

Alteration of flower color in *Solanum lycopersicum* through ectopic expression of a gene
for capsanthin-capsorubin synthase from *Lilium lancifolium*

by
Stevan Jeknic

A PROJECT

submitted to

Oregon State University

University Honors College

in partial fulfillment of
the requirements for the
degree of

Honors Baccalaureate of Science in Chemical Engineering
(Honors Scholar)

Presented May 11, 2015
Commencement June 2015

AN ABSTRACT OF THE THESIS OF

Stevan Jeknic for the degree of Honors Baccalaureate of Science in Chemical Engineering presented on May 11, 2015. Title: *Alteration of flower color in *Solanum lycopersicum* through ectopic expression of a gene for capsanthin-capsorubin synthase from *Lilium lancifolium*.*

Abstract approved:

Louisa Hooven

Tomato flowers (*Solanum lycopersicum*) were used as a model system to investigate flower color modification by alteration of the carotenoid biosynthetic pathway, due to their ease of transformation and short time from seed to flowering. Tomato flowers are naturally bright yellow, primarily due to the accumulation of violaxanthin. Violaxanthin is a precursor of capsanthin-capsorubin synthase (ccs), which catalyzes the conversion of antheraxanthin and violaxanthin, two yellow xanthophylls, into capsanthin and capsorubin, two red κ -xanthophylls, respectively. A capsanthin-capsorubin synthase gene cloned from tiger lily (*Lilium lancifolium*) was expressed in tomatoes under the control of the promoter from a petunia chalcone synthase gene, fused to an enhancer sequence of the cauliflower mosaic virus 35S promoter. All transgenic lines produced flowers with a light orange pigmentation, as opposed to the natural yellow coloration. UHPLC analysis confirmed that the color change coincided with the accumulation of two novel carotenoids, capsanthin and a capsanthin-like carotenoid. A more pronounced color change likely could have been achieved using a stronger promoter or by down-regulating competing pathways. Nevertheless, these results indicate that alteration of the carotenoid biosynthetic pathway with a gene for capsanthin-capsorubin synthase is a possible strategy for producing novel red and orange hues in certain ornamental crops.

Key Words: genetic engineering, capsanthin-capsorubin synthase, flower color, carotenoid biosynthesis, *Solanum lycopersicum*

Corresponding e-mail address: stevan.jeknic1@gmail.com

©Copyright by Stevan Jeknic
May 11, 2015
All Rights Reserved

Alteration of flower color in *Solanum lycopersicum* through ectopic expression of a gene
for capsanthin-capsorubin synthase from *Lilium lancifolium*

by
Stevan Jeknic

A PROJECT

submitted to

Oregon State University

University Honors College

in partial fulfillment of
the requirements for the
degree of

Honors Baccalaureate of Science in Chemical Engineering
(Honors Scholar)

Presented May 11, 2015
Commencement June 2015

Honors Baccalaureate of Science in Chemical Engineering project of Stevan Jeknic
presented on May 11, 2015.

APPROVED:

Louisa Hooven, Mentor, representing Horticulture

Ryan Contreras, Committee Member, representing Horticulture

Hiro Nonogaki, Committee Member, representing Horticulture

Toni Doolen, Dean, University Honors College

I understand that my project will become part of the permanent collection of Oregon State University, University Honors College. My signature below authorizes release of my project to any reader upon request.

Stevan Jeknic, Author

Contents

Introduction	1
1.1 Flower color and genetic engineering	1
1.2 Genetic engineering of anthocyanins	2
1.3 Genetic engineering of carotenoids	3
1.3.1 Occurrence, biosynthesis, and importance of carotenoids	3
1.3.2 Feasibility of altering flower color by using genetic modification of carotenoid biosynthesis	4
1.3.3 Feasibility of altering flower color by using a capsanthin-capsorubin synthase gene from <i>Lilium lancifolium</i>	5
Goals and Objectives	8
Materials and Methods	9
3.1 Genetic material	9
3.2 Plant material and growing conditions	10
3.3 <i>Agrobacterium</i> -mediated transformation of tomato hypocotyl explants	11
3.4 Confirmation of transformation	11
3.4.1 Histochemical assay for GUS activity	11
3.4.2 Detection of <i>Llccs</i> transgene by PCR	12
3.5 UHPLC analysis of carotenoid accumulation in transgenic plant tissue	13
Results	15
4.1 Generation of <i>Llccs</i> -transgenic tomato plants	15
4.2 Phenotypic changes in <i>Llccs</i> -transgenic tomato plants	15
4.3 Transgene expression in <i>Llccs</i> -transgenic tomato plants	16
4.4 UHPLC analysis of carotenoids from <i>Llccs</i> -transgenic tomato plants	17
Discussion	20
Conclusions	22
References	23

List of Figures

1	Partial schematic of the β,β -branch of the carotenoid biosynthetic pathway.	6
2	Partial map of the T-DNA region of the binary transformation vector . . .	9
3	Phenotypic changes in <i>Llccs</i> -transgenic tomato flowers	16
4	Expression analysis of the <i>Llccs</i> transgene under the control of the E35S-PchsA promoter.	17
5	UHPLC chromatograms of pigment extractions from <i>Llccs</i> -transgenic and wild-type flowers	18

List of Tables

1	Media for <i>in vitro</i> cultures and transformations of <i>Solanum lycopersicum</i> L.	10
2	Primers used for expression analysis of <i>Llccs</i> by RT-PCR	13
3	Carotenoid and chlorophyll profile of <i>Llccs</i> -transgenic and wild-type flowers of <i>S. lycopersicum</i>	19

Abstract

Tomato flowers (*Solanum lycopersicum*) were used as a model system to investigate flower color modification by alteration of the carotenoid biosynthetic pathway, due to their ease of transformation and short time from seed to flowering. Tomato flowers are naturally bright yellow, primarily due to the accumulation of violaxanthin. Violaxanthin is a precursor of capsanthin-capsorubin synthase (ccs), which catalyzes the conversion of antheraxanthin and violaxanthin, two yellow xanthophylls, into capsanthin and capsorubin, two red κ -xanthophylls, respectively. A capsanthin-capsorubin synthase gene cloned from tiger lily (*Lilium lancifolium*) was expressed in tomatoes under the control of the promoter from a petunia chalcone synthase gene, fused to an enhancer sequence of the cauliflower mosaic virus 35S promoter. All transgenic lines produced flowers with a light orange pigmentation, as opposed to the natural yellow coloration. UHPLC analysis confirmed that the color change coincided with the accumulation of two novel carotenoids, capsanthin and a capsanthin-like carotenoid. A more pronounced color change likely could have been achieved using a stronger promoter or by down-regulating competing pathways. Nevertheless, these results indicate that alteration of the carotenoid biosynthetic pathway with a gene for capsanthin-capsorubin synthase is a possible strategy for producing novel red and orange hues in certain ornamental crops.

Introduction

1.1 Flower color and genetic engineering

Interesting and novel flower colors are in constant demand in the floricultural industry and ornamental plants are often available in myriad cultivars with a wide array of colors [1]. These cultivars are typically the result of classical breeding methods, which have been successfully used for over a century to select for and amplify desired colors. Classical breeding, however, has failed to produce certain species-color combinations, such as a blue rose or a red iris [1].

Flower color is predominantly due to the accumulation of three types of secondary metabolites: anthocyanins, carotenoids, and betalains [1]. Anthocyanins, which are part of the flavonoid pathway, impart a wide variety of colors, including blue, violet, orange, red, and maroon. Carotenoids, on the other hand, produce only warm colors, such as reds, yellows and oranges, while betalains (which are relatively rare) produce red, pink, and purple hues [2]. The limitations of classical breeding are due to a lack of genes required to produce and accumulate specific pigments in certain species. For example, a red iris does not exist naturally because irises are unable to produce red anthocyanins (pelargonidin- and cyanidin-type), or accumulate high concentrations of red carotenoids (such as lycopene) [2]. As a result of these limitations, high consumer demand for novel flower colors has prompted research into finding alternative ways to generate them [1].

Genetic engineering is one possible alternative that provides an opportunity to expand the number of color determining genes available to any species, allowing the creation of new hues. There are three primary ways that genetic engineering can be used to alter flower

color: (i) introducing genes for enzymes that would otherwise be absent; (ii) up-regulating genes to direct and amplify existing pathways; and (iii) down-regulating genes to suppress existing pathways [3].

