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ORIGINAL PAPER

Alteration of flower color in *Iris germanica* L. 'Fire Bride' through ectopic expression of phytoene synthase gene (*crtB*) from *Pantoea agglomerans*

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Abstract

Key message **Genetic modulation of the carotenogenesis in *I. germanica* 'Fire Bride' by ectopic expression of a *crtB* gene causes several flower parts to develop novel orange and pink colors.**

Abstract Flower color in tall bearded irises (*Iris germanica* L.) is determined by two distinct biochemical pathways; the carotenoid pathway, which imparts yellow, orange and pink hues and the anthocyanin pathway, which produces blue, violet and maroon flowers. Red-flowered *I. germanica* do not exist in nature and conventional breeding methods have thus far failed to produce them. With a goal of developing iris cultivars with red flowers, we transformed a pink iris *I. germanica*, 'Fire Bride', with a bacterial phytoene synthase gene (*crtB*) from *Pantoea agglomerans* under the control of the promoter region of a gene for capsanthin–capsorubin synthase from *Lilium lancifolium* (*Llccs*). This approach aimed to increase the flux of metabolites into the carotenoid biosynthetic pathway and lead to elevated levels of lycopene and darker pink or red flowers. Iris callus tissue ectopically expressing the *crtB* gene exhibited a color change from yellow to pink-orange and red, due to accumulation of lycopene. Transgenic iris plants, regenerated from the *crtB*-transgenic calli, showed prominent color changes in the ovaries (green to orange), flower stalk (green to orange), and anthers (white to pink), while the standards and falls showed no significant differences in color when compared to control plants. HPLC and UHPLC analysis confirmed that the color changes were primarily due to the accumulation of lycopene. In this study, we showed that ectopic expression of a *crtB* can be used to successfully alter the color of certain flower parts in *I. germanica* 'Fire Bride' and produce new flower traits.

Keywords Carotenoids · Tall bearded iris · Flower color · Flower-specific promoter

Abbreviations

DHK	Dihydrokaempferol
DFR	Dihydroflavonol 4-reductase
TP	Transit peptide
GGPP	Geranylgeranyl diphosphate
PSY	Phytoene synthase (plant)
CrtB	Phytoene synthase (bacteria)
PPFD	Photosynthetic photon flux density
PCR	Polymerase chain reaction
RT-PCR	Reverse transcriptase-polymerase chain reaction
HPLC	High-pressure liquid chromatography
UHPLC	Ultra high performance liquid chromatography
PLlccs	Promoter of a gene for capsanthin–capsorubin synthase

Introduction

The genus *Iris* comprises about 300 species of perennial monocotyledonous plants that originated in the temperate regions of the Northern Hemisphere and are cultivated

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around the world. Irises have been known worldwide for centuries for their beautiful flowers, which hold many mythological, artistic, and historical evocations (Kandeler and Ullrich 2009). Consequential of the wide spectrum of flower colors that exist in nature, the name iris is derived from the Greek word for "rainbow". A red iris, however, is not naturally occurring and human efforts over the past 100 years with classical breeding have thus far failed to produce it. This elusive hue has been the subject of human fascination for several decades. For example, since 1954, the world-famous iris garden in Florence (Italy), Giardino dell'Iris, in collaboration with the Italian Iris Society, has held an annual International Iris Competition, which awards a special prize, the "Comune di Firenze Silver Plate", for the flower whose color is the closest to the red color of the iris on the city's banner.

Production of new flower colors such as this one meet consumer demands and has been a driving force among flower breeders for decades. However, some plant species lack a natural ability to produce and accumulate certain types of pigments in their flowers, preventing them from producing given hues. As such, marketing opportunities driven by high consumer demands for such novel flower colors have prompted research into finding alternative ways to generate them.

Genetic engineering provides a potential avenue for the development of a red iris that has not yet been explored. Genetic engineering can be used to create novel flower colors in a number of different ways: (1) by introducing new genes that encode enzymes that are otherwise absent to create new biosynthetic pathways, (2) by redirecting biosynthetic pathways through the up-regulation of genes already present, or (3) by suppressing biosynthetic pathways through the down-regulation of genes (Chandler and Brugliera 2011).

In *Iris germanica*, the best known and most horticulturally important tall bearded iris, flower color is known to be determined by two distinct biochemical pathways, the anthocyanin biosynthetic pathway and the carotenoid biosynthetic pathway (Warburton 1978; Meckenstock 2005). In cyanic iris flowers, the main pigments are delphinidin-type anthocyanins which impart blue, violet and maroon colors, while in acyanic flowers the main pigments are carotenoids, which produce orange, yellow, and pink colors. A red cyanic iris does not naturally exist because of a deficiency in the synthesis of pelargonidin- and cyanidin-type anthocyanins, which are usually responsible for red and orange hues. On the other hand, a red acyanic iris does not exist because red carotenoids, such as lycopene, do not accumulate in large concentrations, thus producing only pink flowers, as opposed to red ones.

Genetic modification of the flavonoid biosynthetic pathway has already been used successfully in several species to produce red flowers (reviewed by Chandler and Tanaka 2007; Nishihara and Nakatsuka 2011). For instance, previous research has shown that plants which accumulate dihydrokaempferol (DHK) can be engineered using a heterologous gene for dihydroflavonol 4-reductase (DFR) with different substrate specificity to produce red flowers (Meyer et al. 1987). However, because this strategy requires hosts that accumulate DHK naturally, it cannot be directly applied to *I. germanica*.

Red flower color has also been achieved in several species by genetic modulation of the anthocyanin biosynthetic pathway through the manipulation of multiple genes (Nakatsuka et al. 2007; Nakamura et al. 2010). These strategies could potentially be applied to *I. germanica*; however, because the genetic information available for irises is limited, it would require cloning and transformation of multiple genes. Furthermore, even if the genetic information was available there exist other roadblocks for using flavonoid manipulation to produce red flowers in *I. germanica*. The primary problem is that anthocyanidins often go through a number of potentially color-changing glycosylations, acylations, and methylations before being stored in the vacuoles (reviewed by Tanaka et al. 2010). These reactions are very difficult to control, which make it problematic to direct the synthesis to a specific anthocyanin. In addition, anthocyanins change color as a result of a variety of factors, such as vacuolar pH (Quattrocchio et al. 2006; Yoshida et al. 2009), co-pigmentation (Takeda et al. 2010; Yabuya et al. 1997), metal ion-complexation (Shiono et al. 2008; Momonoi et al. 2009) as well as cell size and shape (Kay et al. 2003; Dyer et al. 2007), so even if the correct anthocyanin was accumulated, there is no guarantee that it would exhibit the expected color.

For these reasons, we chose to focus on metabolic modifications to the other main group of pigments in iris flowers, carotenoids. There have only been a few successful attempts at alteration of carotenoid biosynthesis in flowers and, to our knowledge, all of these studies dealt with metabolic modification to the carotenoid biosynthetic pathway to introduce astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione) synthesis. For example, Mann et al. (2000) transformed *N. tabacum* using a β-carotene ketolase gene (*crtO*) cloned from the green alga *Haematococcus pluvialis*, and produced plants that accumulated novel κ-carotenoids, including astaxanthin, in the nectary tissues of the flowers. This resulted in the normally pale yellow nectaries becoming bright red. Though these authors achieved color change in a specific flower tissue through modification of carotenoid biosynthesis, their goal was to demonstrate the potential of a new method for producing astaxanthin, an economically important compound, which is widely used as a feed supplement in aquaculture (Mann et al. 2000).

