four more cell lines: CCD2/PSY (CsCCD2L/TpCrtB), CCD2/BCH/PSY (CsCCD2L/OsBCH/TpCrtB), CCD4/PSY (GjCCD4a/TpCrtB), CCD4/BCH/PSY (GjCCD4a/OsBCH/TpCrtB). Except for CCD2/PSY, which appeared slightly orange, all the lines were pale-yellow, a symptom of good substrate consumptions.

Subsequently, the carotenoid profiling of the transgenic lines

highlighted the utilization of lutein and zeaxanthin in the transgenic callus expressing *CsCCD2L* and *GjCCD4a*. Surprisingly, a reduction in β-carotene and β-cryptoxanthin contents were seen only in the lines expressing *GjCCD4a*. Crocetin dialdehyde, crocetin and crocins were quantified in the lines expressing *GjCCD4a* and *CsCCD2L*. In all cases, the introduction of *BCH* allowed a higher accumulation of crocetin and crocins, and the levels were always higher in the lines expressing *GjCCD4a*. Although the levels were much lower than the ones obtained in Nicotiana species, with up to 0.022 mg/g DW in the lines with the CCD4/BCH/PSY combination (Fig. 3C). Picrocrocin was also detected at higher levels in these lines, accumulating 0.042 mg/g and 0.025 mg/g DW, in CCD4/BCH/PSY and CCD2/BCH/PSY, respectively (Fig. 3C).

4.4. Crocin production in potato

Approximately 5000 different types of potatoes are known, the majority of which have been obtained through human selection. Although seven *Solanum* species, including seven subspecies, are cultivated, and almost 200 wild species have been identified, the majority of cultivated variants are from the *Solanum tuberosum* specie (Fernandez-Orozco et al., 2013). It is the world's fourth most important crop, behind corn, wheat, and rice. World production exceeded 300 million tons in 2009, occupying an area of 18 million hectares. The main producers are China and India, which account for more than a third of world production. In addition to being high in vitamin C, potatoes are also a good source of folic acid, niacin, riboflavin, thiamine, and pyridoxine. Potassium,