Multiple efficient methods of transfecting plants with exogenous genetic material exist, including (i) biolistic (microprojectile bombardment) transformation; (ii) direct gene transfer by electroporation or polyethylene glycol (PEG) treatment; and (iii) silicon carbide fiber-mediated transformation. From the large list of options, *Agrobacterium tumefaciens*-mediated transformation is one of the most commonly used and was selected for this project because it offers numerous advantages including (i) integration of a few copies of T-DNA with defined border sequences; (ii) minimal rearrangement of the plant genome; (iii) preferential integration into transcriptionally active regions of the chromosome; (iv) high quality and fertility of transgenic plants; and (v) easy manipulation [4, 5].

The focus of this project was to use *Agrobacterium*-mediated transformations to transfect tomato flowers (*Solanum lycopersicum*) with a gene to modify carotenoid biosynthesis with the goal of accumulating novel pigments. Tomatoes are an excellent model system for numerous reasons (discussed in later sections) and the results of this project will inform similar experiments in other species. This project is one of the few examples of modification of flower color by perturbation of the carotenoid biosynthetic pathway.

1.2 Genetic engineering of anthocyanins

Anthocyanins are a class of flavonoids, which are the most well-studied secondary metabolite in plants [6]. Their entire pathway has been elucidated in higher plants, which has allowed for multiple strategies of genetic modification to produce novel flower colors [6, 7, 8, 9]. In 1987, Meyer et al. were the first to create new flower coloration by showing that petunias that accumulate dihydrokaempferol (DHK) can be engineered using a heterologous gene for dihydroflavonol 4-reductase (DFR) with different substrate specificity to produce red flowers [10]. Soon after, van der Krol et al. (1988) prevented pigmentation in tobacco and petunia flowers by suppressing chalcone synthase (CHS). This strategy produced bright white flowers in both species [11].

Additionally, flower color modification has also been achieved with the simultaneous modification of multiple genes. One such achievement was in *Nicotina tabacum*, where red flowers were produced by redirecting the anthocyanin biosynthetic pathway towards pelargonidin synthesis. This was done by suppressing flavonol synthase (FLS) and flavonoid 3'-hydroxylase (F3'H), and subsequently introducing a gerbera DFR gene [12]. A similar approach was successful in *Torenia hybrid*, where suppression of F3'H and flavonoid 3',5'-hydroxylase (F3'5'H), followed by overexpression of a heterologous DFR gene resulted in pink flowers, as opposed to the natural blue ones [13].

Despite these successes, several challenges to using anthocyanin modification to target a specific color exist. The main concern is that anthocyanins (and their non-sugar containing counterparts, anthocyanidins) often go through a number of potentially color-changing glycosylations, acylations, and methylations before being stored in the vacuoles [14]. These reactions are difficult to control, which makes it problematic to direct the accumulation of a specific pigment [1]. Additionally, anthocyanins change color as a result of a variety of factors, such as vacuolar pH [15, 16], co-pigmentation [17, 18], metal ion-complexation [19, 20], and cell size and shape [21, 22]. Thus, even if the correct anthocyanin was synthesized there is no guarantee that the flower would exhibit the expected color.

1.3 Genetic engineering of carotenoids

Unlike anthocyanin modification, relatively little work has been done investigating modification of the carotenoid biosynthetic pathway in flowers. This project is intended to research a new approach for modifying flower color and demonstrate its potential for use in ornamental species.

1.3.1 Occurrence, biosynthesis, and importance of carotenoids

Carotenoids, synthesized in plants, bacteria, and some fungi, are lipophilic C₄₀ isoprenoids with polyene chains [23, 24, 25]. To date, more than 700 naturally occurring carotenoids have been identified [26]. In plants, carotenoids are integral components of photosynthesis, participating in light-harvesting reactions and protecting the photosynthetic machinery

from photo-oxidation [27, 28, 29]. Additionally, carotenoids are precursors of some plant hormones, such as abscisic acid and strigolactones [30, 31].

The first committed step in the synthesis of carotenoids is the production of phytoene (C₄₀) by the condensation of two geranylgeranyl diphosphate (GGPP) molecules. Phytoene, via four desaturation reactions and enzymatic or light-mediated photo-isomerization, then forms all-*trans*-lycopene. The pathway branches at this point with cyclohexane rings forming on the ends of the lycopene molecule to form α -carotene (one ϵ - and one β -ionone end group) or β -carotene (two β -ionone end groups). Carotenoids with two ϵ -ionone end groups are relatively rare [1].

All of the molecules in the carotenoid biosynthesis pathway are exclusively hydrocarbons until the formation of lycopene; subsequent reactions involve the enzymatic addition of oxygen moieties to produce hydroxy, epoxy, and oxy derivatives. These oxygen-containing carotenoids are known collectively as xanthophylls [32]. Examples include lutein on the β,ϵ -branch, zeaxanthin, antheraxanthin, and violaxanthin on the β,β -branch, and lactucaxanthin on the ϵ,ϵ -branch [1].

1.3.2 Feasibility of altering flower color by using genetic modification of carotenoid biosynthesis

In addition to their other vital functions, carotenoids impart yellow, orange, pink, and red pigmentation to the flowers of many plant species. To date, only a few successful attempts at carotenoid biosynthesis modification in flowers have been achieved, and most of these studies were directed at establishing astaxanthin (3,3'-dihydroxy- β,β -carotene-4,4'-dione) synthesis [33, 34]. For example, Mann et al. (2000) transfected *N. tabacum* with a β -carotene ketolase gene (*crtO*) from *Haematococcus pluvialis*, producing plants with bright red nectaries [34]. Similarly, Ralley et al. (2004) engineered astaxanthin synthesis in *N. tabacum* flowers using two genes, 3,3'- β -hydroxylase (*crtZ*) and 4,4'- β -oxygenase (*crtW*), from marine bacteria (*Paracoccus* species) [35].

Although these authors were able to produce color changes in specific flower tissues, their goal was to demonstrate the potential for plant-based production of an economically important compound [34]. To my knowledge, only three studies have explicitly attempted

to alter flower color for ornamental purposes through modification of the carotenoid biosynthetic pathway, and two of these examples were also focused on producing astaxanthin synthesis. Umemoto et al. (2006) used a *crtW* gene from *Agrobacterium auranticum* to engineer astaxanthin synthesis and transform the pale-yellow color of petunia flowers into shades of deep-yellow and orange [36]. Suzuki et al. (2007) also used *crtW*, but used it in *Lotus japonicus* to produce orange pigmentation in the naturally yellow flowers [27].

Jeknić et al. (2014) used a different carotenoid-based approach to successfully alter flower color. They used a gene for phytoene synthase (*crtB*) from *Pantoea agglomerans* to increase metabolite flux through the carotenoid pathway in *Iris germanica*, thus increasing production and accumulation of all carotenoids naturally present [2]. Similar strategies have previously been used successfully to increase carotenoid production in several plant species and tissues including canola seeds, tomato fruits, potato tubers, and tobacco leaves [37, 38]. The *crtB*-transgenic flowers produced by Jeknić et al. (2014) exhibited color changes in several flower tissues, including the stalk, ovaries, and anthers. The color of the standards and falls (petals and sepals), however, were not noticeably changed [2].

1.3.3 Feasibility of altering flower color by using a capsanthin-capsorubin synthase gene from *Lilium lancifolium*

Two carotenoid pigments that are capable of producing distinct red coloration are capsanthin (3,3'-dihydroxy- β,κ -carotene-6'-one) and capsorubin (3,3'-dihydroxy- κ,κ -carotene-6,6'-dione). The red color of bell peppers (*Capsicum annuum*) and the red-orange color of tiger lilies (*Lilium lancifolium*) are both due to the accumulation of capsanthin and capsorubin [39, 40, 41, 42]. The precursors to capsanthin and capsorubin are two yellow xanthophylls, antheraxanthin and violaxanthin, respectively (Figure 1).

