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Alteration of Flower Color in *Viola cornuta* cv. "Lutea Splendens" through Metabolic Engineering of Capsanthin/Capsorubin Synthesis

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Abstract: Flower color is an important characteristic that determines the commercial value of ornamental plants. The development of modern biotechnology methods such as genetic engineering enables the creation of new flower colors that cannot be achieved with classical methods of hybridization or mutational breeding. This is the first report on the successful *Agrobacterium*-mediated genetic transformation of *Viola cornuta* L. The hypocotyl explants of cv. "Lutea Splendens" variety with yellow flowers were transformed with *A. tumefaciens* carrying empty pWBVec10a vector (*Llccs*$^{-}$) or pWBVec10a/CaMV 35S::*Llccs*::TNos vector (*Llccs*$^{+}$) for capsanthin/capsorubin synthase gene (*Llccs*) from tiger lily (*Lilium lancifolium)*. A comparative study of shoot multiplication, rooting ability during culture in vitro, as well as phenotypic characteristics of untransformed (control) and transgenic *Llccs*$^{-}$ and *Llccs*$^{+}$ plants during ex vitro growth and flowering is presented. Successful integration of *Llccs* transgene allows the synthesis of red pigment capsanthin in petal cells that gives flowers different shades of an orange/reddish color. We demonstrate that the ectopic expression of *Llccs* gene in ornamental plants, such as *V. cornuta* "Lutea Splendens" could successfully be used to change flower color from yellow to different shades of orange.

Keywords: genetic transformation; *Agrobacterium tumefaciens*; capsanthin; horned pansy; flower color change; capsanthin/capsorubin synthase; carotenoid biosynthesis

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1. Introduction

Flower color is a vital characteristic for determining the commercial value of ornamental plants sold as cut flowers and potted plants or used in landscaping and gardening. Flower colors that are not intrinsic for a particular species are especially valued and have been a focus of human efforts for centuries. Classical methods of plant breeding have been successful at producing a variety of colors but are severely limited in producing novel colors due to the limited gene pool available, high heterozygosity, and self-sterility of various species [1]. As such, certain flower colors have yet to be produced in specific species, such as a true blue rose or a red iris. Genetic engineering greatly expands the number of genes that can be used to affect flower color and is a promising alternative approach that has been used successfully multiple times in the past [2].

Flower color is predominantly a consequence of the synthesis and accumulation of three classes of secondary metabolites: anthocyanins, carotenoids, and betalains [3]. To date, the genetic modifications of various steps of all three biosynthetic pathways have been carried out with the purpose of flower color change in different plant species.

Anthocyanins, which are part of the flavonoid biosynthetic pathway, impart a wide variety of colors, including red, blue and violet. The first successful alteration of flower

color was achieved by the genetic modification of the anthocyanin pathway in *Petunia hybrida* [3]. The petunia mutant RL01, which accumulates dihydrokaempferol and shows nearly no flower pigmentation, was transformed with the A_1 gene for dihydroquercetin 4-reductase from *Zea mays*, which can reduce dihydrokaempferol. The transgenic petunia plants developed orange-red flower phenotype due to synthesis and accumulation of the pelargonidin-type derivatives that are not produced in RL01 owing to the limited substrate specificity of the petunia dihydroflavonol 4-reductase [4]. Since then, significant progress in the alteration of flower color has been achieved by genetic modifications of anthocyanin biosynthesis in many different plant species by using various genes or combinations of genes of this pathway [5–8].

Betalains are water-soluble, nitrogen-containing plant pigments that are synthesized from tyrosine and are limited to a single plant order, *Caryophyllales*. This class of pigments contains a wide array of compounds, which are generally classified into two groups: red betacyanins and yellow betaxanthins [9]. Genetic modification of tobacco plants expressing different combinations of betalain pathway genes with altered flower color from naturally pale-violet to red-violet, yellow and orange-pink due to accumulation of betalains in different betacyanin/betaxanthin ratios was recently reported [9]. In the same study, the metabolic engineering for betalain production in an ornamental petunia (*Petunia* × *hybrida* cv. Mitchell) resulted in altered flower color from naturally white to pale-violet, which was due to the accumulation of betanin and isobetanin as the main betalains produced as determined by LC–MS analysis [10].

Carotenoids are an important and diverse group of mostly lipophilic C_{40} isoprenoids with polyene chains that are responsible for yellow, orange and red pigmentation of flowers and fruits in many plant species [11–14]. In plants, carotenoids are present in both photosynthetic and non-photosynthetic tissues. In photosynthetic tissues, such as leaves, carotenoids are found in chloroplasts where they provide numerous functions necessary for the process of photosynthesis, such as the construction of the photosystem, light absorption and protection of the photosynthetic apparatus from photooxidation [15–17]. One important non-photosynthetic role of carotenoids is that they determine the coloration of flowers and fruits in many plant species which attracts pollinators and fruit-eating animals that help dissemination of seeds, respectively [17–19]. In higher plants, carotenoids are synthesized in the plastids de novo by the enzymes encoded by nuclear-encoded genes [19]. The carotenoid biosynthetic pathway in plants is well defined and in the last two decades, all genes of the main carotenoid biosynthetic pathway were identified, cloned, and functionally characterized [19–21]. An abbreviated schematic illustration of the main carotenoid biosynthetic pathway is shown in Figure 1.

The head-to-head condensation of two geranylgeranyl diphosphate (GGPP) molecules catalyzed by phytoene synthase (PSY) yields phytoene (C_{40}) and is the first committed step in the biosynthesis of carotenoids [22,23]. Several 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 [24,25]. A bacterial gene for phytoene synthase (*crtB*) was used to genetically modify *Iris germanica* "Fire Bride". Transgenic iris plants showed prominent color changes in the ovaries, flower stalk, and anthers, while the standards and falls showed no significant differences in color when compared to control plants [26].

Later, [27] used the same *crtB* gene under the control of CaMV 35S promoter to genetically transform *Fortunella hindsii* Swingle. Transgenic *F. hindsii* plants overexpressing *crtB* gene changed flower color from naturally white to light orange due to increased synthesis of carotenoids [27].

Several successful attempts at the alteration of flower color by metabolic modification of carotenogenesis towards the synthesis of keto-carotenoids (κ-carotenoids) such as astaxanthin and other κ-derivatives by ectopic expression of different 4,4′ β-ketolase (*crtW*) genes alone or in combination with 3,3′ β-hydroxylase (*crtZ*) were engineered to date [28–31]. For example, [28] and [29] used *crtW* from *Agrobacterium aurantiacum* to target it to the chromoplasts of pale-yellow petunia. The *crtW*-transgenic petunia plants

developed flowers whose color ranged from deep yellow to orange due to differential accumulation of astaxanthin. Similarly, the transformed *L. japonicus* plants accumulated several novel carotenoids in the flowers, which changed from their natural light-yellow color into deep yellow or orange [30]. More recently, [31] transformed *Nicotiana glauca* with *crtW* and 3,3′ β-hydroxylase genes (*crtZ*) from *Brevundimonas* sp. SD-212. The flowers of transgenic tobacco plants changed from their natural yellow color into red, due to the accumulation of several non-endogenous ketolated and hydroxylated carotenoids.