To our knowledge, only two studies focused specifically on producing novel flower colors through the use of

carotenoid biosynthesis modification. Umemoto et al. (2006) and Umemoto and Toguri (2012), used *crtW* from *Agrobacterium auranticum* (reclassified to *Paracoccus* sp. strain N81106), fused to different transit peptides (TP), to target it to the chromoplasts of pale yellow petunia flowers with the goal of assessing the effect of different TP on gene expression and ensuing color changes. The resulting transgenic plants developed flowers whose color ranged from deep yellow to orange due to differential accumulation of astaxanthin which depended on a TP used to target *crtW*. Similarly, Suzuki et al. (2007) used *crtW* to engineer astaxanthin biosynthesis in *Lotus japonicus*. The transformed plants accumulated several novel carotenoids, including astaxanthin, in the flowers, which changed from their natural yellow color into various shades of orange.

Aside from metabolic engineering of astaxanthin synthesis, another strategy used to manipulate carotenoid biosynthesis in different plant parts, but not in flowers, is the ectopic expression of a gene for phytoene synthase (*Psy* in plants, *crtB* in bacteria) to increase the overall carotenoid content. Phytoene synthase catalyzes the formation of phytoene from two molecules of geranylgeranyl diphosphate (GGPP) and is the first committed step in carotenoid biosynthesis (Camara 1993). Studies have shown that the synthesis of phytoene is a rate-limiting step in many species, and that levels of expression of *Psy* directly correlate to carotenoid accumulation (Giuliano et al. 1993; Welsch et al. 2000). Although genetic engineering with phytoene synthase genes has not yet been used to alter the carotenoid content in flowers, it has been applied successfully to increase carotenoid content in tomato fruits, potato tubers, tobacco leaves, and the seeds of several species (reviewed by Rosati et al. 2009). For instance, Shewmaker et al. (1999) overexpressed *crtB* in canola seeds (*Brassica napus*), which are normally green, using a seed-specific promoter and obtained orange seeds that contained 50 times more carotenoids. To our knowledge, no study to date has used *Psy* or *crtB* to alter color in flower tissues, but these studies suggest it is possible that overexpressing a gene for PSY or CrtB in flowers may result in enhanced pigmentation and novel hues.

In the present study, we attempted to increase the synthesis and accumulation of lycopene in the pink flowers of *I. germanica* 'Fire Bride' by ectopic expression of *crtB* from *Pantoea agglomerans* (formerly *Erwinia herbicola*) with the goal of producing red flowers.

Materials and methods

Plant material and growth conditions

I. germanica 'Fire Bride' plants were transferred from a field to a greenhouse at the end of summer. The main shoots were cut back completely to reduce apical dominance and to encourage young shoots to emerge. The greenhouse was maintained at 16 h days/8 h nights and 25 ± 3 °C/20 ± 3 °C. Light was supplemented with high-pressure sodium lamps (Energy Technics, York, PA, USA) providing a photosynthetic photon flux density (PPFD) of 400–500 μmol m^{-2} s^{-1} at the surface of the growing medium. Plants were fertilized with a controlled-release fertilizer [Nutricot-Type 100 (16N–4.4P–8.3K); Chisso-Asahi, Fertilizer Co., Ltd., Tokyo, Japan].

Establishment and maintenance of suspension cultures

Newly sprouted shoots (~50–100 mm tall) were excised from the stock plants to be used for induction of calli. First, they were washed with tap water until all traces of dirt were removed, and then washed again with an antibacterial liquid soap. They were then immersed in 70 % ethanol for 3 min and subsequently immersed in a 1 % sodium hypochlorite solution containing Tween 20 (2–3 drops/100 mL) and gently shaken on a rotary shaker (100 rpm) for 30 min. In a sterile environment, they were rinsed five times with sterile water and outermost leaves were removed from each shoot and the basal portions (~15–20 mm) were excised. Every leaf layer was carefully separated, sliced into ~3–5 mm-wide pieces, and placed on MS-C medium [MS medium with vitamins (Murashige and Skoog (1962), containing 50 g L^{-1} sucrose, 290 mg L^{-1} proline, 0.5 μM kinetin (Kin), 5.0 μM 2,4-dichlorophenoxyacetic acid (2,4-D), 3 g L^{-1} Phytagel; pH 5.9 (Wang et al. 1999)] to induce callus development. Calli were cultured in the dark at 25 °C and subcultured every 3–4 weeks on MS-C medium. To establish suspension cultures, ~1–2 g of friable calli were transferred to a 250-mL Erlenmeyer flask containing 50 mL of MS-L medium (same composition as MS-C without Phytagel) and incubated in the dark at ~20 °C on a rotary shaker at 100 rpm. Well-established cultures were maintained by subculturing every 3–4 weeks by completely removing the old medium by pipetting and dividing each culture into two new flasks.

Construction of the binary plasmid vector for transformation

We constructed a binary transformation vector that carried the *P. agglomerans* phytoene synthase gene (*crtB*), fused to the TP of the small subunit of ribulosebisphosphate carboxylase–oxygenase (RuBisCO) from tobacco (*Nicotiana sylvestris*) (Pinck et al. 1984), under the control of the promoter region of a gene for capsanthin–capsorubin synthase (*Llccs*) (Jeknić et al. 2012) and the Nos terminator (TNos) (Fig. 1). The source of the *crtB* gene was the plasmid pAC-LYC (Cunningham et al. 1994).

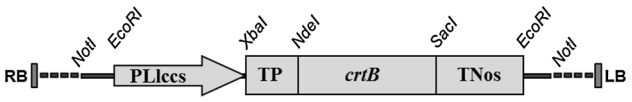

Fig. 1 Binary vector construct pWBVec10a/PLlccs:TP-*crtB*:TNos containing *P. agglomerans* phytoene synthase (*crtB*) gene used for transformation of *I. germanica* 'Fire Bride'. PLlccs, promoter of a capsanthin–capsorubin synthase gene from *L. lancifolium*; TP, RuBisCO small subunit transit peptide of tobacco (*N. sylvestris*); TNos, nopaline synthase terminator

Table 1 The primers used for cloning of a gene for phytoene synthase (CrtB) from *P. agglomerans* and the promoter region (PLlccs) of a gene for capsanthin–capsorubin synthase (*Llccs*) from *L. lancifolium*

Primer	Sequence (5′ → 3′)
crtB-F	CCAATGCATATGAGCCAACCGCCGCTGCTTGACC
crtB-R	ACTGAGCTCCTAAACGGGACGCTGCCAAAGACC
PLlccs-F	AAGCTTGAATTCGCAGCAAACCATGAACCTTTG
PLlccs-R	TCTAGAGTTGGACTGGAGTGGTAGAG
E-crtB-F	TCTGATGATGGCCAGGGTGA
E-crtB-R	TAAACGGGACGCTGCCAAAG
E-18S-F	TAGGTGAACCTGCGGAAGGATCATT
E-18S-R	CAACTTGCGTTCAAAGACTCGATG

Also, included are primers used for the expression analysis by RT-PCR of the *crtB* gene and the gene for 18S ribosomal RNA (*18S rRNA*) in transgenic plants of *I. germanica* 'Fire Bride'

First, we designed a pair of forward and reverse primers (crtB-F and crtB-R, Table 1) in such a way as to introduce the appropriate restriction sites to allow us to fuse the *crtB* gene 'in frame' with cDNA that encodes the TP of small subunit of RuBisCO. The forward primer (crtB-F) was designed to introduce *Nsi*I and *Nde*I restriction sites at the 5′ end, while the reverse primer (crtB-R) was designed to introduce the *Sac*I restriction site at the 3′ end of the *crtB* gene. The c*rtB* gene was amplified using PCR and subcloned into the pGEM®-T Easy vector (Promega, Madison, WI, USA). After sequencing to confirm its identity, the *crtB* gene was excised with *Nde*I and *Sac*I, and ligated into the pUC/*codA* vector (Hayashi et al. 1997), in place of the gene for choline oxidase (*codA*), to generate pUC/CaMV35S:TP-*crtB*:TNos.

