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Crocins-rich tomato extracts showed enhanced protective effects *in vitro*

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ABSTRACT

Crocins are high-value water soluble pigments that have long been recognized for their medicinal properties, and whose demand has increased worldwide in recent years. In the present study transgenic tomato fruits engineered for the production of crocins and *hp3/B^{sh}* tomato mutants with alterations in the carotenoid metabolism, have been combined to increase the levels of crocins in tomato fruits. Tomato fruits from F_4 plants showed high antioxidant capacity and the crocin-rich juice of the produced tomato fruit was protected neuroblastoma cells against oxidative insult, through its ability to activate factor 2 related to nuclear erythroid factor 2 (Nrf2). The bio-accessibility of crocins in the juice showed values similar to the ones observed with saffron. Overall, our results support that crocin-fortified tomatoes could result in higher crocin availability and have additional health-promoting effects and could provide better protection against oxidative stress related chronic diseases in humans.

1. Introduction

Carotenoids are a large family of secondary metabolites that serve as substrates for carotenoid cleavage dioxygenases (CCDs), generating shorter compounds known as apocarotenoids (Ahrazem, Gomez-Gomez, Rodrigo, Avalos, & Limon, 2016). Although apocarotenoids are widespread throughout different kingdoms, including plants and animals, some apocarotenoids are specific for only a few plant species (Rodriguez-Concepcion et al., 2018). Based on their chemical structure, apocarotenoids can be found as volatile or non-volatile compounds (Winterhalter & Rouseff, 2001). Among the non-volatile ones, their length and number on double bonds determine their coloration. Examples of apocarotenoids with intense colors are bixin and crocetin (Rivera-Madrid, Aguilar-Espinosa, Cárdenas-Conejo, & Garza-Caligaris, 2016; Winterhalter & Straubinger, 2000), which accumulate in specific plant species, and both show high antioxidant activities (Hashemzaei et al., 2020; Kurniawati, Soetjipto, & Limantara, 2010).

Many biological processes in the human body, such as the digestion of food, metabolization of alcohol or drugs, as well as the conversion of fats into energy, produce free radicals, which are usually destroyed by the natural antioxidant system (Irshad & Chaudhuri, 2002). In addition, the exposure to environmental factors, such as pollutants, also contributes to the generation of these free radicals (Samet & Wages, 2018). However, under certain conditions, the free radicals can trigger a negative chain reaction in the human body, blocking proper functioning (Kurutas, 2016) and promoting the development of several metabolic

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dysfunctions while contributing to chronic disorders such as coronary heart diseases, neurological disorders or cancers (Sharifi-Rad et al., 2020). Thus, the beneficial effects of the antioxidants present in our diet have been widely studied (S. Li et al., 2014). Increasing evidence from epidemiological and animal feeding studies, as well as from *in vitro* studies, suggests that dietary carotenoids contribute to the beneficial effects of nutrition on the prevention of chronic diseases (Fiedor & Burda, 2014). Both the basic skeleton and further modifications (e.g. hydroxylation, glycosylation) appear to determine the bioavailability and bioactivity of carotenoids in humans (Kopec & Failla, 2018).

Crocins are glucosylated apocarotenoids, consisting in a series of ester compounds of crocetin with gentibiose or glucose molecules added to the ends of the C20 crocetin skeleton, that confer their solubility in water (Bathaie, Farajzade, & Hoshyar, 2014). Recent pharmacological studies have shown that crocins have a variety of beneficial effects on the cardiovascular system, the central nervous system (CNS), and the liver, including anti-tumor, anti-oxidative, anti-inflammatory and detoxification activities (Cerdá-Bernad, Valero-Cases, Pastor, & Frutos, 2022). The main natural sources of bioactive crocins are saffron (*Crocus sativus*) and gardenia (*Gardenia jasminoides*). Crocins are also present in other plant species, e.g. *Buddleja davidii* and *Nyctanthes arbor-tristis*, but in smaller amounts (Ahrazem et al., 2017; Gadgoli & Shelke, 2010). In saffron, Buddleja and gardenia, the pathway for crocin biosynthesis has been fully elucidated, and the key enzymes have also been characterized (Ahrazem et al., 2017; Ahrazem, Rubio-Moraga, et al., 2016; G. Diretto et al., 2019; Gianfranco Diretto et al., 2021; Frusciante et al., 2014; Moraga, Nohales, Perez, & Gomez-Gomez, 2004; Xu et al., 2020). More in detail, the carotenoid dioxygenase enzyme CCD2 in saffron and the CCD4 subfamily in Buddleja and gardenia catalyzed the cleavage of the carotenoid substrate to render crocetin dialdehyde, which is further dehydrogenated and glucosylated, thus generating the different crocin molecules (Pfrander, 1976).