The formation of capsanthin and capsorubin is catalyzed by capsanthin-capsorubin synthase (*ccs*) genes, which have previously been cloned and functionally characterized from two species. The first example was cloned and characterized from *C. annuum* in the 1990s, and it was later cloned from *L. lancifolium* in 2012 [39, 40, 41]. Seemingly contrarily, the NCBI GenBank includes sequences from five other plant species (*Citrus sinensis*, *Capsicum annuum*, *Daucus carota*, *Medicago truncatula*, and *Ricinus communis*) that have been

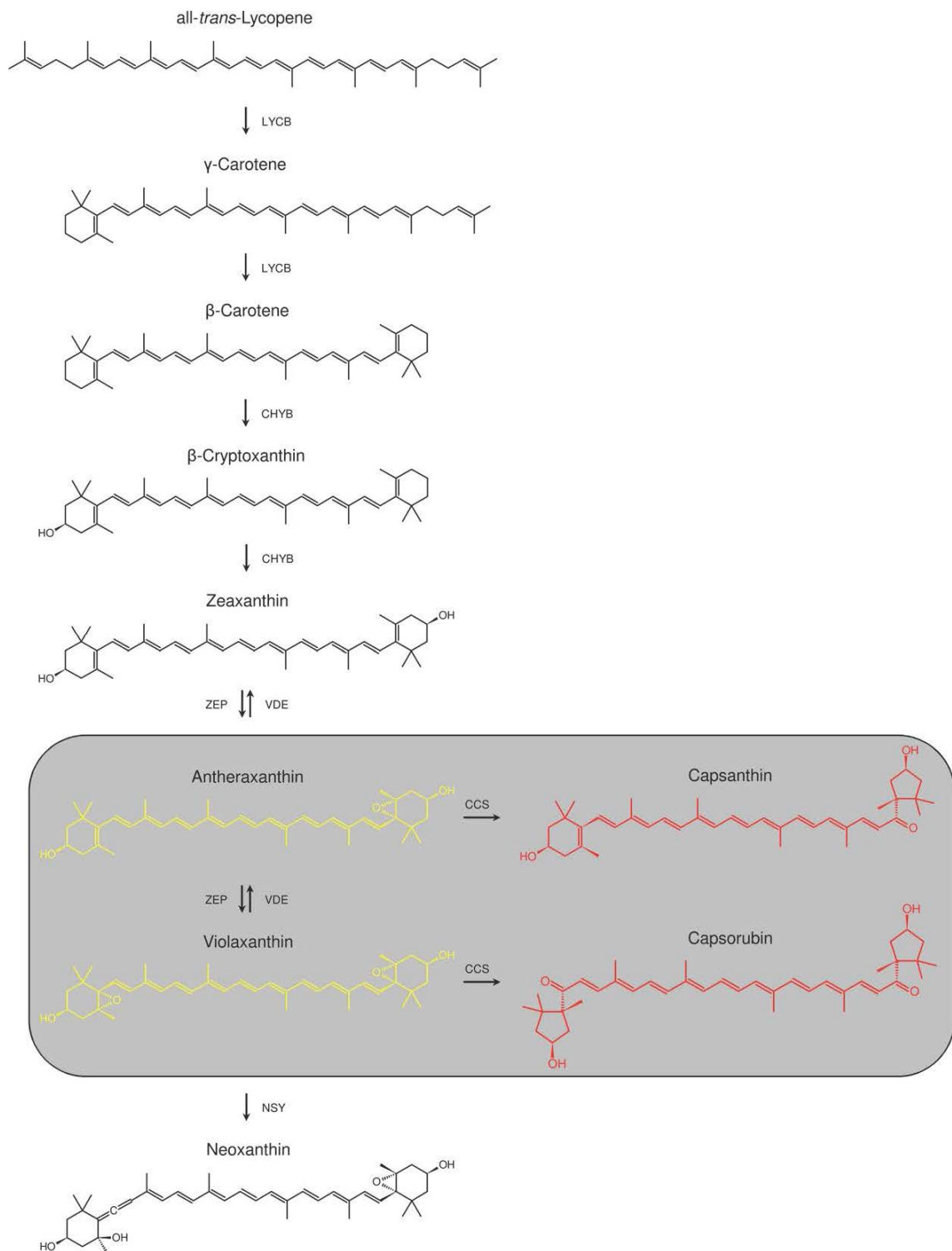


Figure 1: Partial schematic of the β,β -branch of the carotenoid biosynthetic pathway. The conversion of two yellow xanthophylls, antheraxanthin and violaxanthin, into the red κ -xanthophylls capsanthin and capsorubin is boxed. LYCB - lycopene β -cyclase; CHYB - carotene β -hydroxylase; ZEP - zeaxanthin epoxidase; VDE - violaxanthin de-epoxidase; CCS - capsanthin-capsorubin synthase; NSY - neoxanthin synthase.

identified as *ccs* genes. However, it is likely that these sequences were identified based on homology alone, thus no *ccs* genes other than the *ccs* from *C. annuum* or *L. lancifolium* have been truly cloned and functionally characterized.

The natural yellow color of tomato flowers is due to the production/accumulation of antheraxanthin and violaxanthin. Thus, *ccs* genes provide a promising possibility for flower color alteration in tomato flowers [43]. Additionally, tomato is a good candidate for genetic engineering experiments because it is amenable to genetic transformation and quickly grows from a seed to a flowering plant. The results of this project can then be extrapolated to other species that accumulate antheraxanthin and/or violaxanthin in flower tissues.

Goals and Objectives

The goal of this project was the development of a genetic engineering-based method to alter the carotenoid biosynthetic pathway in tomato flowers (*S. lycopersicum*) with the aim of producing flower colors that do not naturally occur. This would serve as a model for using similar strategies in ornamental plants such as irises.

The following phases were used to realize this goal:

1. Creation of a binary transformation vector containing a gene for capsanthin and cap-sorubin synthase from *Lilium lancifolium* (*Llcs*) and stable transfection in *Agrobacterium tumefaciens* strain LBA4404.
2. Use of an *Agrobacterium*-mediated transformation to produce *Llcs*-transgenic tomato cultures.
3. Regeneration of shoots and plants from *Llcs*-transgenic cultures.
4. Stably-transfected plants were grown to flowering and color change in flowers was evaluated by visual inspection and ultra high performance liquid chromatography (UHPLC) analysis.

Materials and Methods

3.1 Genetic material

Genetic material was prepared and provided by Zoran D. Jeknić [1]. The binary vector pWBVec10a was used as the transformation vector and was provided by Dr. Ming-Bo Wang (CSIRO Plant Industry, Black Mountain, Australia). pWBVec10a contains a GUS marker gene expression cassette (CaMV 35S::*uidA*::TNos) and a hygromycin-resistance gene expression cassette (PUBi1::*hpt*::TNos) within the T-DNA borders. Both genes contain an intron to prevent their expression in *A. tumefaciens*, in which intron splicing does not occur [1, 44].

A gene for capsanthin-capsorubin synthase (*Llccs*) was cloned from orange flower buds of *L. lancifolium* as described by Jeknić et al. (2012) [39]. The promoter for a petunia chalcone synthase gene (PchsA) was fused to an enhancer (E35S) sequence of the cauliflower mosaic virus 35S promoter and was used to control *Llccs* gene expression (E35S-PchsA). Expression was terminated by the Nos terminator. The resultant expression cassette (E35S-PchsA::*Llccs*::TNos) was excised and cloned into the pWBVec10a binary vector to produce the transformation vector, which was designated pWBVec10a/E35S-PchsA::*Llccs*::TNos (Figure 2) [1].

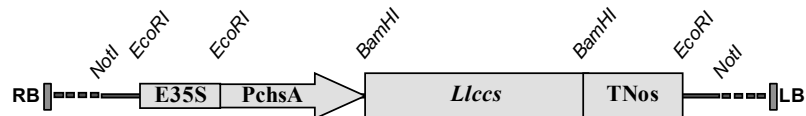


Figure 2: A partial map of the T-DNA region of the PWBVec10A/E35S-PchsA::*Llccs*::TNos binary transformation vector showing the expression cassette containing the gene for capsanthin-capsorubin synthase (*Llccs*) under the control of the E35S-PchsA chimeric promoter and Nos terminator.