Second, we assembled a translational fusion of the TP-*crtB* with the promoter region (PLlccs; −1,877 bp) of a gene for capsanthin–capsorubin synthase (*Llccs*) from *Lilium lancifolium* Thunb. 'Splendens' (GenBank Number GU443955) that included the 5′UTR of *Llccs* (89 bp long out of 94 bp; the last 5 bp were changed to introduce the *Xba*I site). The PLlccs was PCR amplified using the primers PLlccs-F and PLlccs-R (Table 1). The PLlccs-F forward primer was designed in such a way to introduce *Hind*III and *Eco*RI at the 5′ end, while the PLlccs-R reverse primer was designed to introduce *Xba*I at the 3′ end of PLlccs. The PLlccs was amplified using PCR, subcloned into the pGEM®-T Easy vector, and sequenced to confirm its identity. It was then excised with *Hind*III and *Xba*I and inserted into pUC/CaMV35S:TP-*crtB*:TNos in place of the CaMV35S promoter to generate the PLlccs:TP-*crtB*:TNos expression cassette.

To flank it with the *Not*I restriction sites, the PLlccs:TP-*crtB*:TNos expression cassette was excised with *Eco*RI and subcloned into the pGEM®-T Easy vector previously digested with the same enzyme and dephosphorylated using shrimp alkaline phosphatase (UBS, Cleveland, OH, USA) to prevent self-ligation. Finally, the expression cassette was excised with *Not*I and ligated into the pWBVec10a binary transformation vector (Wang et al. 1998), previously digested with *Not*I and dephosphorylated as above to prevent self-ligation. The resulting vector was designated as pWBVec10a/PLlccs:TP-*crtB*:TNos.

Agrobacterium tumefaciens-mediated transformation of *I. germanica* 'Fire Bride'

Plasmid vector pWBVec10a/PLlccs:TP-*crtB*:TNos was transformed into *A. tumefaciens* LBA4404 (Hoekema et al. 1983) using a chemical-based procedure, as described by Walkerpeach and Velten (1994). Transformation of *I. germanica* 'Fire Bride' was done as described before (Jeknić et al. 1999, 2012). *A. tumefaciens* was grown in YEP medium containing 100 mg L^{-1} spectinomycin and 300 mg L^{-1} streptomycin at 28 °C overnight in a rotary shaker at 250 rpm. The bacteria were pelleted by centrifugation and resuspended in twice the original volume using MS-Inf medium (MS basal medium with vitamins containing 50 g L^{-1} sucrose, 10 g L^{-1} glucose, 100 μM acetosyringone; pH 5.2). *A. tumefaciens* was induced in this medium for 1–2 h at 100 rpm prior to transformation. Selection of transgenic cell clumps was done on media containing 10 mg L^{-1} hygromycin. The hygromycin-resistant calli were tested for GUS activity as described by Jefferson (1987). Plant regeneration, acclimatization, and plant growth in greenhouses were done as described before (Jeknić et al. 1999; Jevremović et al. 2013).

Expression analysis of the *crtB* transgene

The total RNA was isolated from different plant parts (standards, falls, stigmas, ovaries, stamens, flower stalk, leaves and rhizome) of control and *crtB*-transgenic plants using an RNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA, USA) following manufacturer's instructions. The concentration and purity of RNA were determined by measuring the absorbance at 260 nm (A_{260}) and 280 nm

(A_{280}) with a NanoDrop 1000 (Thermo Fisher Scientific, Wilmington, DE, USA). All RNA samples were then treated with DNase (Promega) following manufacturer's instructions, and subsequently cleaned up using an RNeasy Plant Mini Kit, following the RNA cleanup protocol. The concentration was determined again, as described above.

Expression analyses of the *crtB* gene in different plant parts were carried out using a reverse transcriptase PCR (RT-PCR). For RT-PCR analysis, 1 µg of total RNA was used as a template for first-strand cDNA synthesis with Omniscript® Reverse Transcription kit (Qiagen Inc., Valencia, CA, USA). The sequences of primers (E-crtB-F, E-crtB-R, E-18S-F and E-18S-R) used for expression analysis are given in Table 1. The PCR analyses were carried out under the following conditions: initial denaturation at 95 °C/3 min, followed by 33 cycles of three-step PCR (95 °C/30 s, 60 °C/30 s, 72 °C/1 min) and a final extension at 72 °C/7 min using GoTaq® Hot Start Polymerase (0.625 units) with Green GoTaq® Flexi Buffer (Promega).

HPLC and UHPLC analysis of carotenoids

For the analyses of carotenoid pigments in callus tissue, carotenoids were extracted from control and *crtB*-transgenic calli as described by Fraser et al. (2000). We used 100 mg of freeze-dried control calli and 50 mg of freeze-dried *crtB*-transgenic calli. For HPLC analysis, a 200-µL aliquot of each sample was evaporated to dryness under a stream of nitrogen and the residue was resuspended in 200 µL of 90 % acetone with an internal standard (vitamin E). HPLC was performed as described by Van Heukelem and Thomas (2005). Samples were mixed on a vortex mixer and placed in the cooling rack of the HPLC system. Then 357 µL of buffer and 143 µL of extract were injected into the system (LC-10A HPLC system with LC solution software; Shimadzu, Kyoto, Japan) using a pretreatment program and mixing in the loop prior to injection.

For the analyses of carotenoid pigments in flowers, standards and falls were collected from *crtB*-transgenic plants as well as from the control plants, frozen in liquid nitrogen, pulverized, and freeze dried. An aliquot of dried powder of each sample was dissolved in 3 mL of 90 % acetone with an internal standard (vitamin E), mixed on a vortex mixer, sonicated on ice, allowed to extract at 4 °C for 20 h, and mixed again. The dissolved samples were then filtered through a 0.2-µm Teflon syringe filter into HPLC vials, and placed in the cooling rack of an UHPLC system. The samples were injected in the UHPLC (Shimadzu LC-30A UHPLC system with Lab Solution software) using a pretreatment program and mixing in the loop with buffer before injection (Van Heukelem and Thomas 2005). All UHPLC analyses were carried out in triplicates.

Results

Production of *crtB*-transgenic plants

Suspension cell cultures of *I. germanica* 'Fire Bride' were used for *A. tumefaciens*-mediated transformation with pWBVec10a/PLlccs:TP-*crtB*:TNos binary vector, that harbored *crtB* under the control of the PLlccs (Fig. 1), as well as an empty vector, pWBVec10a, as a control. Ten independent transgenic lines were regenerated from *crtB*-transgenic calli. PCR analysis of the transgene and GUS staining confirmed that they were stably transformed (data not shown). Transgenic plants were grown to maturity in a greenhouse under long-day photoperiod (16 h days/8 h nights). Within about a year, most of the transgenic plants had flowered.

Carotenoid content and color changes in *crtB*-transgenic calli

Ectopic expression of the *crtB* gene, under the control of the PLlccs, changed the calli's normal yellow color into varying shades of pink-orange and red (Fig. 2a, c). An HPLC profiling of the carotenoids in control (Fig. 2b) and red *crtB*-transgenic calli (Fig. 2d) was carried out to determine the qualitative and quantitative changes of carotenoids.

The most prominent change was that the *crtB*-transgenic calli accumulated lycopene at $\sim$52 µg g^{-1} DW, while control calli contained no or negligible amounts of lycopene. Lycopene was the most abundant carotenoid in *crtB*-transgenic calli. Furthermore, in control calli, the two major carotenoids were lutein and α-carotene which were accumulated at $\sim$12 and $\sim$8 µg g^{-1} DW, respectively. The amount of both of these carotenoids increased approximately threefold in *crtB*-transgenic calli, to $\sim$38 µg g^{-1} DW of lutein and $\sim$26 µg g^{-1} DW of α-carotene. Control calli also accumulated minor amounts of neoxanthin and violaxanthin, $\sim$0.2 and $\sim$0.4 µg g^{-1} DW, respectively. In *crtB*-transgenic calli, however, the accumulation of these two carotenoids increased approximately sevenfold, to $\sim$1.3 and $\sim$3 µg g^{-1} DW, respectively. Finally, *crtB*-transgenic calli accumulated a carotenoid that was very similar to β-carotene (designated as β-carotene-like, Fig. 2d), but which could not be unequivocally identified because it eluted very close to the α-carotene peak, at $\sim$24 µg g^{-1} DW. This carotenoid was absent or only present in negligible amounts in control calli.