Due to their proven health benefits, there is growing interest in the development of food crops with high levels of crocins, designed to exert an optimal bioavailability and beneficial biological effects. Genetic engineering is a powerful tool that is being used to produce crocins in food crops by using the saffron mini-pathway (Ahrazem, Diretto, et al., 2022). Previously, we have used different transgenic approaches to produce crocins and other saffron apocarotenoids, like picrocrocin and crocetin, in tobacco, potato and in tomato fruits using the enzymes from saffron (Ahrazem, Diretto, et al., 2022; Ahrazem, Zhu, et al., 2022; Gómez-Gómez et al., 2022; Huang et al., 2022; Martí et al., 2020). Ectopic and tissue-specific expression of the saffron mini-pathway resulted in a tissue-specific biosynthesis and accumulation of crocins, crocetin, and picrocrocin in tomato fruit (Ahrazem, Diretto, et al., 2022) (Fig. 1), reaching levels close to the ones present in the stigma of saffron (Ahrazem, Diretto, et al., 2022). In these high-crocin lines, the carotenoid content was strongly reduced, suggesting that the introduced mini-pathway was able to divert the fruit metabolic flux towards the production of saffron apocarotenoids. Thus, an increase in the carotenoid pool should help enhance crocin production in tomato. Several spontaneous or induced mutants are available in the tomato germplasm, thus providing valuable genetic materials for additional genetic engineering attempts. In this context, a double The tomato mutant carrying the *hp3/B*^{Sh} mutations (loss and gain of function of, respectively, zeaxanthin epoxidase (ZEP) and β-lycopene cyclase (CYC-B)) was previously generated, which accumulates in the mature fruit higher levels of β-carotene and zeaxanthin (Karniel, Koch, Zamir, & Hirschberg, 2020) than the single mutant *hp3* (Galpaz, Wang, Menda, Zamir, & Hirschberg, 2008), and can be considered as a good platform for the obtention of fruits with enhanced crocin accumulation.

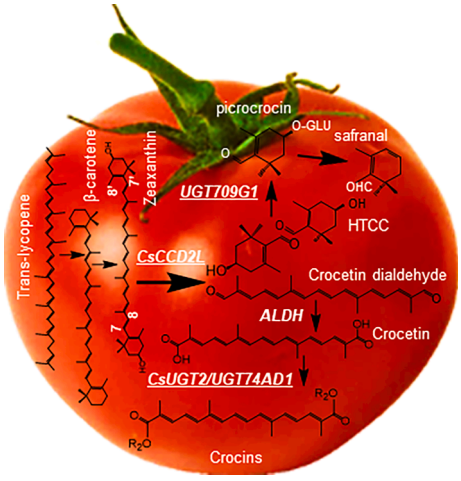

Fig. 1. Schematic representation of the biosynthetic pathway for crocetin, crocins, picrocrocin and safranal generation of transgenic tomato O1_9A. In the scheme, the carotenoid pathway is shown from lycopene to zeaxanthin, the substrate for the CsCCD2L enzyme.

In order to further explore the possibility of improving crocin engineering in tomato fruits, we have produced different genotypes resulting from an initial cross between a previously engineered crocin-tomato line, O1_9A, and the double mutant line *hp3/B*^{Sh}. This approach resulted in transgenic tomatoes accumulating higher levels of saffron apocarotenoids and antioxidant activity than the control O1_9A lines, and the demonstration that extracts from those fruits have protective effects on neuroblastome cells against oxidative insult. Finally, a bioaccessibility test showed that crocins in these tomato fruits are bioaccessible for exercising beneficial effects on human health. Overall, this study demonstrates the potential of tomato fruits, which accumulate carotenoids downstream of lycopene, promoting the accumulation of crocins as valuable compounds.

2. Materials and methods

2.1. Experimental breeding

A conventional breeding scheme was conducted between O1_9A (*Solanum lycopersicum* cv. MM) and *hp3/B*^{sh} (*S. lycopersicum* cv. M82) in a greenhouse, using both cultivars as receptor and acceptor. Flowers from both lines were emasculated two days before anthesis and used in crossing, which involved pollen collected at anthesis from the flowers of the other cultivar. Seeds obtained from the two reciprocal crosses were germinated and grown in 30-plug trays containing sterilized soil under controlled light and temperature conditions consisting of 16 h light (200 μE m^{-2} sec^{-1}) at 25 °C and 8 h dark at 18 °C at constant 60 % relative humidity in a phytotron. After three weeks, seedlings were transferred to individual 5.0 L pots in the greenhouse and fertilized every week with a nutrient solution. F_1 plants obtained from both reciprocal crosses were grown and examined during the fruiting stage based on the production of crocins by the fruits. F1 plants were allowed to self-pollinate to produce seeds for the F_2 population. The obtained seeds were visually checked for differences in color, and yellow-orange seeds were selected for germination (Ahrazem, Diretto, et al., 2022). The selected F_2 seeds of both crosses were grown individually in a phytotron under controlled conditions. At the cotyledon stage, a small piece from the cotyledon was collected and analyzed for the presence of the transgene and the *hp3* mutation by PCR and further sequencing. The presence of the *B*^{Sh} mutation was further followed by analyses of the fruit phenotype, which showed a characteristic orange coloration. Positive seedlings were further grown for flower and fruit phenotypes (Karniel et al., 2020), crocin content and seed collection. Selection was applied up to the F_4 generation. Fruit from lines 6-3A and 1-5F and control 1-4C were used for the rest of the analyses.