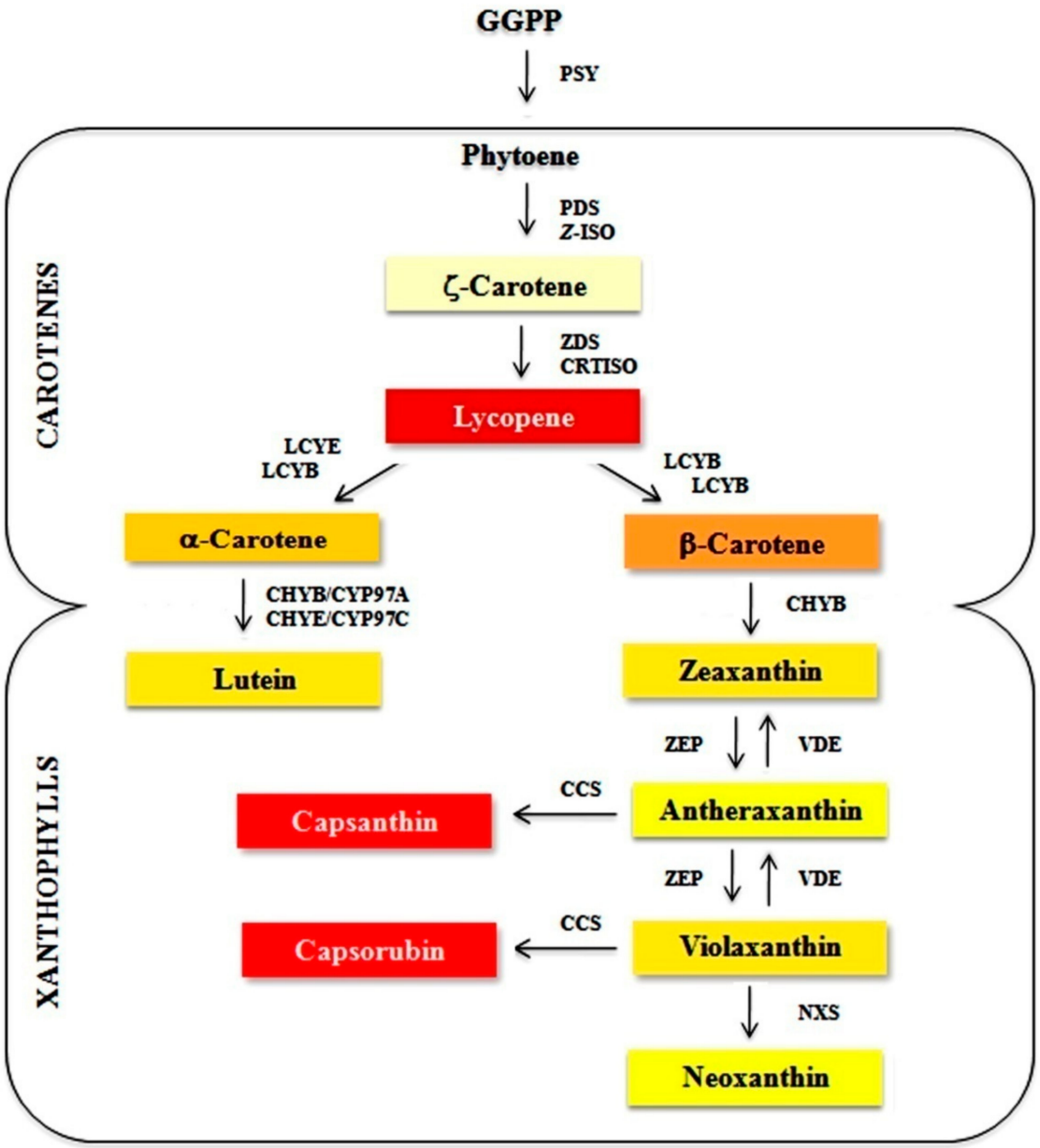

Figure 1. Schematic illustration of carotenoid biosynthesis in plants. GGPP, geranylgeranyl diphosphate; PSY, phytoene synthase; PDS, phytoene desaturase; Z-ISO, 15-cis-ζ-carotene isomerase; ZDS, ζ-carotene desaturase; CRTISO, carotenoid isomerase; LCYB, lycopene β-ring cyclase; LCYE, lycopene ε-ring cyclase; CHYB, β-ring hydroxylase; CHYE, ε-ring hydroxylase; CHYB/CYP97A, cytochrome P450-type β-ring hydroxylase; CHYE/CYP97C, cytochrome P450-type ε-ring hydroxylase; ZEP, zeaxanthin epoxidase; VDE, violaxanthin de-epoxidase; NXS, neoxanthin synthase; CCS, capsanthin/capsorubin synthase.

In some plant species, enzyme capsanthin/capsorubin synthase (CCS) catalysis the conversions of two ubiquitous yellow 5,6-epoxy-xanthophyls, antheraxanthin and violaxanthin, into capsanthin (3,3′-dihydroxy-β,κ-carotene-6′-one) and capsorubin (3,3′-dihydroxy-κ,κ-carotene-6,6′-dione), respectively (Figure 1). Capsanthin and capsorubin are two red κ-xanthophylls which contain, respectively, one and two unusual cyclopentane κ-rings and are the main determinants of orange to red colors in flowers and/or fruits of several species (summarized in Table 1).

Table 1. The list of plant species that synthesize and accumulate capsanthin and/or capsorubin in different plant parts and of those from which the capsanthin/capsorubin synthase genes were cloned.

Plant Species	Capsanthin	Capsorubin	Plant Part	Reference
Aesculus hippocastanum	+	+	anthers	[32]
Aesculus parviflora	+	+	anthers	[33]
Aesculus rubicunda	+	+	anthers	[34]
Asparagus falcatus	+	+	fruit	[35]
Asparagus officinalis	+	+	fruit	[36]
Asparagus tenuifolia	+	n.d.	fruit	[37]
Berberis s sp. (9 species)	+	n.d.	fruit	[38,39]
Capsicum annuum	+	+	fruit	[40–42] *
Cajophoralateritia	n.d.	+	petals	[37]
Cionosicyos macranthus	+	n.d.	fruit	[43]
Cardulovica palmata	+	+	fruit	[44]
Encephalartos altensteinii	+	+	fruit	[37]
Encephalartos villosus	+	n.d.	fruit	[37]
Lilium amabile	+	+	petals	[37]
Lilium davidii	+	+	petals	[45]
Lilium lancifolium (formerly *L. tigrinum*)	+	+	anthers, petals	[46–48] **
Lilium leichtinii	+	+	petals	[45]
Lilium Maxwill (*L. davidii* × *L. leichtinii*)	+	+	petals, anthers, styles	[45]
Lilium pumilum	+	+	petals	[49] ***
Lilium sp.′Saija′	+	n.d.	red tepals	[13]
Pandanus conoideus	+	+	fruit	[50]
Pouteria sapota	+	n.d.	fruit	[51]
Zamia dressleri	+	+	leaf, seed	[44]

* *C. annuum Ccs* gene; ** *L. lancifolium Llccs* gene; *** *L. pumilum Lpccs* gene (GenBank KY508342.1, submitted to NCBI GenBank by: Jeknić, Z., Jeknić, S. and Chen, T.H.H., 2017); +—detected; n.d.—not detected.

To date, only two genes for CCS, one from red bell pepper, *Capsicum annuum* [42] and the other from tiger lily–*Lilium lancifolium* Thunb. *Llccs* [48], have been cloned and functionally characterized. The functionality of the pepper *ccs* gene was demonstrated through the transfection of *Nicotiana benthamiana* with an RNA viral vector that harbored *ccs* cDNA placed under the transcriptional control of a tobacco virus subgenomic promoter. The leaves of the transfected plants developed an orange phenotype and the subsequent TLC and HPLC analyses with an authentic capsanthin standard showed that the color change was due to synthesis and accumulation of high levels of capsanthin in the chloroplasts [52]. Functional characterization of tiger lily *Llccs* was achieved by genetic transformation of the non-embryogenic callus tissue of *Iris germanica* "Hot Property" with *Llccs* cDNA under the control of the constitutive CaMV 35S promoter. The *Llccs*-transgenic iris calli changed color from yellow to different shades of orange-red, due to synthesis and sequestration of capsanthin and capsorubin in their chromoplasts as determined by HPLC and/or UPLC-MS/MS analysis using authentic standards [48]. Genetic transformation of tomato (*Solanum lycopersicum)* with *Llccs* under the control of a chimeric promoter consisting of CaMV 35S enhancer (domain B1 −750 to −90) fused to a −220 bp promoter fragment of a chalcone synthase gene (*Chs A*) from *Petunia hybrida* [53] led to flower color change from wild-type

yellow to different shades of orange [54]. To date, however, no such results have been reported in any ornamental plant.