Phenotypic changes in *crtB*-transgenic plants

Ectopic expression of *crtB* in transgenic iris plants altered the carotenoid biosynthetic pathway and caused a visible

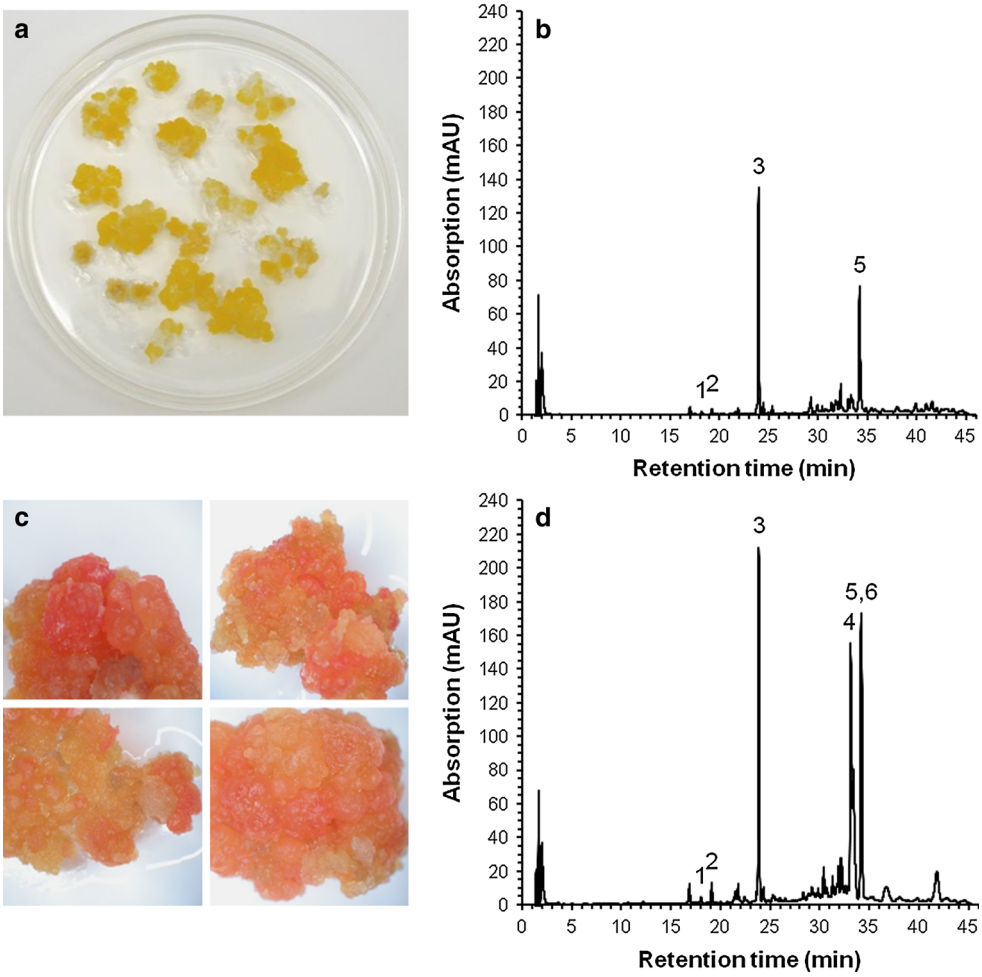

Fig. 2 Color changes in *crtB*-transgenic calli and HPLC profiles of carotenoid pigments in control and *crtB*-transgenic calli of *I. germanica* 'Fire Bride'. **a** Control non-transformed calli; **b** HPLC chromatogram of carotenoid extract from non-transformed calli; **c** representative examples of *crtB*-transgenic calli; **d** HPLC chromatogram of carotenoid extract from *crtB*-transgenic calli. Peaks of carotenoids: (*1*) neoxanthin; (*2*) violaxanthin; (*3*) lutein; (*4*) lycopene; (*5*) α-carotene; and (*6*) β-carotene-like. HPLC analysis was carried on carotenoid extracts obtained from 100 mg of freeze-dried control calli and 50 mg of freeze-dried *crtB*-transgenic calli

increase in pigmentation in certain tissues of *crtB*-transgenic plants. In contrast, control plants, transformed with an empty vector, were visually identical to wild-type plants.

Young *crtB*-transgenic plantlets exhibited color change, from white to pink in the meristematic region and surrounding tissues, as well as in the base of the leaves (Fig. 3). As the plantlets matured, the pink hue of these tissues progressed to a more orange one. The color of the rhizome also changed from yellowish-white in the control plants to pink-red in young transgenic plantlets and to orange in mature, transgenic plants. Both *crtB*-transgenic and control plants were grown to flowering stages (Fig. 4a, b).

In wild-type plants, the basal parts of the standards and falls, especially the abaxial sides, normally retain some chlorophyll and appear light green in color (Fig. 5a, b). Conversely, in the *crtB*-transgenic plants, these tissues changed color to orange (Fig. 5c, d). However, there were no visually significant changes in the color of the rest of the standards and falls.

The ovaries of control plants appear green from the outside and longitudinal cross sections through the ovaries show that the inside tissues are green and light green, while the ovules are white (Fig. 6a). In contrast, the ovaries and ovules of *crtB*-transgenic plants accumulated large amounts of carotenoids and acquired a deep orange color (Fig. 6b).

The stamen of wild-type flowers has a white anther that rests upon a light pink filament (Fig. 6c). In *crtB*-transgenic flowers, the filament became darker and more orange and the anthers became pink (Fig. 6d).

The flower stalk including branches of *crtB*-transgenic plants, which are normally green, also changed color, varying from variegated green-orange to completely orange in different transgenic lines (Figs. 4a, b; 5a, c; 6e, f).

Expression analysis of *crtB* transgene

To determine whether the PLlccs promoter actually provided flower-specific expression of the *crtB* transgene in our transgenic iris plants, we carried out expression analysis of the *crtB* transgene in different plant parts (standards, falls, styles, ovaries, stamens, flower stalk, leaves and rhizome) using a RT-PCR. Under PLlccs, the *crtB*