2.2. Genomic DNA extraction, PCR and sequencing

To detect the presence of the *hp3* mutation in the genome of the inbred tomato plants, genomic DNAs were obtained from leaves using a Plant Genomic DNA Kit (https://www.intronbio.com). The obtained DNA was used as template in a PCR reaction using previously described primers (Galpaz et al., 2008). Amplified bands were purified and sequenced using an automated DNA sequencer (ABI PRISM 3730xl, Perkin Elmer, Macrogen Inc., https://www.macrogen.com).

2.3. Extraction and analysis of apocarotenoids and carotenoids

Carotenoids and apocarotenoids were extracted from freeze dried ripe tomatoes. The tomatoes were pulverized using a blade grinder, and extractions were made in three technical replicates from the obtained powder (50o 10 mg each for carotenoid or apocarotenoid analysis, respectively) in 2 mL tubes. First extractions were done with 75 % MeOH in water for the recovery of polar crocins, and the samples sonicated during 10 min. Next, samples were centrifuged for 10 min at 12,000 g in microcentrifuge, and the supernatant recovered for crocin analysis by HPLC-DAD. The pellets of each sample were extracted by the addition of chloroform and methanol (2:1). Samples were sonicated for 10 min, following centrifugation for 10 min at 12,000 g in microcentrifuge. The organic phase, containing the non polar fraction, was transferred to a clean centrifuge tube and the pellets were re-extracted with chloroform and methanol (2:1), and the process repeated. Organic phases were pooled.

and dried using a Speedvac. Dried samples were resuspended in methyl *tert*-butyl ether prior to chromatographic analysis by HPLC-DAD. Crocins and carotenoids were separated and identified as previously described (Ahrazem, Diretto, et al., 2022).

2.4. Total RNA Isolation, cDNA Synthesis, and Real-Time quantitative PCR analysis

Total RNA was extracted using the Trizol reagent (Zymo Research) following the manufacturer's instructions. RNA concentration was assessed by a microspectrophotometer. First-strand cDNA was synthesized from 1 μg of total RNA using the PrimeScript 1st strand cDNA Synthesis Kit (Takara). qPCR was carried out using the primers previously reported (Ahrazem, Diretto, et al., 2022; Zhu et al., 2020) and the GoTaq® qPCR Master Mix (Promega, Madison, WI, USA), in a Roche LightCycler 480 system using the 2X LightCycler 480 SYBR Green master mix (Roche). Three biological replicates were analyzed, and quantifications were made in triplicate for each of the biological replicates. The Actin gene was used as an internal control. The reaction specificity was confirmed by the negative control and a melting temperature analysis. The data were analyzed using the StepOne software v2.0 (Applied Biosystems).

2.5. Antioxidant activities

DPPH assay was performed as previously described (Ahrazem, Diretto, et al., 2022), with some modifications. Ripe fruits (50 mg lyophilized fruits) were reduced to powder and extracted with 75 % MeOH in water and mixed with 0.2 mM methanolic DPPH. Equal volumes of the methanolic extract and DPPH solution were mixed and kept for 30 min at room temperature in the dark. The absorbance of each mix was measured at 517 nm. The DPPH radical scavenging activity was calculated as (%) = $[1-(A_1/A_0)] \times 100$, where A_0 is the absorbance of the solution without extracts and A_1 absorbance of the solution with extracts.

2.6. Analyses of the protective effect of crocins against oxidative insult

For this aim we used SK-*N*-BE, a cell line widely used as a cellular model for the study of neurodegeneration. $1x10^6$ SK-*N*-BE cells were seeded in a 6 well plate. After 24 h they were pre-treated with tomato extract from the line containing 25 μg/ ml of crocins, or with wild type tomato 1-4c (WT). Crocin extract was dissolved in DMSO. Untreated samples were exposed to the vehicle alone (0.1 % DMSO) and were used as control. After 24 h, H_2O_2 at a concentration of 75 uM was added into the culture medium (Di Meo et al., 2020). After 24 h cells were collected and counted after Trypan Blue staining. All the experiments were performed in triplicate.

2.7. Simulated in-vitro gastric and intestinal digestions

The *in-vitro* gastrointestinal digestion was performed using salivary, gastric and intestinal fluids, prepared as described (Minekus et al., 2014). The procedure included three-steps sequentially simulated: digestion in the mouth, stomach, and the small intestine. Samples were prepared in triplicate, and after the process were frozen and lyophilized for extraction with 75 % MeOH in water and further analyses of crocins by HPLC-DAD as previously described (Ahrazem, Diretto, et al., 2022; López-Jimenez et al., 2021).