Viola cornuta L., which is known in the vernacular as horned pansy, is a perennial ornamental plant that naturally grows in the Pyrenees in Spain and France. There are more than 25 cultivars and hybrids of horned pansies cultivated widely which exhibit a variety of flower color phenotypes in the shades of white, yellow and violet, but not red. The flower color of yellow petals of *Viola spp*. is due to the accumulation of very high levels of yellow xanthophylls, including violaxanthin and antheraxanthin, which makes them suitable candidates for this study [54,55]. In the present study, we sought to develop cultivars of *V*. *cornuta* with orange and red flower phenotypes by metabolic engineering of red κ-xanthophylls, capsanthin and capsorubin, in their flowers. Here we demonstrate that the ectopic expression of *Llccs* gene under the control of CaMV 35S promoter in ornamental plants, such as *V. cornuta* "Lutea Splendens", could successfully be used to change flower color from yellow to different shades of orange.

2. Materials and Methods

2.1. Plant Material

The seeds of *V. cornuta* L. cv. "Lutea Splendens" were purchased from B and T World Seeds (Paguignan, France), case number 24421. They were surface sterilized and germinated as in [56]. The hypocotyl explants from upper part of in vitro germinated seedlings, close to epicotyl, were used as starting plant material for genetic transformation of *V. cornuta*.

2.2. Agrobacterium-Mediated Genetic Transformation and Regeneration of Transgenic Plants

Genetic transformation of *V. cornuta* was performed with *A. tumefaciens* strain LBA4404 harboring the super binary vector pWBVec10a, carrying *Llccs* gene for capsanthin/capsorubin synthase under the control of CaMV 35S promoter (pWBVec10a/CaMV 35S::*Llccs*::TNos). The vector also contained a β-glucuronidase (*uidA*) reporter gene and hygromycin phosphotransferase (*hpt*), both interrupted by an intron, ensuring expression of functional proteins only in plant cells. *Llccs* gene was isolated and cloned from perianth of tiger lily (*Lilium lancifolium*) as described in [48]. The same vector (pWBVec10a) without *Llccs* gene was used as transformation control.

The hypocotyl explants (5 mm) were grown on a shoot induction medium (SIM) composed of half-strength Murashige and Skoog mineral solution [57] with full-strength Fe-NaEDTA complex (5.0 mL/L), full-strength MS vitamins (1 mg/L), 7% agar (Torlak, Belgrade, Serbia), 3% sucrose, myo-inositol (Sigma-Aldrich, St. Louis, MO, USA, 100 mg/L) and supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D, 0.1 mg/L, Sigma-Aldrich, St. Louis, USA) and 6 benzylaminopurine (BA, 2 mg/L, Sigma-Aldrich, St. Louis, MO, USA) as a preculture prior to *A. tumefaciens* inoculation. To determine the selective concentration of hygromycin, the hypocotyl explants (5 mm in length) were grown for two or seven days on SIM, and then transferred to the same medium supplemented with different concentrations of hygromycin (0, 1.0, 2.5, 5.0, 7.5 or 10 mg/L). For each preculture treatment and concentration of hygromycin, 30 hypocotyl explants in three replicates (Petri dishes, Spektar, Čačak, Serbia) were used. After four weeks of culture, callus formation and shoot regeneration were evaluated.

The hypocotyl explants, after preculture of two/seven days, were inoculated with *A. tumefaciens* strain pWBVec10a/CaMV 35S::*Llccs*::TNos (3500 explants in total) and with strain pWBVec10a (100 explants) in bacterial suspension with the optical density (spectrophotometer, Agilent 8453, Santa Clara, CA, USA) of 0.3 on 600 nm for 10 min on a shaker and placed on the co-cultivation medium which was SIM supplemented with acetosyringone (100 μM) and pH 5.2. After two days of co-cultivation in darkness, the explants were transferred on the SIM supplemented with 500 mg/L cefotaxime (Tolycar®, Jugoremedija, Zrenjanin, Serbia) and 1 mg/L hygromycin B (Duchefa Biochemie, Haarlem, The Netherlands) for selection, on which they were grown for four weeks. In the next

two intervals of four weeks, the concentration of cefotaxime was gradually reduced to 400 mg/L and then 300 mg/L, while the concentration of hygromycin was increased, all the way to the selective concentration of 2.5 mg/L, determined by the sensitivity test of untransformed hypocotyl explants to hygromycin. Control hypocotyl explants, after preculture were grown on SIM medium without antibiotics and subcultured at four- weeks intervals.

The obtained regenerated shoots from untransformed callus tissue (control) and proven GUS positive transformed callus tissue-derived after transformation with empty (pWBVec10a) vector (*Llccs*$^{-}$) and with vector carrying *Llccs* gene (*Llccs*$^{+}$) were further grown on $\frac{1}{2}$MS medium supplemented with 0.5 mg/L α-naphthylacetic acid (NAA) and 1.0 mg/L BA without antibiotics for shoot multiplication. Each GUS and PCR-proven *Llccs*$^{-}$ and *Llccs*$^{+}$ regenerated transgenic shoot was further referred and multiplied as separate transgenic lines. Established shoot cultures of each transgenic line were subcultured on fresh nutrient medium every four weeks and grown under 16 h light/8 h dark conditions at 23 ± 2 °C, in vitro. Shoot multiplication was evaluated as the number of newly developed shoots after four weeks of culture in comparison to initially cultured shoot explants. The experiment for evaluation of shoot multiplication of control and *Llccs*$^{-}$ and *Llccs*$^{+}$ transgenic lines was repeated twice with 10 shoots cultured in each of four Erlenmayer (100 mL) filled with 30 mL of culture medium. For evaluation of rooting abilities of regenerated shoots, isolated shoots (2 cm) from control and each transgenic line were cultured on $\frac{1}{2}$MS medium supplemented with 0.1 mg/L NAA, 100 mL filled in 750 mL glass jars and grown for seven weeks. Each experimental group contained five glass jars with 10 shoots and experiments were repeated twice (n = 100). After seven weeks following parameters were evaluated: frequency of root formation, number of roots formed in each shoot as well as the length of the longest root.

The untransformed and transformed plants were planted in containers filled with substrate (Floradur® B Pot Medium-Coarse, Floragard Vertriebs-GmbH, Oldenburg, Germany). To improve acclimatization of *Llccs*$^{+}$ plants, the base of the unrooted shoots was cut and rolled in a mixture of talc and NAA (10:1) before planting. The plants were planted during November each year and grown under greenhouse conditions and flowered at early spring next year.

2.2.1. Confirmation of the Genetic Transformation

Histochemical GUS Assay

The histochemical GUS assay was performed according to [58]. The untransformed and putatively transformed callus tissue and then the leaves of young shoots from in vitro culture were rinsed with 90% cold acetone first in the wells of microtiter plate, and then rinsed with GUS buffer without X-gluc (5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid). Following incubation in GUS staining solution with X-gluc (2 mM, #R0851, Thermo Fisher Scientific, Waltham, USA) at 37 °C for 24 h, callus and leaves were rinsed with rising concentrations of ethanol (30%, 70%, 96%) until colorless due to chlorophyll degradation. Transient expression of *GUS* gene was observed with stereomicroscope.