3.2 Plant material and growing conditions

Tomatoes (*S. lycopersicum*) were grown and transfected as described by Park et al. (2004), with some modifications [45]. Approximately 200 seeds of *S. lycopersicum* L. ‘Money-maker’ (Gourmet Seed Int.) were washed in 70% ethanol for five minutes and in 20% bleach (with one drop Tween 20 per 100 mL) for 15 minutes. Seeds were subsequently rinsed thoroughly with sterile ddH₂O and transferred to Magenta GA-7 boxes with MS-G (germination medium) (Table 1). Boxes were kept at 4°C for seven days to break cold dormancy and encourage uniform germination. Boxes were transferred to a growth chamber at 20°C with a 16-hour photoperiod provided by full-spectrum fluorescent lamps (photosynthetic photon flux density (PPFD) 50 $\mu\text{mol m}^{-2} \text{s}^{-1}$). Seedlings were allowed to grow to approximately seven centimeters tall before being excised. Hypocotyls were removed and cut into 0.5 cm long pieces and cultured on MS-T (pre-cultivation medium) for two days prior to *Agrobacterium*-mediated transformation.

Table 1: Media for *in vitro* cultures and transformations of *Solanum lycopersicum* L.

Medium	Function	Composition
MS-G	Seed germination	1/2-strength MS basal medium [46], 10 g L ⁻¹ sucrose, pH 5.7
MS-T	Pre-cultivation and regeneration	Full strength MS basal medium, 30 g L ⁻¹ sucrose, 1 mg L ⁻¹ zeatin, 0.1 mg L ⁻¹ IAA, pH 5.7
MS-Inf	<i>Agrobacterium</i> infection	Full strength MS basal medium, 50 g L ⁻¹ sucrose, 100 μM acetosyringone, pH 5.2
MS-S	Selection	MS-T medium containing 2–5 mg L ⁻¹ hygromycin and 150–300 mg L ⁻¹ timentin
MS-R	Rooting	Full strength MS basal medium, 30 g L ⁻¹ sucrose, 150–300 mg L ⁻¹ timentin, pH 5.7

Post-transformation, explants were sub-cultured every 3–4 weeks on MS-S (selection medium) until roots began to develop. Rooted shoots were transferred to MS-R (rooting medium) until the plantlets developed extensive roots and were ready to be grown in soil. Plants were grown in a greenhouse with a 16-hour photoperiod and temperatures of 25±3 / 20±3 °C. Plants were grown in four-liter pots in soil consisting of three parts

peat, two parts pumice, and one part sandy loam (v/v). Natural light was supplemented with high-pressure sodium lamps (Energy Technics, York, Pa.) providing a PPFD of 400–500 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Plants were fertilized with Nutricot-Type 100 controlled-release fertilizer with a N/P/K content of 16/4.4/8.3 (Chisso-Asahi Fertilizer Co., Ltd., Tokyo, Japan) [1].

3.3 *Agrobacterium*-mediated transformation of tomato hypocotyl explants

Agrobacterium tumefaciens strain LBA4404 was used to transfect tomato tissues. First, *A. tumefaciens* was transformed with the pWBVec10a/E35S-PchsA::Llccs::TNos plasmid using a chemical-based procedure as described by Walkerpeach and Velten, 1994 [47]. *A. tumefaciens* was grown overnight in YEP medium containing 300 mg L⁻¹ streptomycin and 100 mg L⁻¹ spectinomycin at 28°C in a rotary shaker at 250 rpm. Bacteria were pelleted by centrifugation and resuspended in MS-Inf (*Agrobacterium* infection medium). *A. tumefaciens* was kept in this medium for 1–2 hours at 100 rpm immediately prior to infection. Pre-cultured seedling hypocotyls were exposed to the liquid suspension of *A. tumefaciens* for five minutes and then co-cultured for two days in the dark on MS-T before being thoroughly rinsed with a timentin solution (100–300 mg L⁻¹) and sterile ddH₂O and grown on MS-S for selection. MS-S contained 2–5 mg L⁻¹ hygromycin for selection of transgenic plant tissue and 150–300 g L⁻¹ timentin for prevention of *Agrobacterium* overgrowth.

3.4 Confirmation of transformation

3.4.1 Histochemical assay for GUS activity

As mentioned earlier, the transformation vector contained a GUS marker gene expression cassette to allow for easy detection of transgenic plants. Tissues expressing the GUS gene stain dark blue when exposed to the GUS staining solution. Histochemical GUS assays were performed as described by Jefferson (1987) [48]. GUS staining solution [0.1 M sodium phosphate buffer pH 7.2, 5 mM K₃[Fe(CN)₆], 5 mM K₄[Fe(CN)₆], 10 mM EDTA, 20%

methanol (v/v), 0.01% Triton X-100 (v/v), and 1 mg mL⁻¹ 5-bromo-4-chloro-3-indolyl- β -D-glucuronic acid cyclohexylammonium salt (x-Gluc)] was prepared immediately preceding each test to ensure the solution was highly active.

Slices of regenerated green leaves (\approx 2 mm) and roots (\approx 5 mm) were placed in separate microcentrifuge tubes and covered with at least 100 μ L of staining solution. Samples were infiltrated with GUS staining solution under vacuum for ten minutes at room temperature, and then left overnight at 37°C. Chlorophylls from green leaves were removed with several soaks in 95% ethanol before results were examined. The presence of blue staining (GUS-positive) indicated a stable transformation.

3.4.2 Detection of *Llccs* transgene by PCR

The GUS assay was used to quickly reveal transgenic plantlets, but to confirm stable insertion of the *Llccs* transgene, PCR amplification was used. GUS-positive plantlets that were able to grow on MS-S were selected for RT-PCR testing. Total RNA was isolated from flowers (corollas and stamens) and leaves of wild type and *Llccs*-transgenic tomato plants using an RNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA) following the manufacturer's instructions. The concentration and purity of the extracted RNA was determined by measuring absorbance at 260 nm (A_{260}) and 280 nm (A_{280}) with a Nanodrop 1000 (Thermo Fisher Scientific, Wilmington, DE). To eliminate DNA contamination, RNA samples were treated with DNase I (Promega, Madison, WI) and cleaned using an RNeasy Plant Mini Kit, following manufacturer's instructions for RNA cleanup. Final concentration was determined using a Nanodrop 1000 measuring A_{260} and A_{280} .

To synthesize cDNA for RT-PCR analysis, an Omniscript[©] Reverse Transcription kit (Qiagen Inc., Valencia, CA) was used with 1 μ g of total RNA as a template. The primers used for RT-PCR analysis of *Llccs* (*Llccs*-F and *Llccs*-R) and a gene for ubiquitin synthesis (*Ubi3*-F and *Ubi3*-R) are provided in Table 2. PCR amplification was conducted with initial denaturation at 95°C for 3 min, followed by 33 cycles of three step PCR (95°C for 30 sec, 60°C for 30 sec, 72°C for 1 min), and a final extension at 72°C for 7 minutes using a GoTaq[©] Hot Start Polymerase (0.635 units) with Green GoTaq[©] Flexi Buffer.

Table 2: Forward and reverse primers used for expression analysis of *Llccs* and a gene for ubiquitin synthesis (Ubi3) by RT-PCR.

Primer	Sequence (5' → 3')
Llccs-F	CTCCTCCGCCACCGCTAC
Llccs-R	CGGAGCAGGAGACGGAGGAG
Ubi3-F	GAAAACCCTAACGGGGAAGA
Ubi3-R	GCCTCCAGCCTTGTTGTAAA

3.5 UHPLC analysis of carotenoid accumulation in transgenic plant tissue

Carotenoids were extracted from two grams of wild-type flowers and *Llccs*-transgenic (line L1) flowers. Flowers were powdered in liquid nitrogen with a mortar and pestle and suspended in 50 mL tubes containing 20 mL of solvent extraction mixture (50% hexane, 25% acetone, and 25% ethanol (w/w)). The suspension was mixed and agitated for 15 minutes at room temperature before adding 5 mL of 50 mM Tris-HCL (pH 8.0) containing 1 M NaCl. The suspension was further agitated for 5 minutes and then centrifuged at 3500 rpm for 5 minutes to produce a distinct phase separation. A Pasteur pipette was used to collect the solvent extract (top phase), being careful not to collect any of the denser phase. The extract was evaporated to dryness under a stream of N₂.