3. Results

3.1. Strategy to produce increased crocin levels in tomato fruits

Fruit from the Transgenic O1_9A tomato line accumulated high levels of crocins and other saffron apocarotenoids, such as picrocrocin and its degradation product: safranal (Ahrazem et al., 2022). This transgenic line was generated by expressing three genes from saffron: *CsCCD2L*, *UGT74AD1* and *UGT709G1* (Fig. 1), under fruit-specific promoters in the MoneyMaker (MM) background of tomato. In this paper, line O1_9A was crossed with the *hp3/B^{Sh}* tomato. Pollen from the *hp3/B^{Sh}* mutant was used to pollinate pistils of the O1_9A line, and vice versa, to create F_1 seed populations (Fig. 2). The *hp3* mutation has been reported to be recessive in nature, while the *B^{Sh}* was reported as semi-dominant in nature (Galpaz et al., 2008). Therefore, selection of genetic and phenotypic components started in the F_2 generation, created from self-fertilization of the F_1 populations (Fig. 2A). Genotyping for the *hp3* phenotype among the F_2 lines, which were hygromycin resistant and therefore carry the crocin mini-pathway, was performed on 2-week-old seedlings by PCR. The mutation in the *hp3* allele used is a single base change (Galpaz et al., 2008) to detect the absence or presence of the *hp3* allele and its zygosity. PCR was carried out following band purification and sequence analyses. In the following generations and up to F4, only plants giving orange fruits (the *B^{Sh}* phenotype) with all yellow-orange seeds (phenotype for crocins accumulation), and positive for the presence of *hp3*, were selected for further analyses (Fig. 2A and 2B). In addition, during the selection process of plants from F_2-F_4, fruits were scored for the presence of crocins by cutting the fruits during seed collection (Supplemental Fig. 1). F_4 plants fixed for *hp3/B^{Sh}* and the crocin expression cassette were selected for further analyses: 6-3A and 1-5F. In addition, a control plant producing orange fruits and non-colored seeds was selected (1-4C) as a negative control for crocin accumulation.

3.2. Carotenoid levels in parental and F_4 plants

Pigment analysis of ripe fruit tissues from each F_4 plant showed different carotenoid profiles in the developed lines in comparison to their parental lines (Supplemental Fig. 2 and Fig. 3A). O1_9A parental line showed the highest levels of lycopene (0.4 mg.g^{-1} DW) but the lowest levels of β-carotene (0.038 mg.g^{-1} DW)(Fig. 3A) while the recombinants that include *hp3/B^{Sh}* plants showed the highest levels of lutein (0.042 mg.g^{-1} DW) and zeaxanthin (0.067 mg.g^{-1} DW), reduced levels of lycopene (0.028 mg.g^{-1} DW), and high levels of β-carotene (0.34 mg.g^{-1} DW) (Fig. 3A). Analysis of F4 lines evidenced a clear change in the carotenoid content compared with the parental line

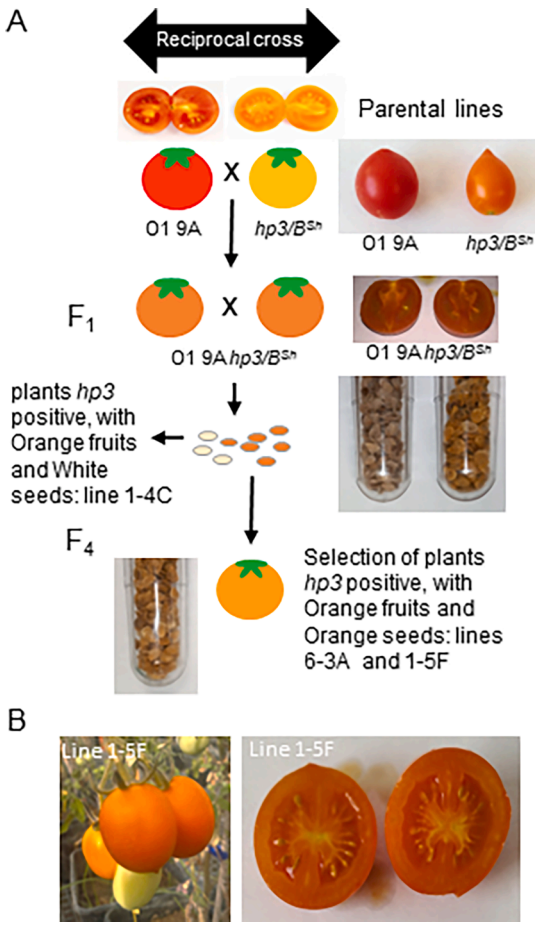

Fig. 2. Developing fruits for high crocin production using the parental plants O1_9A and *hp3/B^Sh*. (A) Schematic representation of the procedure followed for the obtention of tomatoes with fruits accumulating high concentrations of crocins. Phenotypes of parental fruits are shown, together with the phenotype of seeds recovered from resulting F_2 progeny. (B) phenotype of tomato fruits from 1 to 5F plants. In the lefthand picture the tomato fruits are shown on the plant, in the central picture a tomato fruit is shown, and in the righthand picture the longitudinal section is shown.

O1_9A. Indeed, the control line 1-4C, producing non-colored seeds, and selected as a control for not producing crocins, showed a profile similar to the parental line *hp3/B^sh*, but with higher levels of lycopene (0.10 mg.g^{-1} DW), similar β-carotene levels (0.34 mg.g^{-1} DW), but reduced levels of lutein (0.014 mg.g^{-1} DW) and zeaxanthin (0.011 mg.g^{-1} DW). However, in the F_4 lines producing yellow-orange seeds, lines 1-5F and 6-3A, lutein and zeaxanthin were not detected (Fig. 3A).