DNA Isolation and Polymerase Chain Reaction (PCR) Analysis

Plant genomic DNA was isolated from the leaves of untransformed and putatively transformed regenerated shoots from in vitro culture according to [59]. PCR reactions were carried out in a 25 µL mixture containing 2.5 µL DNA (100 ng/µL), 2.5 µL 10× PCR buffer, 1.5 µL dNTPs (10 mM each), 1.25 µL of each primer (5 µM), 0.5 µlTaq DNA polymerase (Fermentas, Thermo Fisher Scientific, Waltham, MA, USA) (5 U/µL) and 15.5 µL of nuclease-free water. PCR primers that were used for amplification of specific fragments are listed in Table 2.

The PCR program for all analyzed genes included initial denaturation (95 °C for 5 min), 36 cycles of denaturation (95 °C for 1 min), annealing (58 °C for 1 min) and extension (72 °C for 2 min) followed by final extension at 72 °C for 10 min. The amplified PCR products were analyzed on 1.5% agarose gel and visualized under UV light after ethidium bromide staining. Efficiency of genetic transformation was evaluated as number of initial explants with shoots (*Llccs*$^{-}$ and *Llccs*$^{+}$) in which PCR analysis confirmed the presence of transgenes in relation to the total number of inoculated explants.

Table 2. The list of primer sequences that were used in current study.

Gene *	GenBank™ Accession Number	Primer Sequence	Product Size (bp)
uidA	A00196.1	F: 5′-GTGACAAAAACCACCCAAGC-3′ R: 5′-CAGCCATGCACACTGATAGT-3′	395
hpt	FJ457012.1	F: 5′-GATGTTGGCGACCTCGTATT-3′ R: 5′-GATTGCTGATCCCCATGTGT-3′	238
Llccs (PCR)	JF304153.1	F: 5′-GTCAGATTCCACCCCTCCAA-3′ R: 5′-AACACTTCTCCTCCTCCAGC-3′	463
Llccs (qPCR)	JF304153.1	F:5′-GTACGACAGACCGAGAAACC-3′ R: 5′-TTGGAATAGCAGCGTTGTGA-3′	164
18S	18S consensus: X16077	F:5′-TGACGGAGAATTAGGGTTCG-3′ R:5′-CAATGGATCCTCGTTAAGGG-3′	190–191

* *uidA*—β-glucuronidase gene, *hpt*—hygromycin phosphotransferase gene, *Llccs*—capsanthin/capsorubin synthase gene; *18S*—18S rRNA, F—forward, R—reverse.

RNA Isolation and Transgene Expression Analysis

The total RNA was isolated from fresh leaves and flower petals of untransformed and transformed acclimatized plants of *V. cornuta*. The RNA from leaves was isolated by method of [60] while from flower petals RNA was isolated by Trizol following manufacturer's instructions (Invitrogen, Carlsbad, CA, USA).

Following DNase treatment (Applied Biosystems, Foster City, USA) reverse transcription was carried out using GeneAmp RNA PCR Core Kit (Applied Biosystems, Foster City, CA, USA). Reactions were performed using 5 µL of RNA (0.1 µg/µL), 1 µL of 10× PCR buffer, 2 µL of 25 mM $MgCl_2$, 1 µL of 10 mM dNTPs, and 0.5 µL of RNAse inhibitor (20 U/µL), 50 U/µL MuLV reverse transcriptase and 50 µM random hexamer primers. RT reactions were performed using the following conditions: 25 °C for 10 min, 37 °C for 30 min and 95 °C for 5 min.

The obtained cDNAs were PCR amplified using specific qPCR *Llccs* primers (Table 2), the amplicons were extracted from agarose gel using GeneJET Gel Extraction Kit (Thermo Fisher Scientific, Waltham, MA, USA) and their concentration was measured using NanoPhotometer® N60 (Implen, Munchen, Germany). The cDNA samples were then serially diluted in a 2×10^8–2×10^2 range to be used as standards for absolute quantification. The *Llccs* expression level was quantified by qPCR using QuantStudio 3 Real-Time PCR System (Applied Biosystems, Foster City, USA). Each of 10 µL reactions comprised of 1 µL cDNA (corresponding to 50 ng RNA), 2 µL Mix EvaGreen® (No Rox, Bio & Sell, Feucht/Nuremberg, Germany) 5×, 0.4 µL of primers mixture (7.5 µM each) and 6.6 µL of nuclease-free water. Primers used for gene amplification were presented in Table 2. qPCR conditions were initial denaturation (95 °C for 10 min), 40 cycles of denaturation (95 °C for 30 s), annealing (56 °C for 30 s) and extension (72 °C for 30 s) followed by final extension at 72 °C for 10 min. The specificity of the amplification of the *Llccs* gene was checked by electrophoretic sizing and by melting curve analysis. Constitutive expression of 18S gene was confirmed in parallel using universal 18S primers [61] which, depending on species, produce 190–191 bp amplicon. All reactions were carried out as biological triplicates. The

results were analyzed using QuantStudioDesign and Analysis Software v1.4.2 (Applied Biosystems, Foster City, CA, USA).

2.3. Analysis of Phenotypic Characteristics of Transformed Plants Grown Ex Vitro

Comparative analysis of phenotypic characteristics was evaluated during ex vitro growth of control, untransformed and *Llccs*$^{-}$ and *Llccs*$^{+}$ transgenic plants. Measurement was carried out in ten plants of three randomly selected lines (30 plants in total) from each treatment, after four months of acclimatization in greenhouse conditions, during flowering. The following parameters were measured: plant height; number of newly formed shoots (branches), leaf and flower diameter (length and width). Leaf parameters were measured in leaves developed on fifth nodes from the top of the plants.

2.4. Analysis of Carotenoid Accumulation in Plant Tissue of V. cornuta

2.4.1. Carotenoid Extraction and UHPLC Analysis

Identification and quantification of carotenoid pigments were carried out by modified method of [62] on Dionex Ultimate 3000 UHPLC system (Thermo Fisher Scientific, Bremen, Germany) configured with a diode array detector (DAD). Carotenoids were extracted from 1 g of frozen flower petals from untransformed and transformed acclimatized plants. The petals were mechanically grounded with liquid nitrogen and resuspended in extraction buffer (hexane, acetone, 96% ethanol; 50:25:25, v:v:v) for 15 min and washed twice with 10 mL of 50 mM TRIS-HCl pH8 with 1 M NaCl. To the organic phase of each sample 2 mL diethyl ether and 2 mL 10% KOH in MeOH solution were added and left overnight for saponification. The next day the saponified extracts were extracted with 2 mL diethyl ether and 5 mL of deionized water, phases were separated and the upper phase was evaporated to the dryness under nitrogen pressure, and redissolved in 2 mL of absolute ethanol. The whole process of carotenoid extraction took place in a room with lower light, and the tubes were covered with aluminum foil to protect the carotenoids from isomerization under light. The samples were filtered through a polytetrafluoroetlylen (PTFE) filter before injection to the UHPLC. Separation of the pigments was performed on Hyperisil gold C18 column (50 $\times$ 2.1 mm) with 1.9 mm particle size (Thermo Fisher Scientific, Waltham, USA) at 30 °C. The mobile phase consisted of water with 0.1% formic acid (A) and acetonitrile (B). The injection volume of the sample was 10 µL while the following solvent gradient was used: 0 min 60% B, 7.5 min 80% B, 9 min 100% B, 12 min 80% B and 15 min 60% B. An absorption was detected at 480 nm. A series of dilutions for the standard curve was made using the capsanthin standard ($\geq$95% purity, Extrasynthese, Lyon, France) and quantification of the samples was performed on the basis of the obtained peaks on the chromatograms.