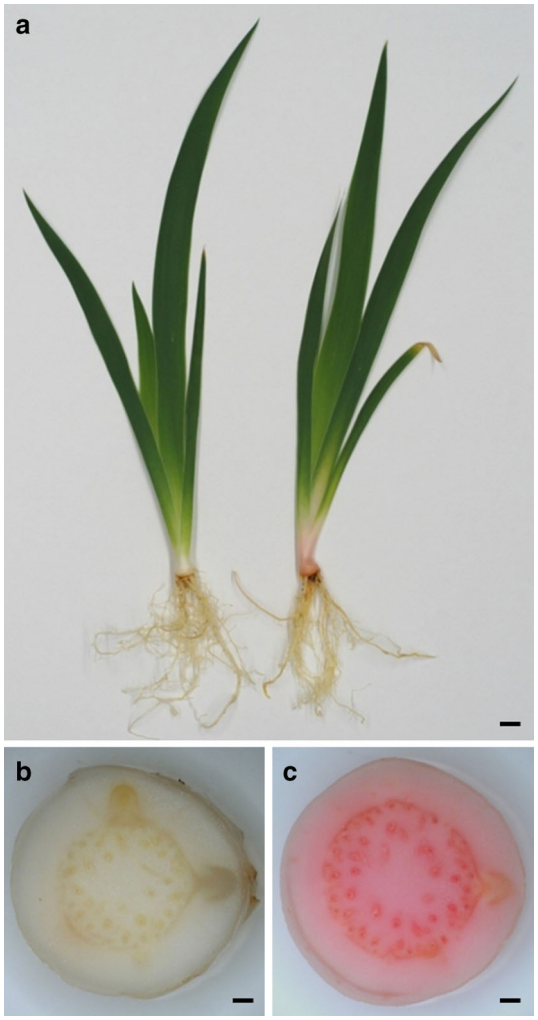

Fig. 3 One-month-old control and *crtB*-transgenic plantlets of *I. germanica* 'Fire Bride'. **a** Control (*left*) and *crtB*-transgenic plantlet (*right*); **b** cross-section through the meristematic region of control plantlet; **c** cross-section through the meristematic region of *crtB*-transgenic plantlet. *Bars* 10 mm (**a**) and 1 mm (**b**, **c**)

gene was expressed primarily in flower tissues (standards, falls, styles, ovaries and stamens) (Fig. 7a). Only traces of the *crtB* transcript were detected in the leaves, and rhizome (Fig. 7a). As expected, no *crtB* transgene expression was detected in wild-type samples, showing that the primers had no selectivity for endogenous phytoene synthase gene(s) (Fig. 7b).

UHPLC analysis of carotenoids in standards and falls

UHPLC analysis was conducted on carotenoid extracts from the standards and falls of *crtB*-transgenic plants, as well as control plants, to determine if there were any changes in carotenoid pigment composition.

As mentioned before, the pink color of the standards and falls in *I. germanica* 'Fire Bride' flowers is due to the accumulation of lycopene. The standards and falls of control plants accumulated lycopene at ~595 μg g^{-1} DW, while the *crtB*-transgenic plants accumulated lycopene in the standards and falls at ~521 μg g^{-1} DW (Fig. 8a, b; Table 2). Analysis of the carotenoid extracts of control plants by UHPLC revealed that very little carotenoids other than lycopene accumulated in the standards and falls of control flowers, though small amounts of neoxanthin, violaxanthin, and lutein were present (Fig. 8a, b; Table 2). Similar to lycopene, the amount of these carotenoids in *crtB*-transgenic plants was also reduced (Table 2). The amount of lutein in *crtB*-transgenic plants was reduced by 48 % compared to that of control plants, while the amounts of neoxanthin and violaxanthin were reduced by 43 and 35 %, respectively (Table 2). Visually, the standards and falls of the *crtB*-transgenic plants looked similar to those of control plants, though it appeared that the pink color was less prominent. The bases of the standards and falls of control plants retain visible amounts of chlorophyll and are light green in color, while in *crtB*-transgenic plants the chlorophyll was not present and they turned orange in color (Figs. 5, 8a, b). The standards and falls of control plants contained ~120 μg g^{-1} chlorophyll *a* and ~31 μg g^{-1} chlorophyll *b*, while those of *crtB*-transgenic plants accumulated no chlorophyll *a* or *b*. Quantitative data showing the contents and composition of the carotenoids and chlorophylls in the standards and falls of the control and *crtB*-transgenic plants are summarized in Table 2.

UHPLC analysis of carotenoids in ovaries and flower stalks

As mentioned earlier, in comparison to control plants, the *crtB*-transgenic plants showed a significant orange pigmentation in normally green flower parts, including the ovaries and flower stalks (Figs. 4, 5, 6).

Detailed carotenoid analysis by UHPLC showed that in the ovaries of *crtB*-transgenic plants the color change was primarily due to the accumulation of high amounts of lycopene and α- and β-carotene (incompletely resolved peaks and hereinafter presented as a single value), which accumulated at ~184 and ~174 μg g^{-1} DW, compared to ~8 and ~52 μg g^{-1} DW, respectively, in control plants (Fig. 8c, d; Table 2). The color change was compounded by an approximately tenfold decrease in chlorophyll *a* and *b*, from ~680 and ~236 μg g^{-1} DW in control plants to ~67 and ~22 μg g^{-1} DW in *crtB*-transgenic plants, respectively (Fig. 8c, d; Table 2). Furthermore, the amounts of neoxanthin, violaxanthin and lutein, all declined by about two to three fold compared to control plants (Fig. 8c, d; Table 2).

Similar results were obtained for the flower stalks of *crtB*-transgenic plants in which the color change was due

Fig. 4 One-year-old control and *crtB*-transgenic greenhouse-grown plants of *I. germanica* 'Fire Bride'. **a** Control plant in full bloom; **b** *crtB*-transgenic plant in full bloom

primarily to the accumulation of lycopene and the sharp decline in concentrations of chlorophyll *a* and *b*. The flower stalks of *crtB*-transgenic plants accumulated lycopene at ~45 μg g^{-1} DW, while the flower stalks of control plants accumulated no or negligible amounts of lycopene (Fig. 8e, f; Table 2). The amounts of chlorophylls *a* and *b* in *crtB*-transgenic plants declined compared to control by about eight- and ninefold, respectively (Fig. 8e, f; Table 2). In contrast to ovaries, the amounts of α- and β-carotene in the flower stalks of *crtB*-transgenic plants declined about ~27 % compared to control (Fig. 8e, f; Table 2). However, similar to ovaries, the amounts of neoxanthin, violaxanthin, and lutein in flower stalks of *crtB*-transgenic plants also declined in comparison to control. Quantitative data showing the contents and composition of the carotenoids and chlorophylls in the ovaries and flower stalks of control and *crtB*-transgenic plants are summarized in Table 2.

Discussion

Flower color is one of the most important quality traits in ornamental plants because of its influence on the diversity of floricultural crops, consumer preferences, and marketing opportunities. Here, we present the results of an attempt to generate red flowers in a pink *I. germanica* cultivar, 'Fire Bride', by metabolic modulation of the carotenoid biosynthetic pathway through the ectopic expression of a bacterial gene for phytoene synthase (*crtB*) from *P. agglomerans*.

Carotenoids are essential for light harvesting, photoprotection of the photosynthetic apparatus, and in the synthesis of some plant hormones (abscisic acid and strigolactones) in addition to determining the color of some flowers and fruits (Niyogi 2000; Nambara and Marion-Poll 2005; Dun et al. 2009). Therefore, genetic modification of the carotenoid biosynthetic pathway should be carried out using promoters that ensure spatial and temporal regulation of these alterations to avoid, or minimize, the adverse effects these changes might have on regeneration, different physiological processes, and overall vegetative growth of the transgenic plants. It is also helpful to use "foreign" genes with low homology to endogenous genes, to avoid complications associated with gene silencing, i.e., cosuppression, which was encountered by Fray et al. (1995) with the overexpression of the endogenous gene for PSYI in tomato. It has been shown in tomato that these complications can be avoided using a gene for CrtB with low homology (<35 %) to endogenous genes for PSY, thus preventing cosuppression and gene silencing (Fraser et al. 2002).