3.3. Crocin production in the hybrid lines

From each line, at least three biological replicates represented by independent ripe fruits were harvested and screened for crocin composition and levels by HPLC-DAD (Fig. 3 and Supplemental Fig. 3). Whereas crocins did not accumulate in tomato *hp3/B^sh* fruits, nor in 1-4C fruit, significant levels of crocins were detected in the fruits of 1-5F and 6-3A, with 1-5F (22.6 mg.g^{-1} DW) being the line with higher levels of crocins (Fig. 3B). The main crocin that accumulates in the obtained F_4 fruits and in the parental O1_9A was *trans*-3Gg [(crocetin-(β-D-gentiobiosyl)-(β-D-glucosyl)-ester)], followed by *trans*-4GG [(crocetin-di-(β-D-gentiobiosyl)-ester)] (Supplemental Fig. 3). We further analyzed the content of crocins in the fresh fruits, analyzing the content in th peel, in the juice and in the tomato paste obtained after juice and peel removal (Supplemental Fig. 4).

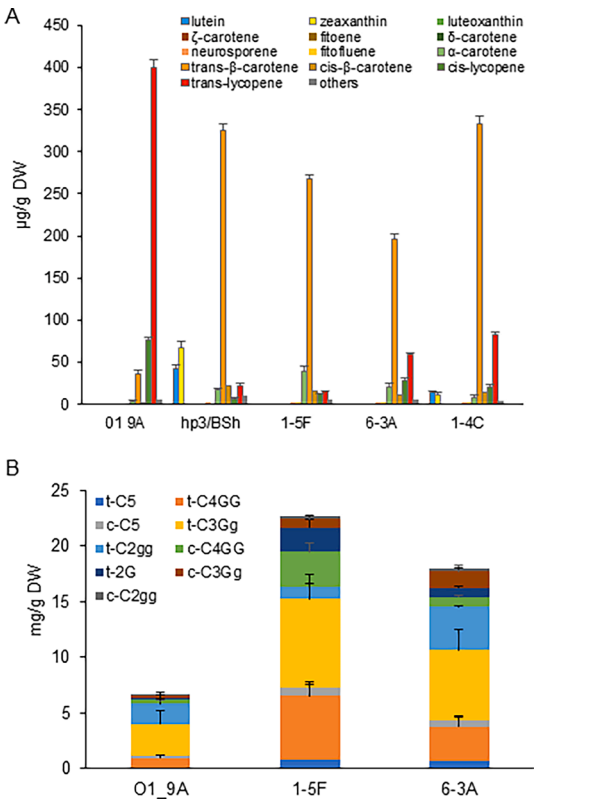

Fig. 3. Carotenoid and crocin levels in tomato fruits from parental and selected plants. (A) Carotenoid levels in ripened fruits of parental plants and positive (1-5F and 6-3A) and negative (1-4C) fruits for crocin accumulation. (B) Crocin levels were quantified in several ripe fruits harvested from the developed positive plants and parental line O1_9A. Data for all samples are the average of three biological replicates. DW, dry weight.

3.4. Expression analyses of carotenogenic genes

According to the results obtained in terms of crocins and other carotenoid contents in the selected lines constitutively expressing *CsCCD2L*, 1-5F, 6-3A and 1-4C fruits were used to analyze the transcriptional differences at carotenoid pathway levels. The carotenoid biosynthetic pathway genes analyzed were: *GGPPs*, geranyl–geranyl pyrophosphate synthase; *PSY1*, chromoplast-specific phytoene synthase; *PSY2*, chloroplast-specific phytoene synthase; *PDS*, phytoene desaturase; *ZDS*, carotene desaturase; *ZDS-iso* carotenoid isomerase, *CRTISO*, carotenoid isomerase; *CrtL-2*, chromoplast-specific lycopene b-cyclase; *CrtL-1*, chloroplast-specific lycopene b-cyclase; *CYP97C11*, lycopene e-cyclase; *CrtR-b1*, chloroplast-specific β-ring hydroxylase; and *CrtR-b2*, chromoplast-specific β-ring hydroxylase. Major changes were observed for 6-3A fruit in comparison with 1-4C fruit, whose expression levels for each analyzed transcript were set to 1 (Fig. 4). Notably, most of the carotenogenic genes: *PSY1*, *PDS*, *ZDS-iso*, *CRTISO*, *CrtL-2*, *CrtR-b1*, and *CrtR-b2,* were significantly down-regulated (Fig. 4). The reduction of the expression of these genes in the carotenoid pathway could explain the lower total carotenoid levels detected in 6-3A fruit (237.64 ± 16.47 µg.g^{-1}) in comparison with control fruit 1-4C (494.31 ± 24.84 µg.g^{-1}). In addition, the reduced expression of *CrtR-b1*, and *CrtR-b2,* whose protein product catalyzed the production of zeaxanthin as a substrate for CsCCD2L, could be the reason for the reduced levels of crocins in the 6-3A fruits, compared with those from 1 to 5F fruit. By contrast, the expression level for CrtL-1 was up-regulated in 6-3A fruits (Fig. 4). The same was observed in the 1-5F fruits, compared with 1-4C (Fig. 4). On the contrary, the other carotenogenic genes did not show significative differences (Fig. 4), suggesting that the overall observed reduction in the carotenoid content of 1-5F fruits (303.98 ± 12.87 µg.g^{-1}) is likely due to