2.4.2. Histological Analysis of *V. cornuta* Tissue

The piece of fresh plant material (callus, leaf, flower parts) of control and/or transformed tissue were placed on glass slides and the preparations were made by transversal or longitudinal sections of plant material by hand using razor blade or by "squash" technique. For detailed analysis of chromoplasts in epidermal cells of flower corolla, the adhesive tape stripping technique was used. A thin sample of tissues was placed on microscopic slides in a drop of glycerol and covered with coverslips before analysis. All samples were analyzed by light microscope (Nikon Eclipse E100) and photographed by MicroCam SP 5.1 Bresser.

2.4.3. Colorimetric Measurements (CIE L*a*b* System)

The color intensity of fresh flower petals from untransformed and transformed acclimatized plants was measured with colorimeter (ChromaMeter CR-400, Konica Minolta Co. Ltd., Tokyo, Japan). The color properties (L*—lightness, a*—redness and greenness, b*—yellowness and blueness, C*—purity of color and h*—hues of color) were expressed numerically by directly reading the values from the instrument. Flower color was evaluated in 9–13 flowers from each transgenic line and repeated twice.

2.5. Statistical Analysis

All data were statistically analyzed by STATISTICA 8.0 and are presented as mean ± standard error (SE). Statistical analysis included analysis of variance (ANOVA) and comparison of mean values using Fisher's LSD test at the level of significance $p \leq 0.01$ or $p \leq 0.05$.

3. Results

3.1. Genetic Transformation and Regeneration of Transgenic *V. cornuta* Plants

3.1.1. Transformation and Regeneration of *V. cornuta* Plants from Hypocotyl Explants

Introduction of *Llccs* gene into *V. cornuta* cv. "Lutea Splendens" was conducted by *A. tumefaciens*-mediated transformation using hypocotyl explants. The hypocotyls were inoculated with agrobacteria bearing either the *Llccs* gene (referred through the text as *Llccs*$^+$) or an empty vector (*Llccs*$^-$). In addition to *Llccs*$^-$ transformants, untransformed explants and regenerants (referred to as control) were also used for comparisons. When hypocotyl explants of *V. cornuta* were inoculated with *A. tumefaciens* strains immediately after isolation, most of them were necrotized after cultivation on a selective medium. To overcome this problem, the hypocotyl explants were grown for two or seven days on a shoot induction medium as preculture. We tested the sensitivity of hypocotyl explants after the preculture to several graded concentrations of hygromycin before inoculation with *A. tumefaciens* strains (Figure 2). Callus formation and shoot development on hypocotyl explants were inhibited at a high (>7.5 mg/L) concentration of hygromycin, so for further selection, concentrations of hygromycin 2.5 and 1.0 mg/L were used.

Following the necessary preculture steps, inoculation and co-cultivation, the inoculated explants were set on a selective medium supplemented with cefotaxime and hygromycin, whereas the control explants were regenerating on an antibiotics-free shoot induction medium. The calli formed on all three groups of hypocotyls (control, *Llccs*$^-$ and *Llccs*$^+$), mostly at the cut edges of hypocotyl explants (Figure 3a–c), albeit at different frequencies (Table 3). In the control, explant callus formation was observed several days after isolation of hypocotyl explants and growth on culture medium, while in inoculated explants callus formation started after 15 days of culture. Calli formed on control and *Llccs*$^-$ inoculated explants were compact and green (Figure 3a,b), while the calli derived from *Llccs*$^+$ inoculated explants gene had, besides green, orange and yellow parts (Figure 3c). All control explants (100%) formed calli after two months of culture, while only 21.1% of *Llccs*$^-$ and 15.4% of *Llccs*$^+$ explants formed calli (Table 3).

Shoots were regenerated indirectly from the formed calli (Figure 3d–f). The regeneration of shoots from control calli was observed after 15 days of growth on medium (Figure 3d), while on inoculated explants regeneration of shoots started three weeks after the inoculation (Figure 3e,f). After two months of culture, shoots regenerated on 32%, 2% and 0.2% of control, *Llccs*$^-$ and *Llccs*$^+$ explants, respectively (Table 3).

3.1.2. Confirmation of Genetic Transformation with *Llccs* Gene

The confirmation of genetic transformation was performed by histochemical GUS assay (*uidA* reporter gene, Figure 4) and PCR analysis of genomic DNA (*uidA* reporter gene, *htp*, selectable marker and *Llccs* gene, Figure 5). The histochemical GUS assay was performed on calli samples and regenerated shoots derived from control, *Llccs*$^-$ and *Llccs*$^+$ inoculated explants (Figure 4). The untransformed callus (Figure 4a) and leaf (Figure 4d) remained unstained, while the pieces of callus tissue of *Llccs*$^-$ (Figure 4b) and *Llccs*$^+$ (Figure 4c) samples stained blue, completely or partially, thus confirming successful transformation as *uidA* expression. The leaves of *Llccs*$^-$ (Figure 4e) and *Llccs*$^+$ (Figure 4f) regenerated shoots also stained intensely blue. About 75% of the analyzed potentially transformed *Llccs*$^-$ and *Llccs*$^+$ calli were stained in blue while 97.3% of regenerated *Llccs*$^-$ and *Llccs*$^+$ shoots were stained in blue after the GUS assay.

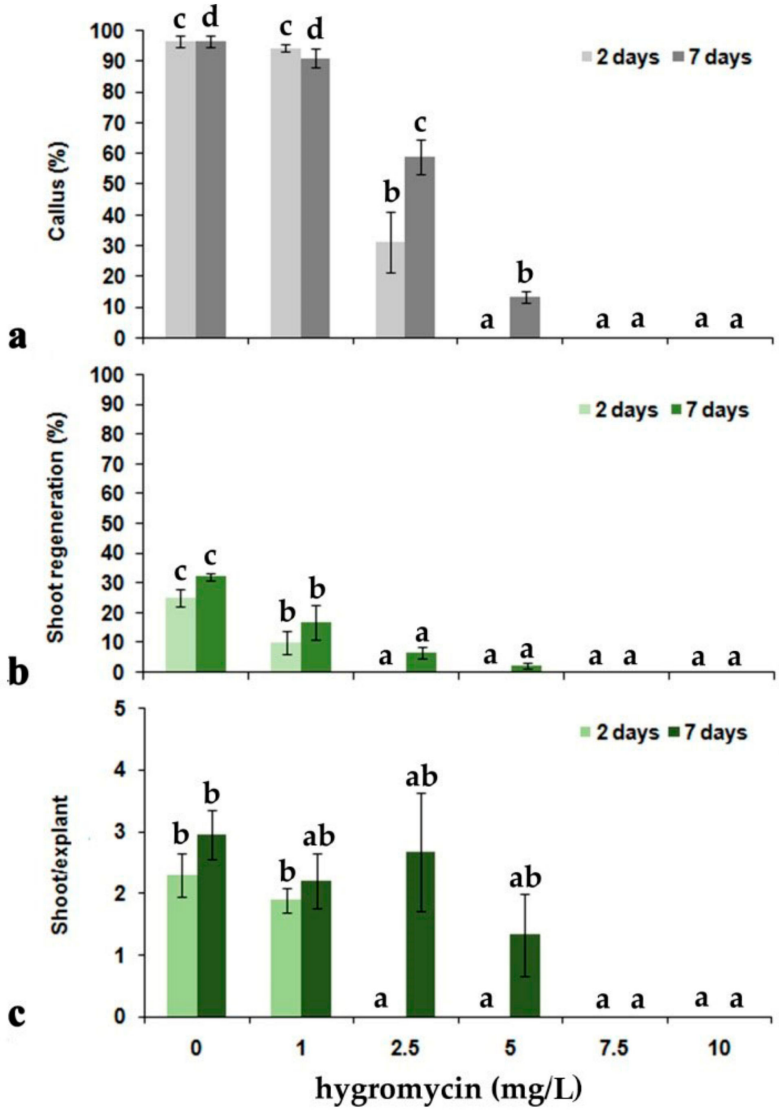

Figure 2. Morphogenetic response of uninoculated hypocotyl explants of *V. cornuta* to different concentrations of hygromycin after two and seven days of preculture. (**a**) Callus formation on hypocotyl explants; (**b**) Shoot development; (**c**) Average number of developed shoots/hypocotyl explant. The data represent mean values ± standard error. Mean values on different concentrations of hygromycin within the same preculture treatment are compared and marked with different letters when significantly different based on the Fisher's LSD test ($p \leq 0.01$).