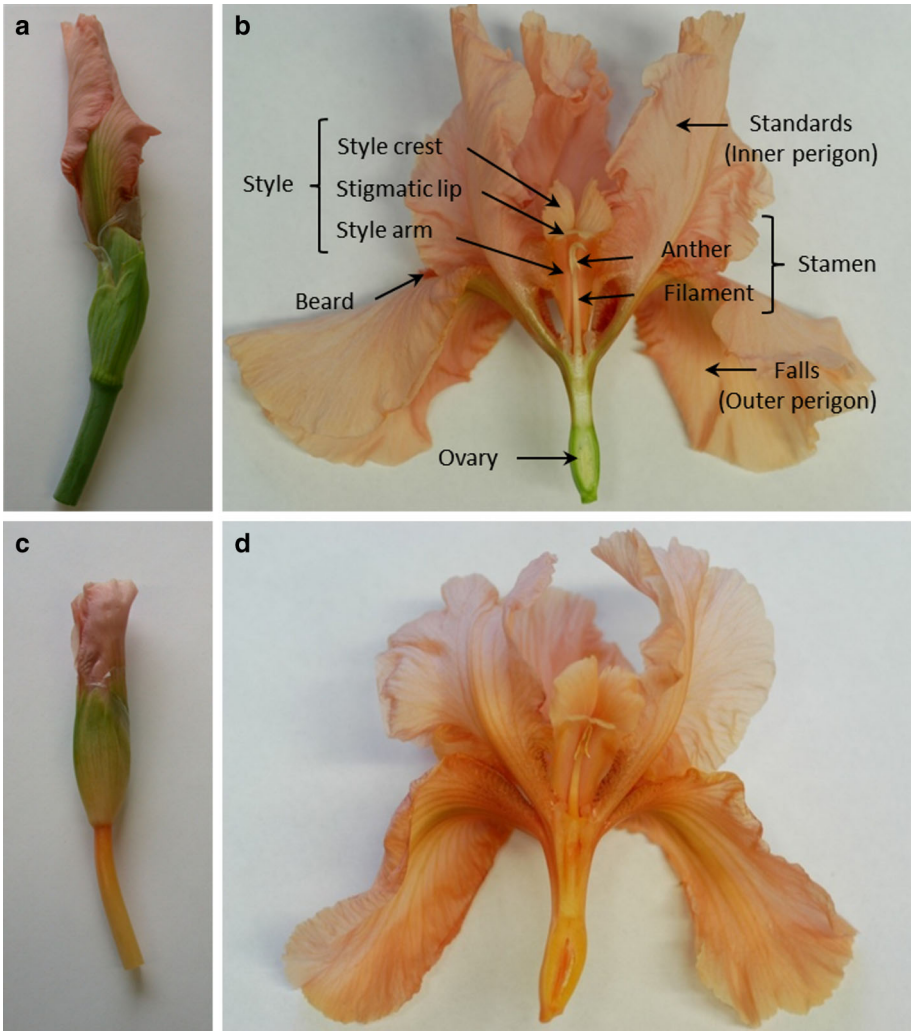

Fig. 5 Flower color changes of greenhouse-grown *crtB*-transgenic *I. germanica* 'Fire Bride' plants. **a** Flower bud of control plant; **b** open flower of control plant with bracts and one fall removed and longitudinally cut-open ovary to reveal other flower parts structures; **c** flower bud of *crtB*-transgenic plant; **d** open flower of *crtB*-transgenic plant with bracts and one fall removed and longitudinally cut-open ovary to reveal other flower parts and structures

For these reasons, we attempted to reduce the potential negative consequences of modifying the carotenoid biosynthetic pathway using a bacterial phytoene synthase gene (*crtB* from *P. agglomerans*) under the control of a flower-specific promoter. Unfortunately, no information is currently available about native flower-specific promoters in *I. germanica*. Thus, we selected the promoter region of a gene for capsanthin–capsorubin synthase (PLlccs) from *L. lancifolium*, which is responsible for the flower-specific expression of that gene in tiger lily (Jeknić et al. 2012).

Expression analysis showed that the *crtB* transgene regulated by PLlccs was primarily expressed in different flower parts, and only a trace amount of the *crtB* transcript was detectable in the leaves and rhizomes of transgenic plants (Fig. 7). This indicates that PLlccs is a suitable promoter for directing flower-specific expression of a transgene in iris.

PLlccs-controlled ectopic expression of *crtB* caused increased synthesis and accumulation of carotenoids in callus tissue. Wild-type *I. germanica* calli are yellow due to the accumulation of yellow xanthophylls such as lutein, α-carotene, violaxanthin, zeaxanthin, and neoxanthin (Fig. 2). In contrast, *crtB*-transgenic calli exhibited various shades of pink-orange and red, primarily due to the accumulation of lycopene (Fig. 2). This confirms that the ectopic expression of *crtB* in iris callus tissue caused a major perturbation of the carotenoid biosynthetic pathway and directed the flux of metabolites towards lycopene synthesis. In addition to the dramatic increase in lycopene accumulation, lutein and α-carotene accumulation increased approximately threefold, while the accumulation of neoxanthin and violaxanthin increased approximately sevenfold.

In comparison to control plants, the *crtB*-transgenic plants showed significant orange pigmentation in normally green flower parts, including the flower stalk, wall of ovaries, bracts, and the basal parts of the standards and falls (Figs. 4, 5, 6). The color changes in ovaries and flower stalks of *crtB*-transgenic plants were primarily due to the dramatic accumulation of lycopene (Table 2). In addition,

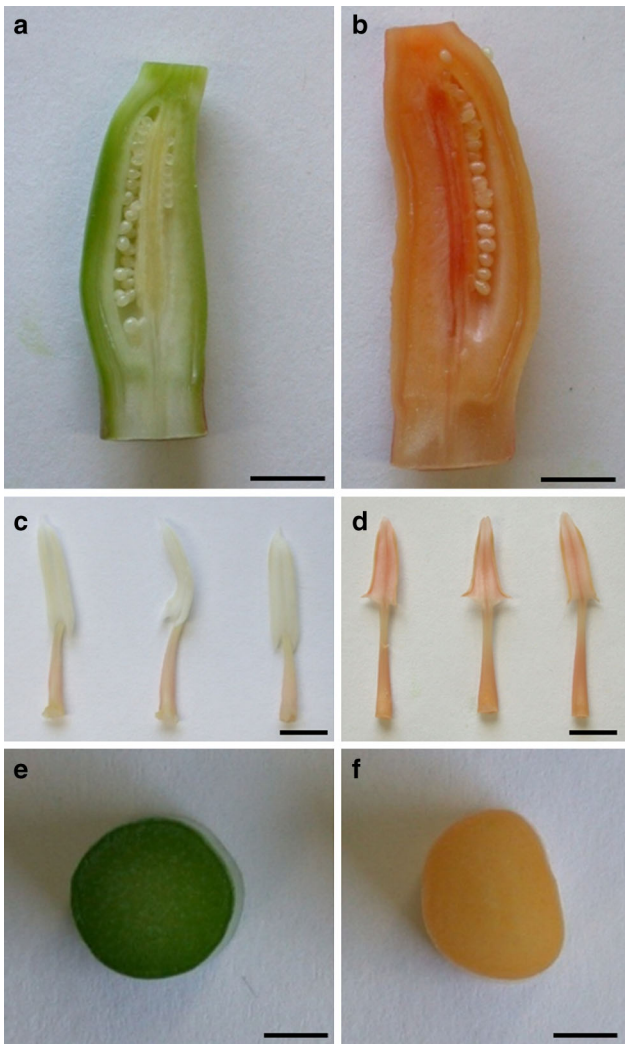

Fig. 6 Color changes in different flower parts of *crtB*-transgenic *I. germanica* 'Fire Bride' plants. Longitudinally cut-open ovary (**a**), stamen (**c**) and cross-cut flower stalk (**e**) of control plants; longitudinally cut-open ovary (**b**), stamen (**d**) and cross-cut flower stalk (**f**) of *crtB*-transgenic plants. *Bars* 5 mm

in both ovaries and flower stalks, the acquisition of orange color was compounded by a sharp decrease in chlorophylls *a* and *b*. The high level of carotenoid accumulation in the ovaries and flower stalks of *crtB*-transgenic plants suggests that CrtB was functionally integrated into the carotenoid biosynthetic pathway and was effective in enhancing production of carotenoids in these flower parts. Furthermore, similar to what we found in callus tissues, the ectopic expression of *crtB* in ovaries and flower stalks redirected the flux of metabolites towards lycopene synthesis.