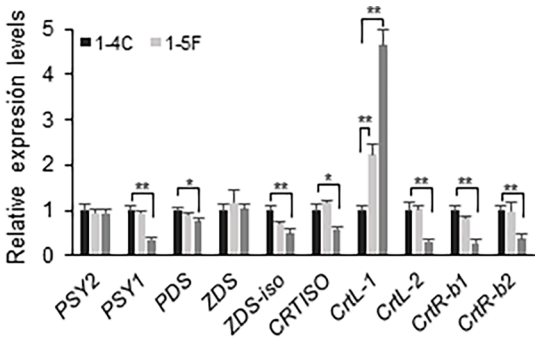

Fig. 4. Transcript levels of genes encoding carotenoid biosynthetic pathway enzymes in inbred tomato fruits. Transcript levels were measured by qRT-PCR for genes encoding carotenoid biosynthetic pathway enzymes in fruits from lines accumulating crocins, 1-5F and 6-3A compared to control 1-4C. Tomato Actin gene was used for normalization. The genes of the carotenoid biosynthetic pathway were: *PSY1*, phytoene synthase 1; *PSY2*, phytoene synthase 2; *PDS*, phytoene desaturase; *Z-ISO*, ζ-carotene isomerase; *ZDS*, ζ-carotene desaturase; *CRTISO*, carotenoid isomerase; *CrtL-1*, lycopene β-cyclase 1; *CrtL-2*, lycopene β-cyclase 2; *CrtR-b1*, β-carotene hydroxylase 1; *CrtR-b2*, β-carotene hydroxylase 2. Student's *t*-test significance compared to 1-4C: (*) P-value < 0.05; (**) P-value < 0.01.

the diverted flux of the endogenous pathway now leading to the formation of crocins.

3.5. Increased antioxidant activity in the developed tomato fruits

Previous work showed an increased antioxidant activity for all the tomato lines accumulating crocins compared to the WT fruits that did not produce crocins (Ahrazem, Diretto, et al., 2022). Therefore, we tested the antioxidant activity of the obtained materials and compared them with those of the parental lines (Supplemental Fig. 5). All the transgenic fruits accumulating crocins showed high antioxidant activity based on their radical scavenging activity (Supplemental Fig. 5). Compared with the fruits of *hp3/B*^{sh} and 1-4C, the antioxidant capacity of the 6-3A and 1-5F fruit was approximately 3 to 7-fold higher. In general, lines with high content in crocins showed higher antioxidant activity.

Further, we have analyzed the protective effect of crocins against oxidative insult. To this aim we used SK-*N*-BE, a cell line widely used as a cellular model for the study of neurodegeneration and we challenged it with H_2O_2 as toxic agent (Di Meo et al., 2020). To ascertain that tomato extracts did not induce cell death, SK-*N*-BE cells were treated with extracts from tomato fruits that do not produce crocins and with equivalent extracts containing crocins at 25 μg.mL^{-1}. The results showed that neither of the two tomato extracts reduced cell viability (Fig. 5A). However, to determine whether crocins played a role in protecting SK-*N*-BE from H_2O_2-induced cell death, cells were pre-treated for 24 h with crocins (25 μg.mL^{-1}) and then challenged with H_2O_2 (75 μM) for the following 24 h. Analysis of cell vitality revealed that the oxidant sensitivity of SK-*N*-BE cells was completely abolished by pre-treatment with crocins (Fig. 5A). It has been previously shown that crocins possess antioxidant properties through their ability to activate factor 2 related to nuclear erythroid factor 2 (Nrf2) (Liang et al., 2020), which activates antioxidant enzymes and inhibits oxidative stress-induced damage (Kaspar, Niture, & Jaiswal, 2009). Thus, in order to analyze the activation of this pathway by crocin treatment, we investigated the activation of two main downstream enzymes: the Glutathione Reductase (GSR) and Glucose-6-phosphate dehydrogenase (G6PD), both of particular interest since they are responsible for the maintenance of intracellular NADPH and GSH pool, respectively. The obtained data showed that the activities of neither enzyme was improved by the treatment with crocins (Fig. 5B) while H_2O_2-activated decline of antioxidant enzymes was inhibited by crocin treatments (Fig. 5B).