Half of the sample was resuspended in 2 mL of methyl-*tert*-butyl ether (MTBE) and saponified as described by Lee et al. (2001) [49], rinsed three times with water, filtered through an Isolute[®] sodium sulfate (Na₂SO₄) drying cartridge (Biotage, LLC, Charlotte, NC), and evaporated to dryness under a stream of N₂. The residue was resuspended in 1 mL of ethanol and filtered through a polytetrafluoroethylene (PTFE) 0.45 μm filter to produce samples ready for UHPLC analysis. The other half of the sample was not saponified and was just resuspended and filtered as described above to produce samples ready for UHPLC analysis.

Capsanthin and capsorubin standards were purchased from CaroteNature (Lupsingen, Switzerland). To prepare reference solutions, 1 mg of capsanthin or capsorubin standard was dissolved in 50 mL of ethanol and filtered through a PTFE 0.45 μm filter.

Samples and standards were injected into a Shimadzu LC-30A UHPLC system (with

Lab Solution software) using a pretreatment program and mixing in the loop with buffer. Injection was done as described by Van Heukelem and Thomas (2005) [50]. All UHPLC analyses were done in triplicate.

Results

4.1 Generation of *Llccs*-transgenic tomato plants

Several *Agrobacterium*-mediated transformations were attempted, resulting in three independent transgenic lines (designated L1, L2, and L3) that were grown to flowering. The relatively low transformation efficiency was partly due to tomato tissues being highly sensitive to hygromycin, even at low concentrations (2–5 mg L⁻¹). MS-S, the selection media, contained no less than 2 mg L⁻¹ of hygromycin to prevent false positives.

4.2 Phenotypic changes in *Llccs*-transgenic tomato plants

The only phenotypic changes observed in full-grown plants were in the flowers. Flowers (corollas and stamens) of *Llccs*-transgenic tomato plants changed color from their natural yellow to varying shades of orange (Figure 3). Color changes were more pronounced in transgenic lines L1 and L2, while L3 was lighter in color. The corollas of L1 and L2 were deep orange, while the stamens were red-orange. Seeds were collected from *Llccs*-transgenic plants and germinated to confirm that the *Llccs* transgene and phenotypic changes were observed in subsequent generations.

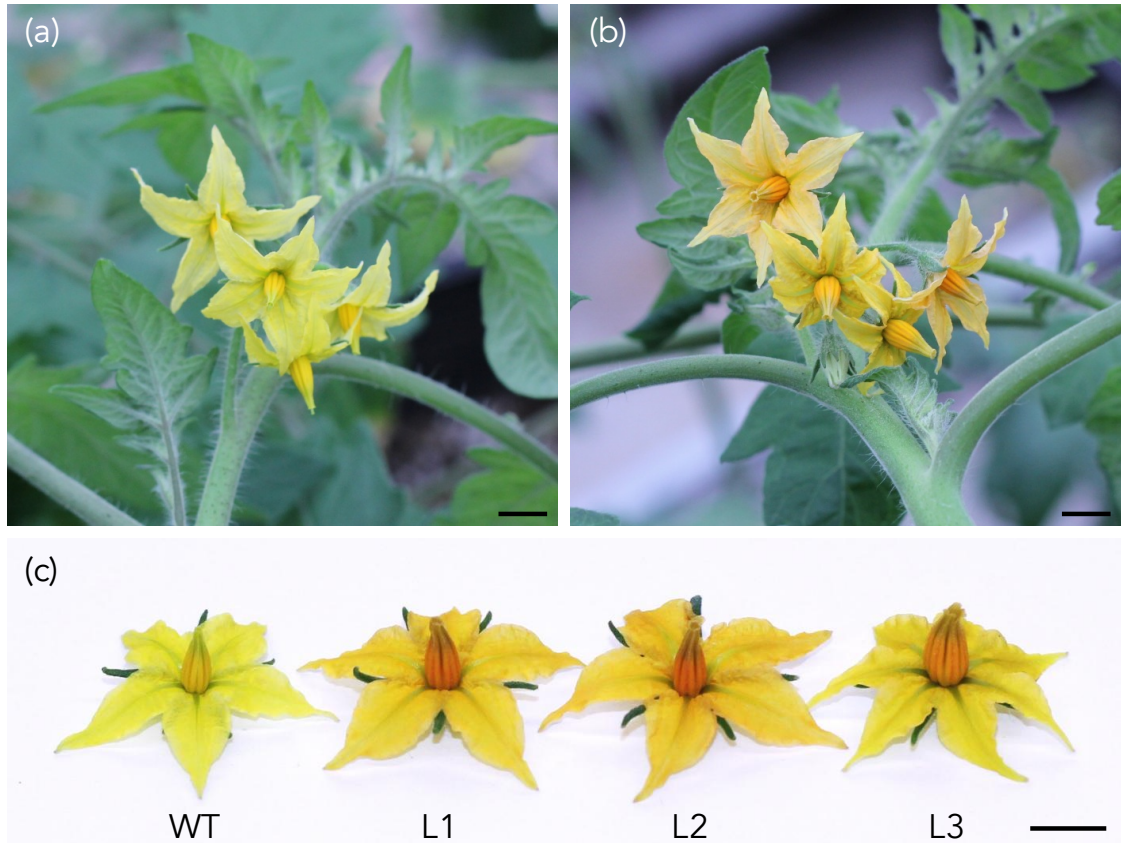


Figure 3: Phenotypic changes observed in *Llccs*-transgenic tomato flowers. Wild-type flower cluster (a); *Llccs*-transgenic line L2 flower cluster (b); and individual flowers of wild-type (WT) plants and *Llccs*-transgenic lines L1, L2, and L3 (c). Bars: 5 mm (a, b) and 1 cm (c)

4.3 Transgene expression in *Llccs*-transgenic tomato plants

Expression analysis by RT-PCR was performed on the flowers (corollas and stamens) and vegetative tissues (leaves) to determine whether the E35S-PchsA promoter was able to drive flower-specific expression of the *Llccs* transgene in *S. lycopersicum*. Expression analysis revealed that the *Llccs* transgene was expressed in both the flowers and vegetative tissue of *Llccs*-transgenic plants (Figure 4), thus E35S-PchsA is not completely flower-specific in *S. lycopersicum*. Higher expression levels were present in the leaves of transgenic lines L1 and L2 when compared to L3, while the flowers had approximately equivalent expression levels.

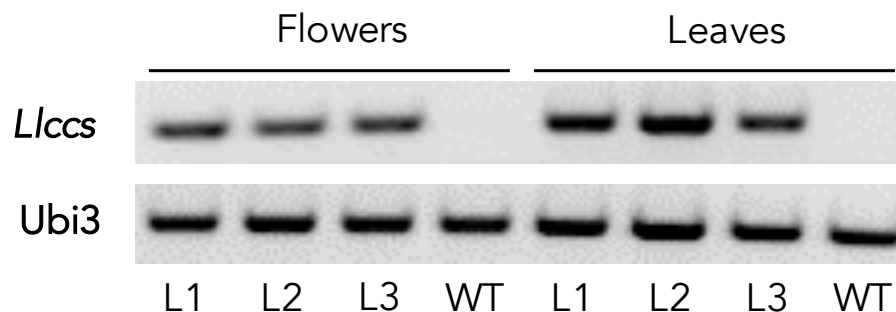


Figure 4: Expression analysis of the *Llccs* gene under the control of the E35S-PchsA promoter in flowers and leaves of *Llccs*-transgenic and wild-type *S. lycopersicum*. Transgenic lines are designated L1, L2, and L3, while WT is the wild-type. A gene for ubiquitin synthesis (*Ubi3*) was used as a control.

4.4 UHPLC analysis of carotenoids from *Llccs*-transgenic tomato plants

UHPLC analysis was used to determine the carotenoid and chlorophyll content of wild-type and *Llccs*-transgenic (L1) tomato flowers. In higher plants, most carotenoids are present in esterified forms in the chromoplasts. This can alter peak times and make identification difficult. Therefore, both saponified and non-saponified samples were tested to ensure representative results.