In contrast to callus tissue and the green flower parts mentioned above where the color changes were mainly due to a significant accumulation of lycopene, the UHPLC analysis of the carotenoid extracts from the standards and falls of *crtB*-transgenic and control plants revealed that there was no increase in lycopene content in these flower parts, despite expression analysis by RT-PCR clearly showing the expression of the *crtB* transgene in these tissues (Figs. 7, 8a, b; Table 2). In fact, contrary to our original expectations, the standards and falls of *crtB*-transgenic plants actually accumulated 14 % less lycopene than the standards and falls of control plants (Table 2). Moreover, the amount of several xanthophylls that accumulated in small amounts in standards and falls of control plants (lutein, neoxanthin, and violaxanthin) was also reduced in *crtB*-transgenic plants (Table 2).

It is unlikely that the reduction in lycopene content in the standards and falls was due to cosuppression of the endogenous gene(s) for PSY in the *crtB*-transgenic plants because we specifically used a bacterial phytoene synthase gene (*crtB*) to reduce the chances of cosuppression. It is possible that the reduced carotenoid content in the standards and falls of *crtB*-transgenic plants was due to some of the early precursors of carotenoid biosynthesis being consumed in the tissues preluding the standards and falls, such as the flower stalks and ovaries. The dramatic color changes observed in these tissues support this possibility.

While the use of a bacterial *crt*B gene for genetic modulation of carotenogenesis in higher plants has its advantages, primarily in preventing cosuppression of endogenous gene(s) for PSY, little is known about suborganellar localization and functional integration of CrtB

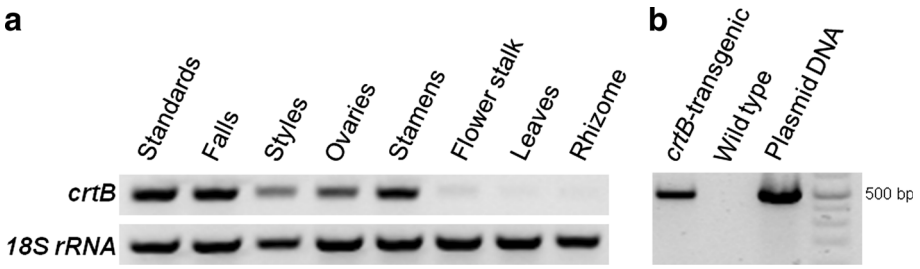

Fig. 7 Analysis of the expression of *crtB* gene under the control of PLlccs in different plant parts (standards, falls, styles, ovaries, stamens, flower stalk, leaves and rhizome) of *crtB*-transgenic *I. germanica* 'Fire Bride' plants by RT-PCR (**a**). Relative levels of expression of the *crtB* transgene were normalized with respect to expression of the *18S rRNA* gene; relevant RT-PCR controls: *crtB*-transgenic plants (standards and falls), wild-type plants (standards and falls), and plasmid DNA (**b**)

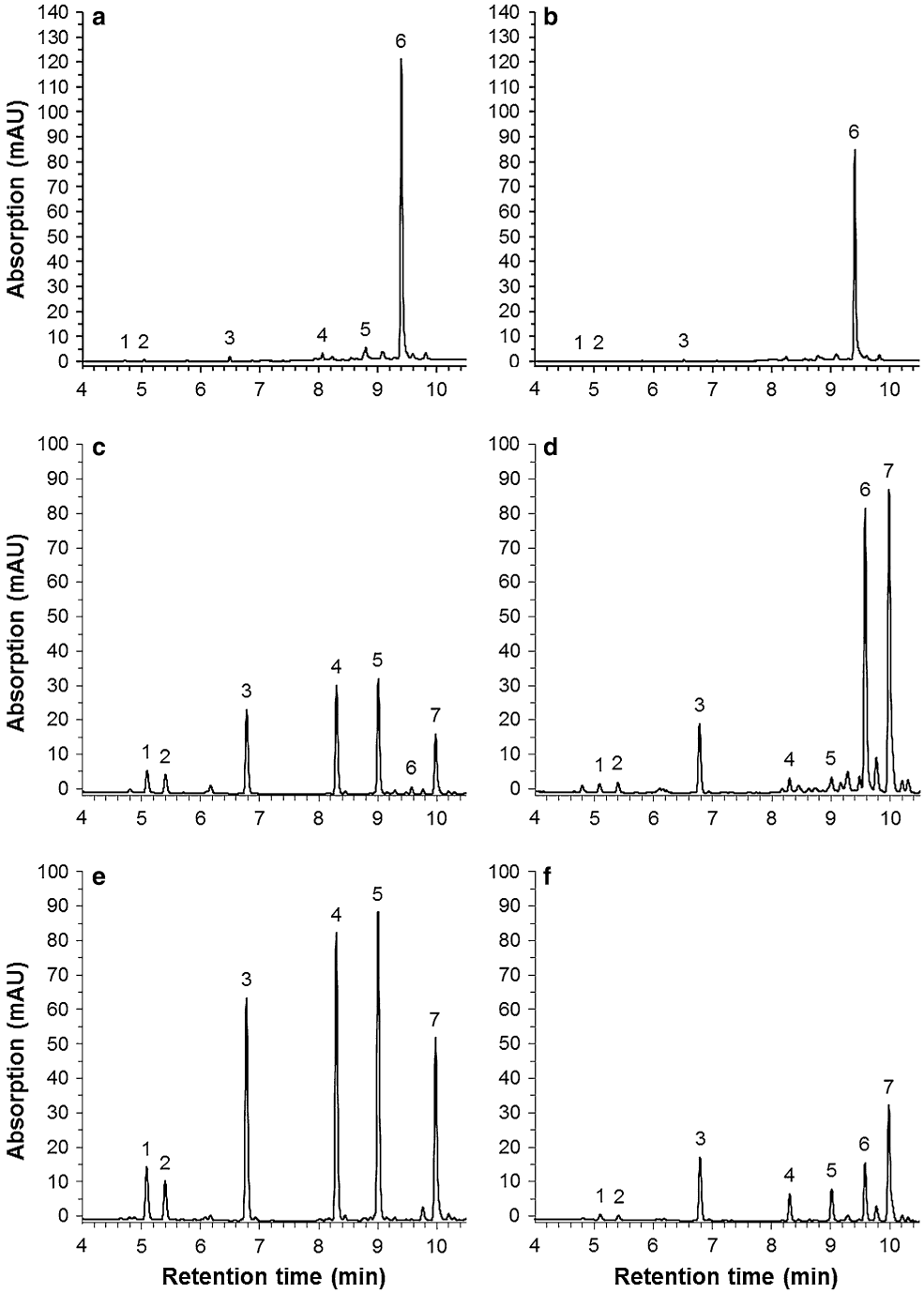

Fig. 8 UHPLC chromatograms of carotenoid extracts from different flower parts of control and *crtB*-transgenic *I. germanica* 'Fire Bride' plants. Standards and falls (**a**), ovaries (**c**), and flower stalks (**e**) of control plants; standards and falls (**b**), ovaries (**d**) and flower stalks (**f**) of *crtB*-transgenic plants. Peaks: (*1*) neoxanthin; (*2*) violaxanthin; (*3*) lutein; (*4*) chlorophyll *b*; (*5*) chlorophyll *a*; (*6*) lycopene; and (*7*) α- plus β-carotene

inside different types of plastids of higher plants. The use of *Psy* genes from different plant species had differential effects on enhancing the synthesis and accumulation of carotenoids in rice (Paine et al. 2005). For example, the "Golden Rice 2" that was developed by introducing a gene for PSYI from maize (*Zea mays*) in combination with the *Erwinia uredovora* (reclassified to *Pantoea ananatis*) carotene desaturase gene (*crtI*) showed increase in total carotenoids of up to 23-fold compared to the original "Golden Rice" transformed with daffodil (*Narcissus pseudonarcissus*) *Psy* in combination with the *E. uredovora crtI* (Ye et al. 2000; Paine et al. 2005). It has been shown recently, that different allelic variants of PSYI from maize, which differ from each other in only 1–2 amino acids, exhibit differential plastid localization that might direct the biosynthesis of carotenoids to different suborganellar locations and could be linked to their activity in promoting carotenoid biosynthesis (Shumskaya et al. 2012;