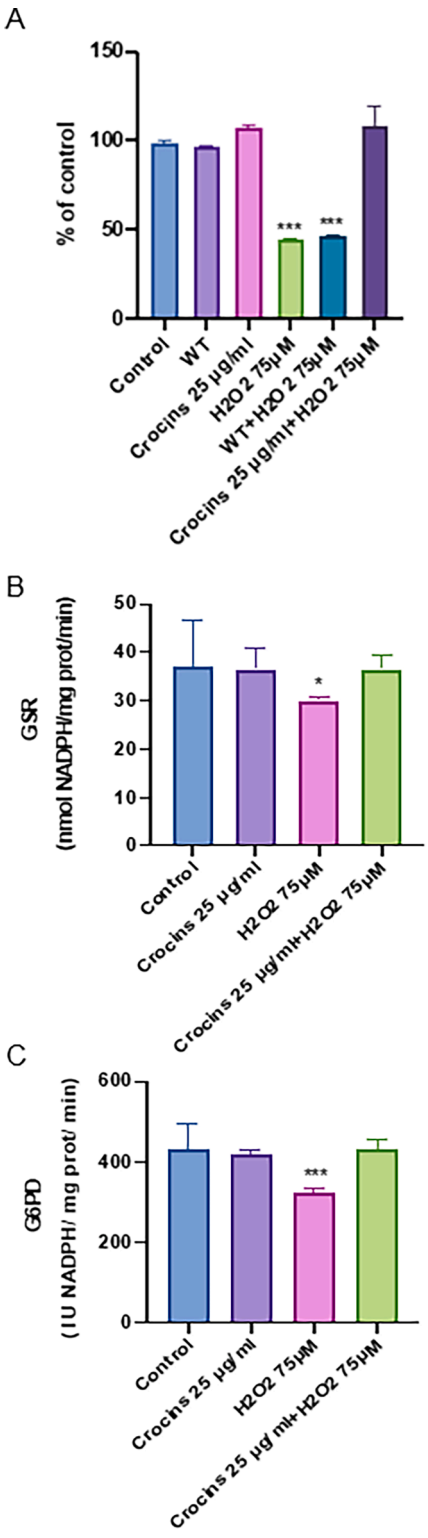

Fig. 5. The protective effects of crocins from tomato extracts on H_2O_2-induced cytotoxicity in SK-*N*-BE cells. (A) Effects of crocins on cell viability. SK-*N*-BE cells treated with crocins (administered 24 h before H_2O_2) were incubated in the presence or absence of H_2O_2 for 24 h. Pretreatment with crocins protects cells from H_2O_2 damage, hence restoring cell viability. (B) Effect of crocins on the activity of Glutathione Reductase (GSR) on SK-*N*-BE cells treated with H_2O_2. (C) Effect of crocins on the activity of Glucose 6 phosphate dehydrogenase (G6PD) on SK-*N*-BE cells treated with H_2O_2. The bars represent ± the average ± SD of independent experiments (n = 3). Statistically significant difference compared to control cells: (*) $p < 0.05$, (***) $p < 0.001$.

Collectively, our results show that crocin extracts from the tomato inbred lines could prevent the cytotoxicity of oxidative insults by inhibiting oxidative stress.

3.6. Simulated in vitro gastric and intestinal digestions

The *in vitro* digestion model mimics the physiological processes that are taking place in the gastrointestinal tract of the human digestive system (transit time, pH and enzymatic conditions), and has been widely used to study the bio-accessibility of different compounds produced in tomato fruits (Li, Deng, Liu, Loewen, & Tsao, 2014). Digestion resulted in 46 % of bioaccessibility for crocins. This value was in line with previous studies analyzing crocins from an aqueous saffron extract (50 mg. L^{-1}) that were found to be bioaccessible by almost 55 % (Kyriakoudi, Tsimidou, O'Callaghan, Galvin, & O'Brien, 2013), and a bioaccessibity of 40.77 % for crocins present in a commercial saffron extract (Kell et al., 2017). Losses of crocin content can be attributed to the effects of the pH during the digestion process, as well as to the physiological temperature of the human body (37 °C), rather than to the presence of digestive enzymes (pepsin, pancreatin) and bile salts (Kyriakoudi, O'Callaghan, Galvin, Tsimidou, & O'Brien, 2015).

4. Discussion

Crocins have a clearly beneficial role in human health; therefore, much attention has been recently focused on their metabolism, and on the possibility of transferring the biosynthetic pathway into plants of agronomical and nutritional interest (Liu et al., 2020). The expression of the saffron mini-pathway for crocin biosynthesis in tomato fruits causes the accumulation of crocins at high levels (Ahrazem, Diretto, et al., 2022). However, the synthesis of crocins in the fruit of tomato could still be limited by low substrate availability. Therefore, an increase in the substrate pool is expected to result in higher levels of crocins. In order to modulate tomato fruit carotenoid profiles, sources of natural variation have been identified and accurately characterized, including the chromoplast-specific allele of lycopene beta cyclase (*LYC-B*) known as Beta (Ronen, Carmel-Goren, Zamir, & Hirschberg, 2000), which leads to orange-colored tomato fruits, and alleles of carotenoid high-pigment 3 (*hp3*), which accumulate 30 % more carotenoids in the mature fruit due to mutation in the zeaxanthin epoxidase gene (ZEP) (Galpaz et al., 2008), leading to fruits with an intensified red color. The cross of both tomato mutants resulted in the double mutant *hp3/B^{sh}* that produces tomatoes with low levels of lycopene but high levels of β-carotene and zeaxanthin (Karniel et al., 2020). This double mutant was crossed with a stable tomato line engineered to produce crocins. The resulting F_4 plants showed fruits with a carotenoid profile similar to the *hp3/B^{sh}* fruits in terms of β-carotene accumulation but showed relatively higher levels of lycopene. In addition, lutein and zeaxanthin were not detected, but these fruits accumulate higher contents in crocins compared with fruits from line O1_9A. In fruit of a selected control 1-4C plant, the levels of lycopene and β-carotene were similar to lines 6-3A and 1-5F, but accumulated higher levels of lutein and zeaxanthin, which were undetectable in 6-3A and 1-5F fruit, likely due to their conversion to crocins by the action of the CsCCD2L enzyme, as previously observed in the tomato fruits producing crocins in a Money Maker background (Ahrazem, Diretto, et al., 2022). Analyses of expression of the carotenogenic genes in the fruit of F_4 crocin-engineered in a *Hp3/B^{sh}* mutant background showed that lower expression levels were associated with a reduction of carotenoid precursor for crocin biosynthesis and, consequently, with a reduced accumulation of crocins in the fruits, but the levels were higher than the produce in the parental line O1_9A.