Two peaks were observed in *Llccs*-transgenic flowers that were not present in wild-type samples. The peaks were identified as belonging to capsanthin (Peak 5 at 7.7 min) and a capsanthin-like xanthophyll (Peak 1 at 5.8 min) (Figure 5). A capsorubin peak was not detected. Identifications were done by comparing the retention times and spectral properties to those of authentic xanthophyll standards.

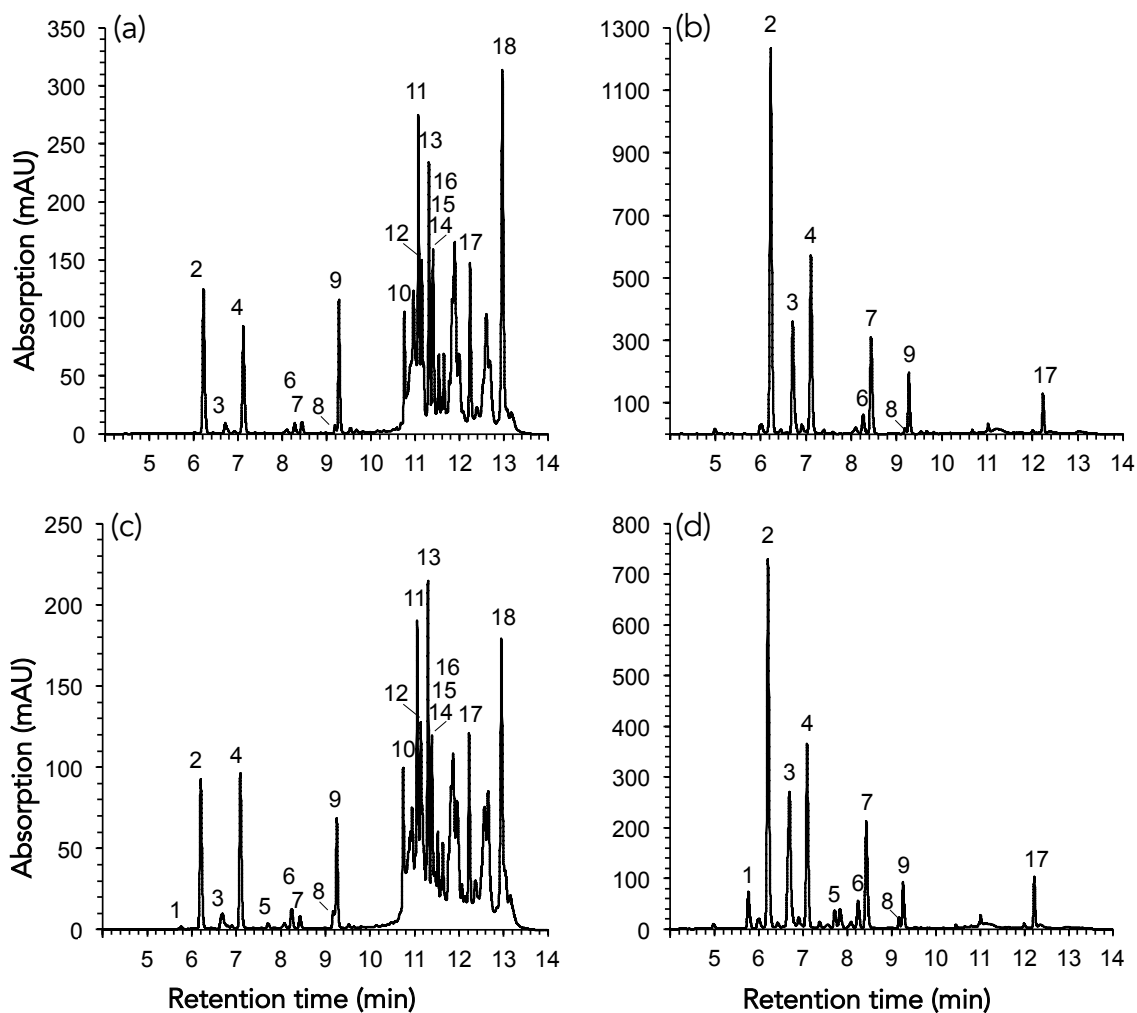


Figure 5: UHPLC chromatograms of pigment extractions from *Llccs*-transgenic (L1) and wild-type tomato flowers. Wild-type non-saponified (a); *Llccs*-transgenic non-saponified (b); wild-type saponified (c); and *Llccs*-transgenic saponified (d). Peak numbers (bold indicates presence in *Llccs*-transgenic samples only): (1) **capsanthin-like**; (2) violaxanthin-like; (3) neoxanthin; (4) violaxanthin; (5) **capsanthin**; (6) lutein-like; (7) neoxanthin-like; (8) zeaxanthin; (9) lutein; (10) chlorophyll b; (11) violaxanthin-like; (12) neoxanthin-like; (13) chlorophyll a; (14) neoxanthin-like; (15) neoxanthin-like; (16) violaxanthin-like; (17) $\alpha+\beta$ -carotene; (18) violaxanthin-like.

Table 3: Carotenoid and chlorophyll content ($\mu\text{g g}^{-1}$ dry mass) in saponified and non-saponified pigment extractions from *Llccs*-transgenic (L1) and wild-type tomato flowers, determined using UHPLC with authentic standards. Bold indicates carotenoids present only in *Llccs*-transgenic samples. Capsorubin was not detected. *n.d. - not detected.

Peak number	Carotenoid ID	Wildtype		<i>Llccs</i> -transgenic	
		Non-saponified	Saponified	Non-saponified	Saponified
1	Capsanthin-like	n.d.*	n.d.	0.14 \pm 0.00	5.58 \pm 0.00
2	Violaxanthin-like	11.33 \pm 0.04	107.27 \pm 0.47	8.76 \pm 0.02	68.18 \pm 0.06
3	Neoxanthin	1.17 \pm 0.01	37.02 \pm 0.11	1.35 \pm 0.00	37.66 \pm 0.08
4	Violaxanthin	8.50 \pm 0.00	53.76 \pm 0.16	9.05 \pm 0.03	35.44 \pm 0.03
5	Capsanthin	n.d.	n.d.	0.30 \pm 0.00	2.95 \pm 0.00
6	Lutein-like	0.57 \pm 0.00	4.19 \pm 0.01	0.86 \pm 0.00	3.89 \pm 0.01
7	Neoxanthin-like	1.02 \pm 0.00	30.62 \pm 0.09	0.86 \pm 0.00	22.07 \pm 0.03
8	Zeaxanthin	0.32 \pm 0.00	0.88 \pm 0.01	0.44 \pm 0.00	1.07 \pm 0.00
9	Lutein	5.26 \pm 0.01	9.66 \pm 0.02	3.40 \pm 0.03	5.08 \pm 0.01
10	Chlorophyll b	12.86 \pm 0.65	n.d.	12.09 \pm 0.57	n.d.
11	Violaxanthin-like	16.61 \pm 2.16	n.d.	12.22 \pm 1.65	n.d.
12	Neoxanthin-like	8.26 \pm 0.87	n.d.	7.33 \pm 1.03	n.d.
13	Chlorophyll a	85.02 \pm 1.52	n.d.	80.43 \pm 0.72	n.d.
14	Neoxanthin-like	11.58 \pm 0.10	n.d.	3.71 \pm 0.04	n.d.
15	Neoxanthin-like	3.86 \pm 0.31	n.d.	3.71 \pm 0.04	n.d.
16	Violaxanthin-like	3.74 \pm 0.25	n.d.	2.62 \pm 0.07	n.d.
17	α + β carotene	2.42 \pm 0.08	2.27 \pm 0.09	1.95 \pm 0.07	1.85 \pm 0.04
18	Violaxanthin-like	28.83 \pm 0.07	n.d.	16.25 \pm 0.13	n.d.

Discussion

The yellow color of tomato flowers is primarily due to the accumulation of violaxanthin, the precursor of the red κ -xanthophyll, capsorubin. As shown in Figure 1, in order to produce violaxanthin, the flower must also produce antheraxanthin, the precursor of the red κ -xanthophyll, capsanthin. Thus, both violaxanthin and antheraxanthin are produced in tomato flowers, and transformation with the *Llccs* gene was expected lead to the accumulation of both capsanthin and capsorubin and the presence of red hues in the flowers.