Table 3. Regeneration of control, *Llccs*$^{-}$ and *Llccs*$^{+}$ transgenic *V. cornuta* hypocotyl explants.

Treatment	Callus Formation (%)	Shoot Regeneration (%)
Control	100	32.00 ± 4.90 *
Llccs^{-}	21.00 ± 1.00	2.00 ± 1.15
Llccs^{+}	15.40 ± 2.35	0.20 ± 0.20

* Data represent mean ± standard error.

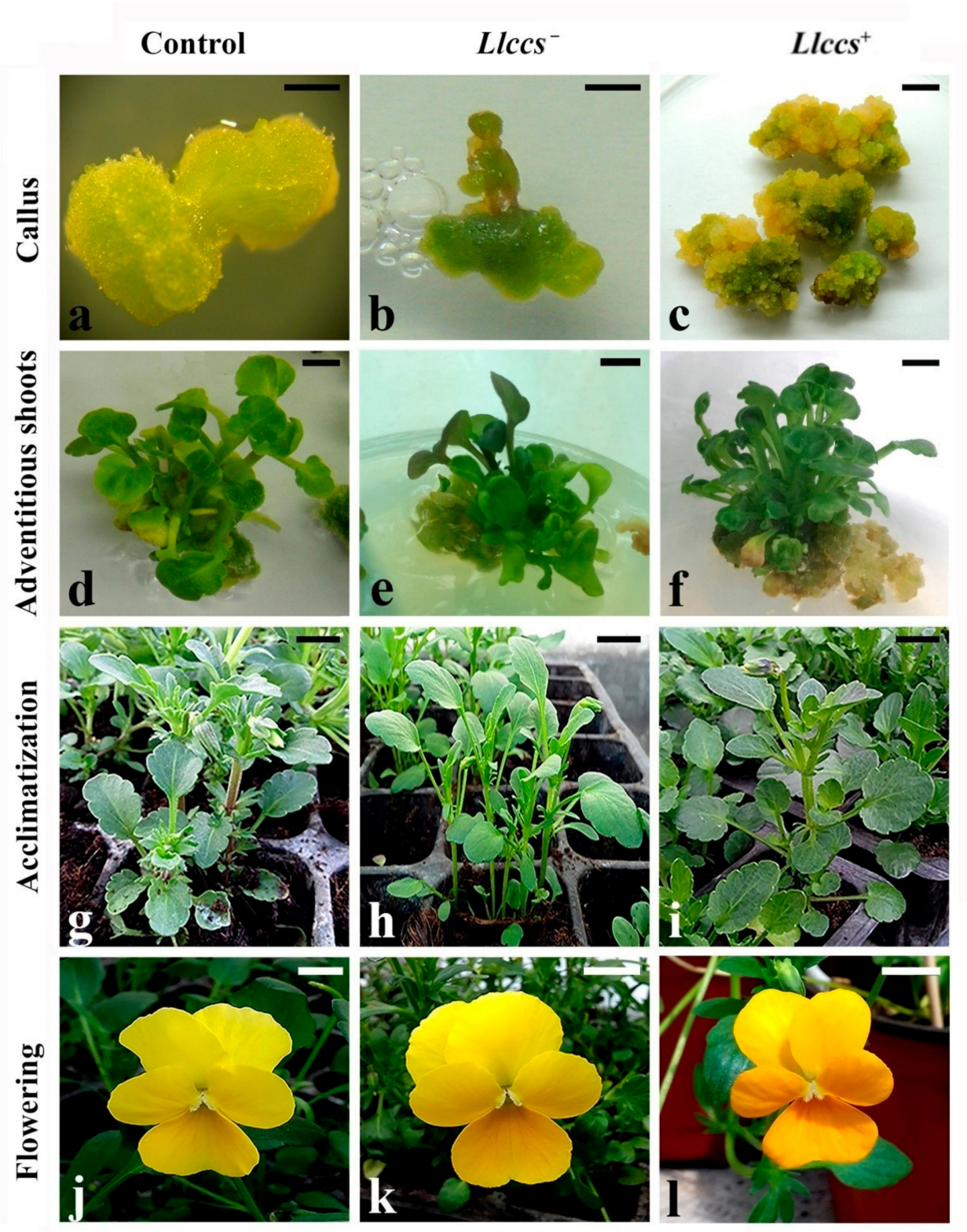

Figure 3. Plant regeneration of *Viola cornuta* cv. "Lutea Splendens". (**a**–**c**) Callus formation on hypocotyl explants; (**d**–**f**) Shoot cultures of control and transgenic plants regenerated two months after genetic transformation; (**g**–**i**) Acclimatization of regenerated plants; (**j**–**l**) Flowering of regenerated control and transgenic plants. Bars: (**a**,**b**), 1 mm; (**c**), 5 mm, (**d**–**l**), 10 mm.

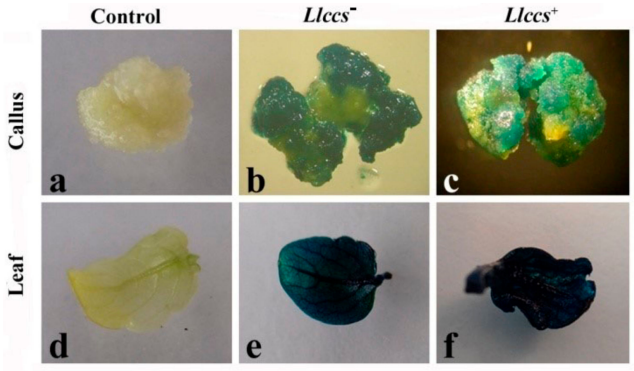

Figure 4. Histochemical GUS assay of control and transgenic *Llccs*$^{-}$ and *Llccs*$^{+}$ calli (**a**–**c**) and leaves (**d**–**f**).

Confirmation of genetic transformation was achieved by PCR analysis of genomic DNA isolated from the leaves of transgenic lines (Figure 5). In the *Llccs*$^{-}$ transgenic leaves, the presence of amplification product of selectable marker, hygromycin phosphotransferase (*hpt*), as well as a reporter gene (*uidA*) was confirmed in all seven selected transgenic shoot cultures (Figure 5a). The presence of amplification product of *Llccs* gene for capsanthin/capsorubin synthase was confirmed in 29 *Llccs*$^{+}$ transgenic shoot cultures out of 30 analyzed (all data are presented as Figure S1), in addition to *hpt* and *uidA* (Figure 5b).