The 1-5F fruits accumulated up to 2.1 % fruit dry weight, similar to levels found in Gardenia fruits (2.24 %), and 10 fold less to the ones reported in saffron (25.24 %) (Song, Wang, Zheng, Liu, & Zhang, 2021). Considering that a common dose of a standardized saffron extract (affron®) used for different treatments is<28 mg/day (Lopresti, Drummond, Inarejos-García, & Prodanov, 2018; Lopresti & Smith, 2022), this dose could be achieved with about ~ 60–70 g fresh weight of the F_4 fruits or consuming about 70–90 mL of juice. In addition, fruits from our improved lines showed increased antioxidant capacities relative to the fruits from the parental lines O1_9A and *hp3/B^{sh}*, confirming the positive association between crocin levels and antioxidant capacity. Higher levels of antioxidants and improved antioxidant capacities in plant extracts have been shown to be positively associated with effective protection against H_2O_2 stress induced in neuroblastoma cell models (Marino, Battaglini, Moles, & Ciofani, 2022; Zhai, Brockmüller, Kubatka, Shakibaei, & Büsselberg, 2020). One of the important mechanisms by which crocin exerts its biological effects is its ability to modulate the redox status of organisms (Mousavi, Tayarani, & Parsaee, 2010). The well-known antioxidant properties of crocins and their protective effects in different pathological conditions in different tissues such as the heart, liver, lungs, pancreas, and brain are through the Nrf2 signaling pathway (Khoshandam, Razavi, & Hosseinzadeh, 2022). Moreover, multiple studies have shown that affecting the Nrf2 signaling pathway by different mechanisms such as inducing anti-oxidant enzymes can prevent some diseases such as cancer, diabetes, and ulcerative colitis among others (Hashemzaei et al., 2020). Among the enzymes affected by the Nrf2 pathway, we tested the enzymatic activities of GSR and G6PD to determine their involvement in the underlying antioxidant mechanisms of crocins in H_2O_2-injured SK-*N*-BE cells. The results showed that the H_2O_2-activated decline of antioxidant enzymes was inhibited by crocin treatments, indicating that the polar fractions enriched in crocins from the fruits of the developed lines could protect neuronal cells from H_2O_2-induced oxidative damage.

To fully exert their biological properties, crocins need to be available for absorption in the target tissue. No subject in this phrase. The evaluation of the *in vitro* bioaccessibility of crocins in the tomato fruits were subjected to *in vitro* gastrointestinal digestion following the procedure developed by InfoGest (Minekus et al., 2014). The results obtained with the tomato powder (45.5 % bioaccessibility) were similar to a previous work using saffron extracts directly (50 %, bioaccessibility) (Kyriakoudi et al., 2013). The observed differences could be due to the fact that in the work with saffron extracts, the gastric digestion was completed in 1 h compared to the 2 h of the InfoGest protocol. In this regard, another study with a standardized saffron extract (affron®) using the same duration and the bioaccessibility (40 %) (Almodóvar et al., 2020), was close to the one reported with the tomato fruits.

5. Conclusion

The high demand for natural compounds to serve as healthy antioxidants requires plants to be green factories for the economical production of high-value metabolites, including crocins. These compounds possess preventive and therapeutic properties against a wide variety of diseases. We have shown that crocin concentration in engineered tomatoes can be further increased using tomato mutants with higher levels of proper carotenoid substrates for crocin biosynthesis. The crocin-rich extracts of these tomatoes were active as antioxidants and showed a bioaccessibility similar to crocins from the saffron spice. Therefore, w hope this study will provide the basis for the clinical application of these tomatoes in the treatment of the inflammatory symptoms associated to different diseases.

Ethics Statements

There were no human subjects and mammalian animal experiments in our research.

Data availability

The data supporting our findings are available in the manuscript file or from the corresponding author upon request.

CRediT authorship contribution statement

L.G-G., O.A., G.D., and A.G., conceived and designed the research; J. A., E.N., and L.M., crosses and selection of lines, including genotyping, L. M, S.C., S.F., G.D., O.A., A.R-M., M. L-G., B.O-A, R.C., P.B., and L.G-G, carried out the experiments. A. L-J., analyzed the sequencing data. L. G-G and O.A., wrote the manuscript. G.D., and T.G. revised the manuscript. All authors read and approved the final manuscript.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2023.105432.

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