Due to a number of advantages, such as simple *Agrobacterium*-mediated transformation and short time from explant to flowering, tomato is an excellent candidate to test the ability of *Llccs* to alter flower color. However, tomato tissues were discovered to have one large drawback; they are highly sensitive to hygromycin, even at very low concentrations (2–5 mg L⁻¹). The pWBVec10a transformation vector used for all transformations carries the *hpt* gene for hygromycin resistance, thus hygromycin was used as the selection agent, which resulted in a limited recovery of stable transformants. Future experiments with *S. lycopersicum* should use a different selection agent.

Despite this, three independent *Llccs*-transgenic plants were eventually generated by transfecting seedling explants and grown to maturity in a greenhouse. Wild-type tomato plants were started from seeds, grown to maturity, and used as controls. The color of the flowers (corollas and stamens) of all three independent transgenic lines developed varying shades of a novel orange color when compared to wild-type plants. Transgenic lines L1 and L2 exhibited a more pronounced color change than line L3. UHPLC analysis showed that this change in flower color was correlated with, and likely caused by, the accumulation of two non-native κ -xanthophylls, capsanthin and a capsanthin-like xanthophyll. Surprisingly,

capsorubin was not detected.

There are a number of reasons that capsorubin may not have been accumulated, despite its precursor, violaxanthin, accumulating in high concentrations in flower tissues. Although it is known that *Llccs* can produce capsorubin [39], it may do so at a very slow rate or it may have a very low affinity for violaxanthin. Alternatively, it is possible that any capsorubin produced may have been immediately altered by endogenous enzymes and was thus never accumulated. It is not surprising that capsanthin was produced, despite no antheraxanthin accumulating, as antheraxanthin had to be produced for violaxanthin to accumulate in flowers.

UHPLC analysis also revealed that violaxanthin was still present in large quantities in the flowers of *Llccs*-transgenic tomatoes. This suggests that the color change could have been much more significant had a stronger promoter been used or a higher copy number achieved. Additionally, zeaxanthin epoxidase (ZEP), which converts antheraxanthin, the precursor of capsanthin, into violaxanthin, achieved a complete conversion of antheraxanthin in wild-type flowers. This suggests that it competes significantly with the introduced *Llccs* gene for antheraxanthin in transgenic flowers. Therefore, down-regulation of ZEP could increase the amount of antheraxanthin available to *Llccs*, and lead to more capsanthin accumulation and a more pronounced color change.

In conclusion, these results indicate that the *Llccs* gene is capable of altering flower color in *S. lycopersicum* through the accumulation of novel xanthophylls. This is in agreement with previous results that modification of the carotenoid biosynthetic pathway can be a successful strategy for altering flower color [2, 27, 36]. The strategy of using *Llccs* should be directly applicable to important ornamental species that accumulate antheraxanthin and/or violaxanthin in flower tissues, such as *Iris germanica* and *Iris hollandica*.

Conclusions

1. *Agrobacterium*-mediated genetic transformation can be used to stably transfect tomato plants (*S. lycopersicum*).
2. Tomatoes are very sensitive to hygromycin, even at low concentrations. A different selection agent should be used to increase recovery of transformants.
3. The E35S-PchsA promoter was not capable of driving entirely flower-specific expression in tomatoes.
4. Expression of *Llccs* in tomato flowers causes the accumulation of two novel xanthophylls, capsanthin and a capsanthin-like carotenoid. The presence of these two xanthophylls led to a novel orange pigmentation in flower tissues (corollas and stamens).
5. No capsorubin was accumulated in tomato flowers. It is possible that *Llccs* has low affinity for violaxanthin or a very slow conversion rate to capsorubin. More detailed investigations will be necessary to determine the exact cause.
6. The use of a stronger promoter likely would have increased the conversion of antheraxanthin into capsanthin, leading to darker red pigmentation. Additionally, down-regulation of ZEP would reduce the competition for antheraxanthin, possibly leading to increased capsanthin accumulation.
7. Based on these results, it should be possible to use *Llccs* to alter flower color in other species that accumulate violaxanthin and/or antheraxanthin in flower tissues.

References

- [1] Zoran D. Jeknić. *Alteration of flower color of Iris germanica L. through genetic modification of carotenoid biosynthetic pathway*. PhD thesis, University of Belgrade, 2013.
- [2] Z. Jeknić, S. Jeknić, S. Jevremović, A. Subotić, and T.H.H. Chen. Alteration of flower color in *Iris germanica* l. ‘Fire Bride’ through ectopic expression of phytoene synthase gene (*crtB*) from *Pantoea agglomerans*. *Plant Cell Rep.*, 33:1307–1321, 2014.
- [3] S. Chandler and F. Brugliera. Genetic modification in floriculture. *Biotechnol. Lett.*, 33:207–214, 2011.
- [4] T. Komari, Y. Hiei, Y. Ishida, T. Kumashiro, and T. Kubo. Advances in cereal gene transfer. *Plant Biotechnol.*, 1:161–165, 1998.
- [5] S. Tingay, D. McElroy, R. Kalla, S. Fieg, M. Wang, S. Thornton, and R. Brettell. *Agrobacterium tumefaciens*-mediated barley transformation. *Plant J.*, 11:1369–1375., 1997.
- [6] M. Nishihara and T. Nakatsuka. Genetic engineering of flavonoid pigments to modify flower color in floricultural plants. *Biotechnol. Lett.*, 33:433–441, 2011.
- [7] S. Chandler and Y. Tanaka. Genetic modification in floriculture. *Crti. Rev. Plant Sci.*, 26:169–197, 2007.
- [8] Y. Tanaka, S. Tsdua, and T. Kusumi. Metabolic engineering to modify flower color. *Plant Cell Physiol.*, 39:1119–1126, 1998.
- [9] G. Forkmann and S. Martens. Metabolic engineering and application of flavonoids. *Curr. Opin. Biotech.*, 12:155–160, 2001.
- [10] P. Meyer, I. Heidmann, G. Forkmann, and H. Saedler. A new petunia flower colour generated by transformation of a mutant with a maize gene. *Nature*, 330:677–678, 1987.
- [11] A.R. van der Krol, P.E. Lenting, J. Veenstra, I.M. van der Meer, R.E. Koes, A.G.M. Gerats, J.N.M. Mol, and A.R. Stuitje. An anti-sense chalcone synthase gene in transgenic plants inhibits flower pigmentation. *Nature*, 333:866–869, 1988.
- [12] T. Nakatsuka, Y. Abe, Y. Kakizaki, S. Yamamura, and M. Nishihara. Production of red-flowered plants by genetic engineering of multiple flavonoid biosynthetic genes. *Plant Cell Rep.*, 26:1951–1959, 2007.