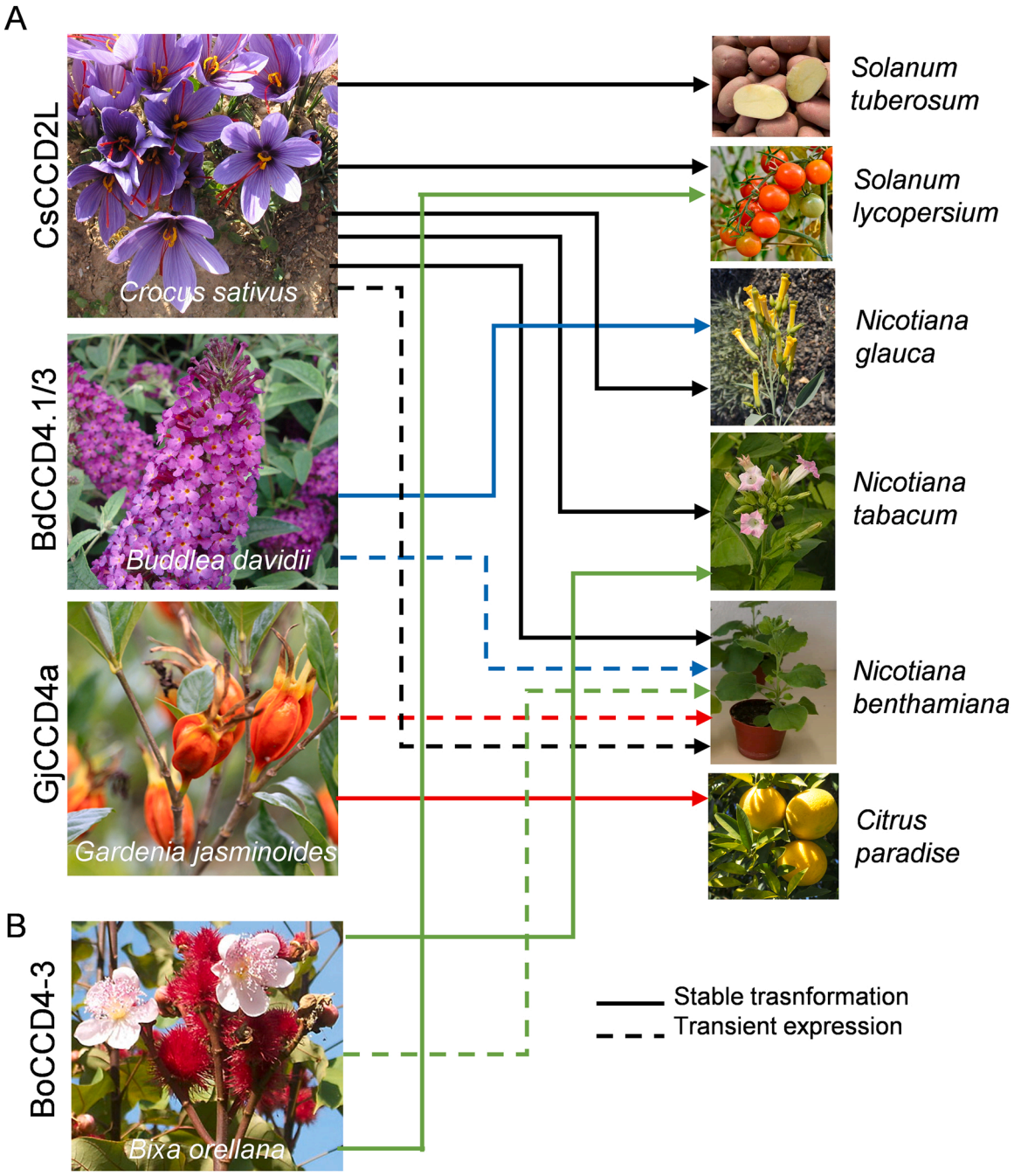

Fig. 4. Plant sources of CCDs used for crocins production in different plant systems. (A) Plant species producing the apocarotenoids crocins and picrocrocin and strategies used for their production in other plant species. (B) *Bixa orellana* does not produce crocins, but the BoCCD4–3 enzyme produced crocins when was introduced in other plant species.

phosphorus, and calcium are the minerals found in raw potatoes in the highest amounts (Camire et al., 2009). Currently, potato is a major food in the human diet, and moreover has other uses as a fodder plant and industrial supplier of feed for livestock and raw material for the starch and alcohol industry. On the contrary, potato is devoid of carotenoids, particularly α- and β-carotene; for this reason, studies of genetic engineering in potato breeding programs with the goal of increasing its carotenoid production have been reported in the literature (Diretto et al., 2007; Farré et al., 2011). Recently, by establishing stable transgenic lines of *S. tuberosum* cv. Desirée that express *CsCCD2L*, *UGT74AD1*, and *UGT709G1* genes under the control of the patatin promoter (Gómez-Gómez et al., 2022), we were able to produce crocins (Fig. 3A) and picrocrocin apocarotenoids-enriched potato tubers. Different transgenic tubers accumulated crocins and picrocrocin ranging from 0.019 to 0.360 mg/g and 0.105–0.800 mg/g DW, respectively, being crocetin, and crocin with one glucose molecule, and crocin t-2 C [(crocetin)-(β-D-glucosyl)-(β-D-glucosyl)-ester)] the main detected compounds. Additionally, the transgenic tubers displayed increased antioxidant capacity than the wild type, up to roughly 4-fold more, which is a promising finding for the potential health advantages of these lines. Furthermore, different cooking techniques were used to better investigate the behavior of these apocarotenoids following processing, and each procedure showed a substantial impact on the retention of these metabolites. More specifically, it was discovered that boiled potatoes (97.23%) had a higher bioaccessibility of these metabolites than raw, baked, and fried ones (80.97%, 78.96%, and 76.18%, respectively). Thus, it was concluded that boiling seems to be the best approach to preserve crocins almost intact, and potato tuber appeared to be a more appropriate matrix than an aqueous extract of saffron in terms of bioaccessibility.

5. Concluding remarks and future perspectives

Two main transformation strategies, stable and transient, have been followed for the production of crocins and picrocrocin in naturally non-producing plants (Fig. 4A and B). The selection of fruit specific promoters for the production of these apocarotenoids in tomato fruits has been very effective for the obtention of quantities identical or close to the ones produced by saffron (Fig. 3A) (80.590–230.360 mg/g DW) (Zhang et al., 2019), gardenia (11.190 mg/g DW) (Liu et al., 2022), or Buddleja (12.760 mg/g DW) (Diretto et al., 2021), followed by the use of a virus-driven system in *N. benthamiana* (Fig. 3A and B).

Both approaches offer advantages for the production of these bioactive metabolites. Tomato fruit is the ideal sink tissue for crocins and picrocrocin production because it is intrinsically adapted to isoprenoid production. In addition, the transformed seeds can be store indefinitely and crocins can be produce on demand. By the other hand, while plant virus-derived vectors boost the expression as a consequence of viral genome amplification and systemically spread the genes of interest throughout the whole plant, with the additional benefit to achieve crocins-engineered tissues in less than two weeks (Rodriguez-Concepcion and Daròs, 2022). Assuming similar costs for developing the two systems, the production cost for 1 kg of crocins using the virus system could reach around USD 520, whereas the equivalent cost in tomato fruits is substantially lower, at less than USD 35. Thus, the saffron apocarotenoids produced using these systems could provide approximate 10–150-fold cost saving, as presently the production cost of saffron spice is in the range of USD 3000- 6000 per kilogram.

Overall, a total of four different CCDs has been assayed, which showed different preferences for the carotenoid substrates. This aspect is of particular interest, since a broad substrate recognition will be useful in terms of higher versatility for selection of the plant platforms suitable for the increased production of these valuable apocarotenoids, although efficiency of these enzymes and biochemical characterization should be more detailed investigated, in order to determine key factors affecting their stability and catalytic activity.

Declaration of Competing Interest

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

Data availability

No data was used for the research described in the article.

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