Table 2 Composition and content of carotenoids and chlorophylls (μg g^{-1} DW) in standards and falls, ovaries and flower stalks of control and *crtB*-transgenic *I. germanica* 'Fire Bride' plants

Peak no. and ID[a]		Perigon (standards and falls)		Ovaries		Flower stalks	
		Control	*crtB*-transgenic	Control	*crtB*-transgenic	Control	*crtB*-transgenic
1	Neoxanthin	3.25 ± 0.1	1.41 ± 0.1	25.98 ± 1.2	7.07 ± 0.1	37.40 ± 1.2	4.35 ± 0.5
2	Violaxanthin	3.09 ± 0.1	1.09 ± 0.1	16.60 ± 0.7	6.44 ± 0.1	21.17 ± 0.7	3.17 ± 0.3
3	Lutein	5.41 ± 0.1	2.60 ± 0.2	62.95 ± 3.0	37.47 ± 0.3	103.42 ± 3.1	34.41 ± 2.3
4	Chlorophyll *b*	30.76 ± 2.8	n.d.	236.85 ± 11.2	22.03 ± 0.2	381.08 ± 12.1	40.91 ± 5.3
5	Chlorophyll *a*	119.33 ± 11.9	n.d.	680.89 ± 31.8	67.04 ± 1.2	1100.32 ± 34.8	134.11 ± 16.1
6	Lycopene	595.20 ± 33.3	521.60 ± 19.1	8.52 ± 1.1	184.04 ± 3.9	n.d.	45.64 ± 0.6
7	α + β carotene	n.d.	n.d.	52.28 ± 2.4	174.19 ± 3.7	95.91 ± 2.2	70.19 ± 4.2

n.d. Not-detected

[a] Peak No. and ID correspond to those in Fig. 8

Shumskaya and Wurtzel 2013). In addition, bacterial carotenoid biosynthetic genes do not contain N-terminal target pre-sequences; therefore, it is necessary to use TPs to target them to the plastids. Most often the TP derived from the small subunit of RuBisCO from pea (*Pisum sativum*) has been used to target the bacterial enzymes into plastids (Rosati et al. 2009). To direct CrtB to the chromoplasts of the standards and falls of *I. germanica* 'Fire Bride', the *crtB* gene was fused to the TP sequence of the RuBisCO small subunit from tobacco (Fig. 1). The change of carotenoid content and the concomitant alteration of color in ovaries, flower stalks and anthers indicate that the TP was recognized and efficiently targeted CrtB into chloroplasts and plastids (most likely leucoplasts and/or other non-colored plastids in white anthers) in these organs. However, the efficiency of this TP to target CrtB into the chromoplasts of the standards and falls, as opposed to chloroplasts and other types of plastids, and its possible effect on the ensuing sub-compartmentalization of the CrtB has not been investigated. Umemoto et al. (2006) and Umemoto and Toguri (2012) used *crtW* augmented with different TPs at its N-terminus to target it into chromoplasts of *Petunia* flowers and found that there was a correlation between the TP sequence used relative to the expression of the transgene and the color values in the transformants. Therefore, it is possible that the CrtB produced in the standards and falls of *crtB*-transgenic iris plants was not targeted and/or localized properly or efficiently in the chromoplasts of these tissues resulting in the CrtB having no significant effect on the carotenogenesis in these flower parts.

If we assume that the CrtB was properly targeted and functionally integrated in chromoplasts of standards and falls, one potential reason that more lycopene did not accumulate in these flower parts of *crtB*-transgenic plants is that the synthesis of phytoene may not be the rate-limiting step in carotenoid biosynthesis in these flower parts. As mentioned before, PSY has been shown to be the rate-limiting step in different parts of many plants, including rice and corn endosperms, and canola, flax and *Arabidopsis* seeds (reviewed by Rosati et al. 2009). However, it has been shown previously that one of the principal mechanisms that controls the carotenogenesis in different species is the transcriptional regulation of different steps in the carotenoid, and broadly in the isoprenoid, biosynthetic pathways (reviewed by Hirschberg 2001; Cazzonelli and Pogson 2010; Cazzonelli 2011). For example, it has been shown that the overexpression of the 1-deoxy-D-xylulose-5-phosphate synthase (DXS), which is the first step in the MEP (2-C-methyl-D-erythritol 4-phosphate) pathway, leads to increased synthesis and accumulation of carotenoids in several species including *Arabidopsis*, tomato and potato, which indicates that it is one of the rate-limiting steps in carotenoid biosynthesis (Estévez et al. 2001; Enfissi et al. 2005; Morris et al. 2006). Our results suggest that a similar situation may exist in the standards and falls of *I. germanica* 'Fire Bride,' where isoprenoid and/or carotenoid biosynthetic enzymes other than PSY may have a larger impact on the flux of metabolites through the pathway and might be the rate-limiting step(s).

Another potential reason that more lycopene did not accumulate in the standards and falls of *crtB*-transgenic plants, under assumption that the CrtB was correctly targeted and functionally integrated, is that the carotenoid storage mechanisms in the chromoplasts of the standards and falls may have been already saturated, consequently limiting further increases of lycopene in these tissues in *crtB*-transgenic plants. The capacity of chromoplasts, which serve as a metabolic sink for sequestration and storage of carotenoids, affects the level of carotenoid accumulation (Li and Yuan 2013). It has been shown previously that increased β-carotene accumulation in orange curd tissue of *Brassica oleracea* is the result of a novel gene mutation that creates additional carotenoid storage mechanisms through the differentiation

of proplastids and/or other non-colored plastids into chromoplasts (Li et al. 2001; Lu et al. 2006). Furthermore, transgenic tomato plants overexpressing the carotenoid-binding protein, fibrillin, which provides additional carotenoid sequestration capacity, had increased levels of carotenoid accumulation (Simkin et al. 2007). In retrospect, genetic transformation of pink irises with either of these two genes, possibly in coordination with a gene for CrtB or PSY, might create a metabolic sink to sequester more lycopene, which may ultimately lead to red iris flowers.

In summary, the ectopic expression of the *crtB* gene in *I. germanica* pink cultivar 'Fire Bride' caused several flower parts to develop different degrees of novel orange and pink colors, demonstrating its utility in altering the color of specific flower parts. Further studies will be needed to clarify why ectopic expression of the *crtB* gene in *I. germanica* 'Fire Bride' did not lead to increased lycopene content in standards and falls and the generation of red flowers.

Author contribution Zoran Jeknić—designed and generated the *crtB* constructs, carried out iris transformations, performed expression analyses of the *crtB* transgene, coordinated pigment analysis, and wrote the manuscript. Stevan Jeknić—performed the tissue culture work, maintained the plants in the greenhouse, phenotypically characterized the iris transformants, carried out statistical analysis of the data, and contributed to writing the manuscript. Slađana Jevremović—directed the regeneration of transgenic iris plants, helped select the transgenic lines to be used for subsequent characterizations, analyzed data, and edited the manuscript. Angelina Subotić—helped with phenotypic characterization of the iris transformants, analysis and interpretation of the data, and edited the manuscript. Tony H.H. Chen—guided the research, coordinated the results, and edited the manuscript.

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Conflict of interest The authors declare that they have no conflict of interest.

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