- [13] N. Nakamura, M. Fukuchi-Mizutani, Y. Fukui, K. Ishiguro, K. Suzuki, H. Suzuki, K. Okazaki, D. Shibata, and Y. Tanaka. Generation of pink flower varieties from blue *Torenia hybrida* by redirecting the flavonoid biosynthetic pathway from delphinidin to pelargonidin. *Plant Biotechnol.*, 27:375–383, 2010.
- [14] Y. Tanaka, F. Brugliera, G. Kale, M. Senior, B. Dyson, N. Nakamura, Y. Katsumoto, and S. Chandler. Flower color modification by engineering of the flavonoid biosynthetic pathway: practical perspectives. *Biosci. Biotechnol. Biochem.*, 74:1760–1769, 2010.
- [15] F. Quattrocchio, W. Verwij, A. Kroon, C. Spelt, J. Mol, and R. Koes. PH4 of petunia is an R2R3 MYB protein that activates vacuolar acidification through interactions with basic-helix-loop-helix transcription factors of the anthocyanin pathway. *Plant Cell.*, 18:1274–1291, 2006.
- [16] K. Yoshida, N. Miki, K. Momonoi, M. Kawachi, K. Katou, Y. Okazaki, N. Uozumi, M. Maeshima, and T. Kondo. Synchrony between flower opening and petal-color change from red to blue in morning glory, *Ipomoea tricolor* cv. Heavenly Blue. *Proc. Jpn. Acad. Ser. B. Phys. Biol. Sci.*, 85:187–197, 2009.
- [17] T. Yabuya, M. Nakamura, T. Iwashina, M. Yamaguchi, and T. Takehara. Anthocyanin-flavone copigmentation in bluish purple flowers of japanese garden iris (*Iris ensata* thunb.). *Euphytica*, 98:163–167, 1997.
- [18] K. Takeda, A. Fujii, Y. Senda, and T. Iwashina. Greenish blue flower colour of *Strongyloodon macrobotrys*. *Biochem. Syst. Ecol.*, 38:630–633, 2010.
- [19] M. Shiono, N. Matsugaki, and K. Takeda. Structure of commelinin, a blue complex pigment from the blue flowers of *Commelina communis*. *Proc. Jpn. Acad. Ser. B. Phys. Biol. Sci.*, 84:452–456, 2008.
- [20] K. Momonoi, K. Yoshida, S. Mano, H. Takahashi, C. Nakamori, K. Shoji, A. Nitta, and M. Nishimura. A vacuolar iron transporter in tulip, TgVit1, is responsible for blue coloration in petal cells through iron accumulation. *Plant J.*, 59:437–447, 2009.
- [21] Q.O.N. Kay, Daoud H.S., and Stirton C.H. Pigment distribution, light reflection and cell structure in petals. *Bot. J. Linn. Soc.*, 83:57–84, 2003.
- [22] A.G. Dyer, H.M Whitney, S.E.J. Arnold, B.J. Glover, and L. Chittka. Mutations perturbing petal cell shape and anthocyanin synthesis influence bumblebee perception of *Antirrhinum majus* flower colour. *Arthropod.-Plant Inte.*, 1:45–55, 20071.
- [23] J. Hirschberg, M. Cohen, M. Harker, T. Lotan, V. Mann, and I. Pecker. Molecular genetics of the carotenoid biosynthesis pathway in plants and algae. *Pure Appl. Chem.*, 69:2151–2158, 1997.
- [24] F. X. Cunningham Jr. Regulation of carotenoid synthesis and accumulation in plants. *Pure Appl. Chem.*, 74:1409–1417, 2002.
- [25] M. Rodríguez Concepción. Supply of precursors for carotenoid biosynthesis in plants. *Arch. Biochem. Biophys.*, 504(118-122), 2010.
- [26] G. Britton, S. Liaaen-Jensen, and H. Pfander. *Carotenoids Handbook*. Birkhäuser, Basel, Switzerland, 2004.

- [27] S. Suzuki, M. Nishihara, T. Nakatsuka, N. Misawa, I. Ogiwara, and S. Yamamura. Flower color alteration in *Lotus japonicus* by modification of the carotenoid biosynthetic pathway. *Plant Cell Rep.*, 26:951–959, 2007.
- [28] K. Niyogi. Safety valves for photosynthesis. *Curr. Opin. Plant Biol.*, 3:455–460, 2000.
- [29] C.A. Howitt and B.J. Pogson. Carotenoid accumulation and function in seeds and non-green tissues. *Plant Cell Environ.*, 29:435–445, 2006.
- [30] E.A. Dun, P.B. Brewer, and C.A. Beveridge. Strigolactones: discovery of the elusive shoot branching hormone. *Trends Plant Sci.*, 14:364–372, 2009.
- [31] E. Nambara and A. Marion-Poll. Abscisic acid biosynthesis and catabolism. *Ann. Rev. Biol.*, 56:165–185, 2005.
- [32] V.G. Ladygin. Biosynthesis of carotenoids in plastids of plants. *Biochemistry (Moscow)*, 65:1113–1128, 2000.
- [33] J. Hirschberg. Carotenoid biosynthesis in flowering plants. *Curr. Opin. Plant Biol.*, 4:210–218, 2001.
- [34] V. Mann, M. Harker, I. Pecker, and J. Hirschberg. Metabolic engineering of astaxanthin production in tobacco flowers. *Nat. Biotechnol.*, 18:888–892, 2000.
- [35] L. Ralley, E.M.A. Enfissi, N. Misawa, W. Schuch, P.M. Bramley, and P.D. Fraser. Metabolic engineering of ketocarotenoid formation in higher plants. *Plant J.*, 39:477–486, 2004.
- [36] N. Umemoto, M. Takano, H. Shimada, K. Mamiya, and T. Toguri. Flower color modification by xanthophyll biosynthetic genes in petunia. *Plant Cell Physiol.*, 47 Suppl:S110, 2006.
- [37] C.K. Shewmaker, J.A. Sheehy, M. Daley, S. Colburn, and D.Y. Ke. Seed-specific over-expression of phytoene synthase: increase in carotenoids and other metabolic effects. *Plant J.*, 20:401–412, 1999.
- [38] C. Rosati, G. Diretto, and G. Giuliano. Biosynthesis and engineering of carotenoids and apocarotenoids in plants: state of the art and future prospects. *Biotechnol. Genet. Eng.*, 26:151–174, 2009.
- [39] Z. Jeknić, J.T. Morré, S. Jeknić, S. Jevremović, A. Subotić, and T.H.H. Chen. Cloning and functional characterization of a gene for capsanthin-capsorubin synthase from tiger lily (*Lilium lancifolium* thunb. ‘splendens’). *Plant Cell Physiol.*, 53:1899–1912, 2012.
- [40] F. Bouvier, P. Hugueney, A. d’Harlingue, M. Kuntz, and B. Camara. Xanthophyll biosynthesis in chromoplasts: isolation and molecular cloning of an enzyme catalyzing the converison of 5,6-epoxycarotenoid into ketcarotenoid. *Plant J.*, 6:45–54, 1994.
- [41] M.H. Kumagai, Y. Keller, F. Bouvier, D. Clry, and B. Camara. Functional integration of non-native carotenoids into chloroplasts by viral-derived expression of capsanthin-capsorubin synthase in *Nicotiana benthamiana*. *Plant J.*, 14:305–315, 1998.

- [42] A.S. Mialoundama, D. Heintz, N. Jadid, P. Nkeng, A. Rahier, J. Deli, B. Camara, and B. Bouvier. Characterization of plant carotenoid cyclases as members of the flavo-protein family functioning with no net redox change. *Plant Physiol.*, 153:437–447, 2010.
- [43] C. Zhu, C. Bai, G. Sanahuja, D. Yuan, G. Farré, S. Naqvi, L. Shi, T. Capell, and P. Christou. The regulation of carotenoid pigmentation in flowers. *Arch. Biochem. Biophys.*, 504:132–141, 2010.
- [44] M.B Wang, Z.Y. Li, P.R. Matthews, N.M. Upadhyaya, and P.M Waterhous. Improved vectors for *Agrobacterium tumefaciens*-mediated transformation of monocot plants. *Acta Hortic.*, 461:401–407, 1998.
- [45] P. Eung-Jun, Z. Jeknić, A. Sakamoto, J. DeNoma, R. Yuwansiri, N. Murata, and T.H.H. Chen. Genetic engineering of glycinebetaine synthesis in tomato protects seeds, plants, and flowers from chilling damage. *The Plant Journal*, 40(4):474–487, 2004.
- [46] T. Murashige and F. Skoog. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.*, 15:473–497, 1962.
- [47] C.R. Walkerpeach and J. Velten. *Agrobacterium*-mediated gene transfer to plant cells: cointegrate and binary vector systems. In S.B. Gelvin and R.A. Schilperoort, editors, *Plant molecular biology manual*, number 2, pages B1/1–B1/19, Dordrecht, 1994. Kluwer Academic.
- [48] R.A Jefferson. Assaying chimeric genes in plants: the GUS gene fusion system. *Plant Mol. Biol. Rep.*, 5(387–405), 1987.
- [49] H.S. Lee, W.S. Castle, and G.A. Coates. High-performance liquid chromatography for the characterization of carotenoids in the new sweet orange (Earlygold) grown in Florida, USA. *J. Chromatogr.*, 913:371–377, 2001.
- [50] L. Van Heukelem and C.S. Thomas. The HPLC method. In S. Hooker et al., editor, *The Second SeaWiFS HPLC Analysis Round-Robin Experiment (SeaHARRE-2)*, volume 2005-212785, pages 86–92. NASA Technical Memorandum, 2005.