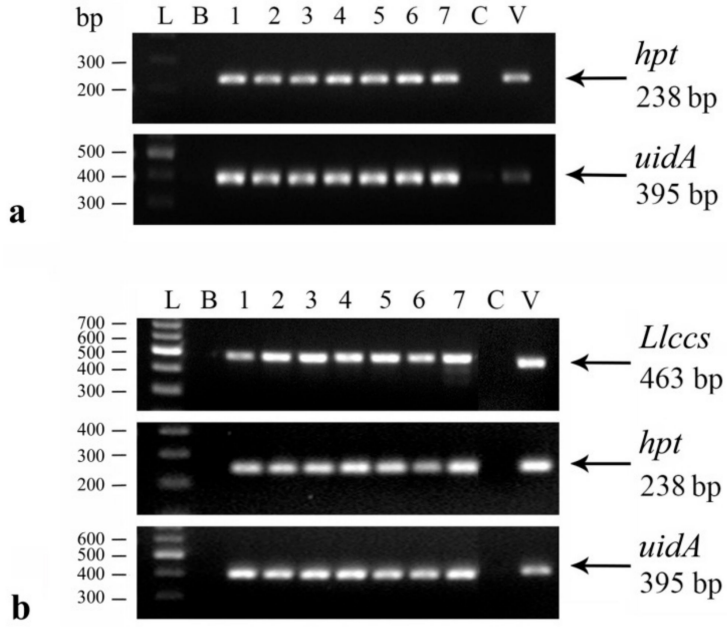

Figure 5. PCR analysis of genomic DNA of selected *Llccs*$^{-}$ transgenic shoots (**a**) and *Llccs*$^{+}$ shoots (**b**). *hpt*—hygromycin phosphotransferase; *uidA*—β-glucuronidase; *Llccs*—capsanthin/capsorubin synthase; L—ladder (DNA ladder, 100 bp); B—blank; C—control (untransformed sample); V– vector: (**a**) pWBVec10a or (**b**) pWBVec10a/CaMV 35S::*Llccs*::TNos; 1–7—selected transformants.

The efficiency of genetic transformation was evaluated after confirmation of genetic transformation by PCR analysis in comparison to an initial number of inoculated explants, and it was 2% for *Llccs*$^{-}$ while for *Llccs*$^{+}$ it was 0.33%.

3.1.3. Shoot Multiplication, Rooting and Acclimatization of Transgenic *V. cornuta* Plants

In the next step of our research, we analyzed growth characteristics of transgenic shoots (*Llccs*$^{-}$ and *Llccs*$^{+}$) in comparison to control shoot cultures, grown on $\frac{1}{2}$MS medium supplemented with NAA and BAP (0.5 and 1.0 mg/L, respectively). The shoot multiplication and rooting ability of *V. cornuta* transgenic shoots in comparison to control cultures are presented in Table 4.

Table 4. Multiplication and rooting of control, *Llccs*$^{-}$ and *Llccs*$^{+}$ transgenic *V. cornuta* shoots in culture in vitro.

Line	Shoot Multiplication		Rooting	
	N° of Shoots	Frequency (%)	N° of Roots/Shoot	Length of Longest Root (mm)
Control	1.95 ± 0.04 ^{c}	100 ^{a}	5.51 ± 0.11 ^{b}	2.75 ± 0.10 ^{b}
Llccs^{-}	1.52 ± 0.20 ^{b}	90.73 ± 2.59 ^{a}	9.28 ± 0.90 ^{c}	8.50 ± 0.64 ^{c}
Llccs^{+}	1.03 ± 0.09 ^{a}	28.65 ± 6.45 ^{b}	2.08 ± 0.21 ^{a}	0.85 ± 0.18 ^{a}

The data in the table represent mean values ± standard error. Mean values within the same column marked with different letters statistically differ based on the Fisher's LSD test ($p \leq 0.05$).

Shoot multiplication and rooting ability of control and transgenic shoots differed significantly. The number of newly formed axillary shoots in transgenic shoot cultures was significantly lower as compared to the untransformed control (Table 4). The lowest shoot multiplication was observed in *Llccs*$^{+}$ transgenic cultures. Besides shoot multiplication, the rooting ability of *Llccs*$^{+}$ transgenic shoots, measured as the frequency of root formation, the number of roots formed per shoot and the length of developed roots was significantly lower in comparison to *Llccs*$^{-}$ and untransformed control shoots (Table 4). Contrary to *Llccs*$^{+}$ transgenic shoots, we recorded a significant increase in the number and length of developed roots in *Llccs*$^{-}$ transgenic shoots in comparison to control untransformed shoots (Table 4).

Fully developed untransformed and transgenic plants were successfully acclimatized to the greenhouse conditions. The success of acclimatization varied from 67% for control plants (Figure 3g), 79% for *Llccs*$^{-}$ transgenic plants (Figure 3h), and 60% for *Llccs*$^{+}$ transgenic plants (Figure 3i). The greenhouse-grown plant flowered during the spring and summer.

3.2. Phenotypic Characteristics of Transgenic V. cornuta Plants Grown Ex Vitro

3.2.1. Morphometric Parameters of Transgenic *V. cornuta* Plants Grown Ex Vitro

Comparative analysis of phenotypic characteristics of ex vitro grown *Llccs*$^{-}$ and *Llccs*$^{+}$ transgenic lines in comparison to untransformed plants is presented in Figure 6. All plants branched and developed new axillary shoots during growth under greenhouse conditions (Figure 6a). The mean number of newly formed axillary shoots varied in transgenic lines from three to six shoots per plant. In *Llccs*$^{-}$ plants, a significantly higher number of axillary shoots (~6) was observed as compared to the control plants (~2). All transgenic plants were taller than untransformed plants (Figure 6b). The width of the leaves of all transgenic lines was somewhat narrower than the leaves of control plants (Figure 6c). The *Llccs*$^{+}$ transgenic plants had significantly longer leaves (~14 mm) than the *Llccs*$^{-}$ plants (~11 mm) and untransformed control plants (~12 mm) (Figure 6d).

However, the most visible and significant changes were observed in size (Figure 6e,f) and colorization of the *Llccs*$^{+}$ flowers in comparison to control and *Llccs*$^{-}$ flowers (Figure 3j–l; Figure 7). The flowers of *Llccs*$^{-}$ transgenic plants were smaller (~9%), with the *Llccs*$^{+}$ flowers being the smallest, about ~30% smaller than flowers of the untransformed plants (Figure 6e,f).

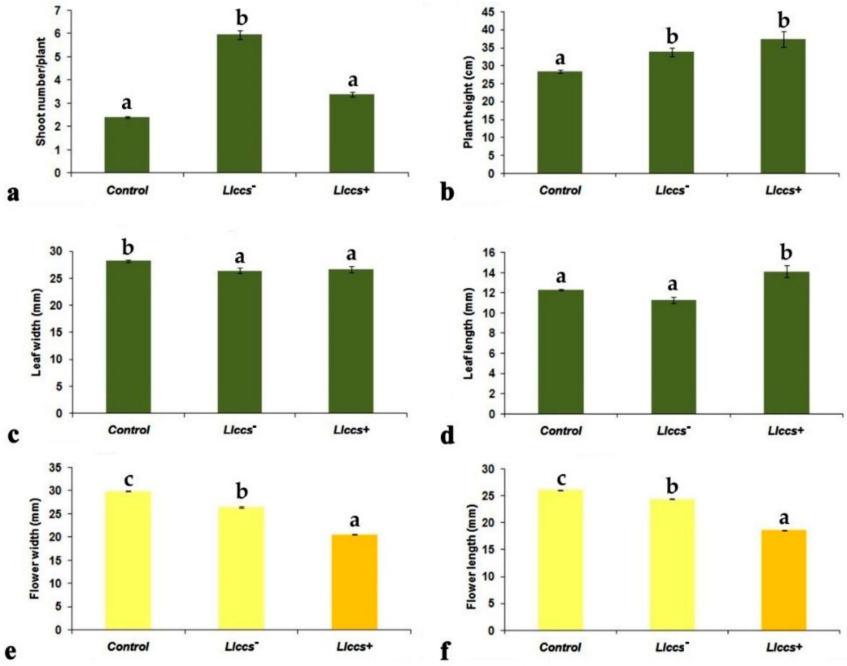

Figure 6. Morphological characteristics of control and transgenic (*Llccs*$^{-}$ and *Llccs*$^{+}$) greenhouse-grown *V. cornuta* plants. (**a**) Number of newly developed shoot/plant; (**b**) Plant height; (**c**) Leaf width; (**d**) Leaf length; (**e**) Flower width; (**f**) Flower length. The data represent mean values ± standard error. Mean values within the same histogram marked with different letters differ significantly based on the Fisher's LSD test ($p \leq 0.01$). Presented data include ten plants of three randomly selected lines—a total of 30 plants for each treatment.

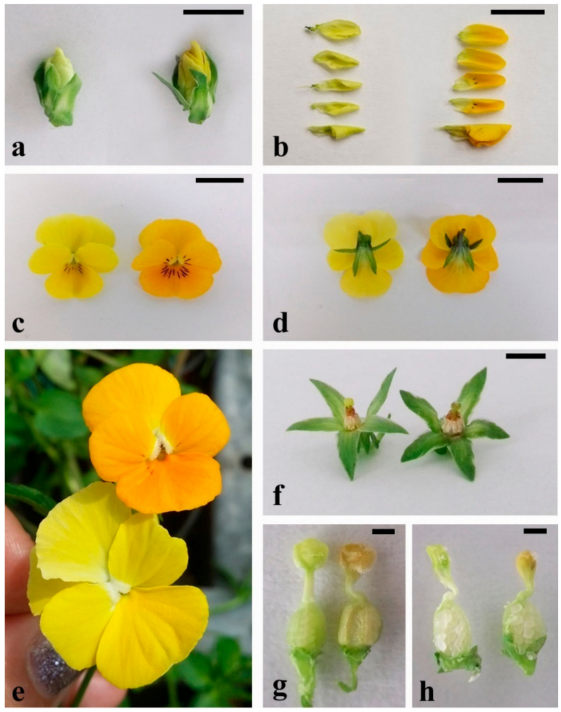

Figure 7. Flower color change of greenhouse-grown *V. cornuta* untransformed (left) and *Llccs*$^{+}$ transgenic plants (right). (**a**) Flower buds; (**b**) Isolated petals of flower buds; (**c**) Fully open flowers; (**d**) Back side of the flowers; (**e**) Flowers of control and *Llccs*$^{+}$ plants in a greenhouse; (**f**) Flowers free of petals; (**g**) Pistils; (**h**) Longitudinal section through the pistils. Bars: (**a**,**e**,**f**)—10 mm; (**b**–**d**)—20 mm; (**g**,**h**)—3 mm.

3.2.2. Flower Color Alteration in Transgenic *V. cornuta* Plants Grown Ex Vitro

The most evident characteristic of the *Llccs*$^{+}$ flowers was the alteration of the flower color. No changes in flower color in *Llccs*$^{-}$ plants were observed as compared to control plants (Figure 3). The difference in flower color between control and *Llccs*$^{+}$ plants was observable immediately with the appearance of the first flower buds (Figure 7a). While the petals of the control flower buds were pale yellow-green, the petals of the *Llccs*$^{+}$ flower buds were bright yellow-orange (Figure 7b). The color change was even more apparent in fully open flowers, where the flowers of control plants were yellow, while *Llccs*$^{+}$ plants had flowers in different shades of orange (Figure 7c,d). The stigma of the pistil also changed color from light green to light orange-brown (Figure 7e–h).

3.2.3. qPCR Quantification of *Llccs* Expression

The expression level of the capsanthin/capsorubin synthase *(Llccs)* transgene in flowering plants was determined by quantitative PCR. As expected, the *Llccs* transcripts were not detected in the leaves and flower petals of untransformed plants or *Llccs*$^{-}$ transformants (Figure 8a,b). In the *Llccs*$^{+}$ plants, however, the *Llccs* transcripts were detected, albeit at different levels in different organs and among individual regenerants (Figure 8a,b). The copy number of *Llccs* transcripts per ng RNA in the leaves of *Llccs*$^{+}$ transformants ranged from 76 to 420, while in flower petals it varied from 8 to 192 (Figure 8a,b).

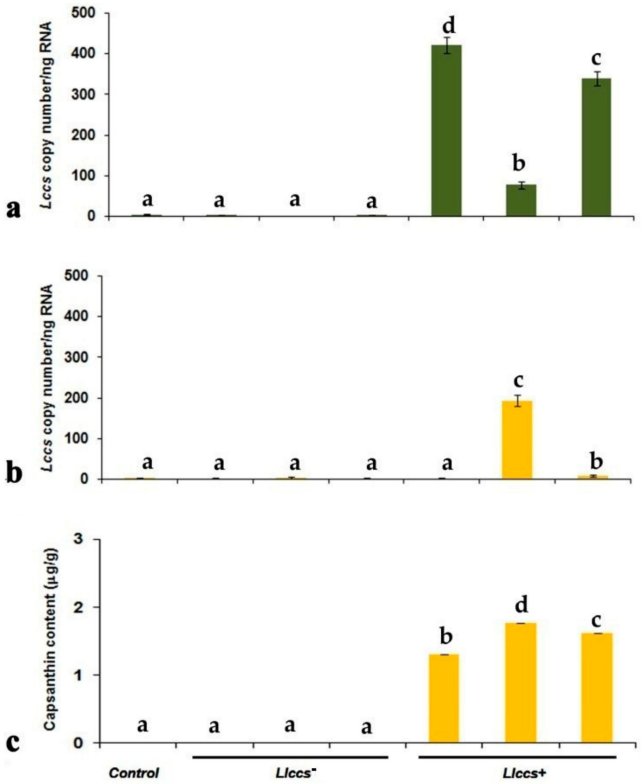

Figure 8. Absolute quantification of *Llccs* expression by qPCR in leaves (**a**) and flower petals (**b**) and capsanthin content in petals (**c**) of control and selected *Llccs*$^{-}$ and *Llccs*$^{+}$ *V. cornuta* plants. Both measurements were conducted with one control and three *Llccs*$^{-}$ (lines 30, 37 and 41) and *Llccs*$^{+}$ (lines 2, 3 and 7) plants. The qPCR quantifications were performed in three technical replicates; data represented the mean values ± standard errors, where the same letters indicate statistically homogenous groups based on the Fisher's LSD test ($p \leq 0.01$).

3.3. Qualitative and Quantitative Analysis of Newly Synthesized Pigment in Llccs⁺ Transgenic Plants

3.3.1. Detection of the Pigment in Plant Tissue

The accumulation of newly synthesized pigment was first observed in the calli cells of *Llccs*⁺ transgenic cultures (Figure 9a–c). In untransformed calli (Figure 9a) and in *Llccs*⁻ calli (Figure 9b), cells had a large number of chloroplasts with chlorophyll, giving the tissue a green color. In *Llccs*⁺ calli cells (Figure 9c), however, many chromoplasts filled with orange-red pigment were observed in addition to green chloroplasts. On control and transgenic leaf cross-sections (Figure 9d–f), no differences were observed in tissue color, probably because chlorophyll masked the presence of red pigmentation in the *Llccs*⁺ leaves. The major difference in color was observed in the conical epidermal cells of the flower petals: while in the petals of untransformed (Figure 9g) and *Llccs*⁻ plants (Figure 9h) yellow chromoplasts were present, the epidermal cells of *Llccs*⁺ flowers (Figure 9i) were rich in chromoplasts filled with orange-red pigment. There were no differences in epidermal cell shape between untransformed and transgenic plants.

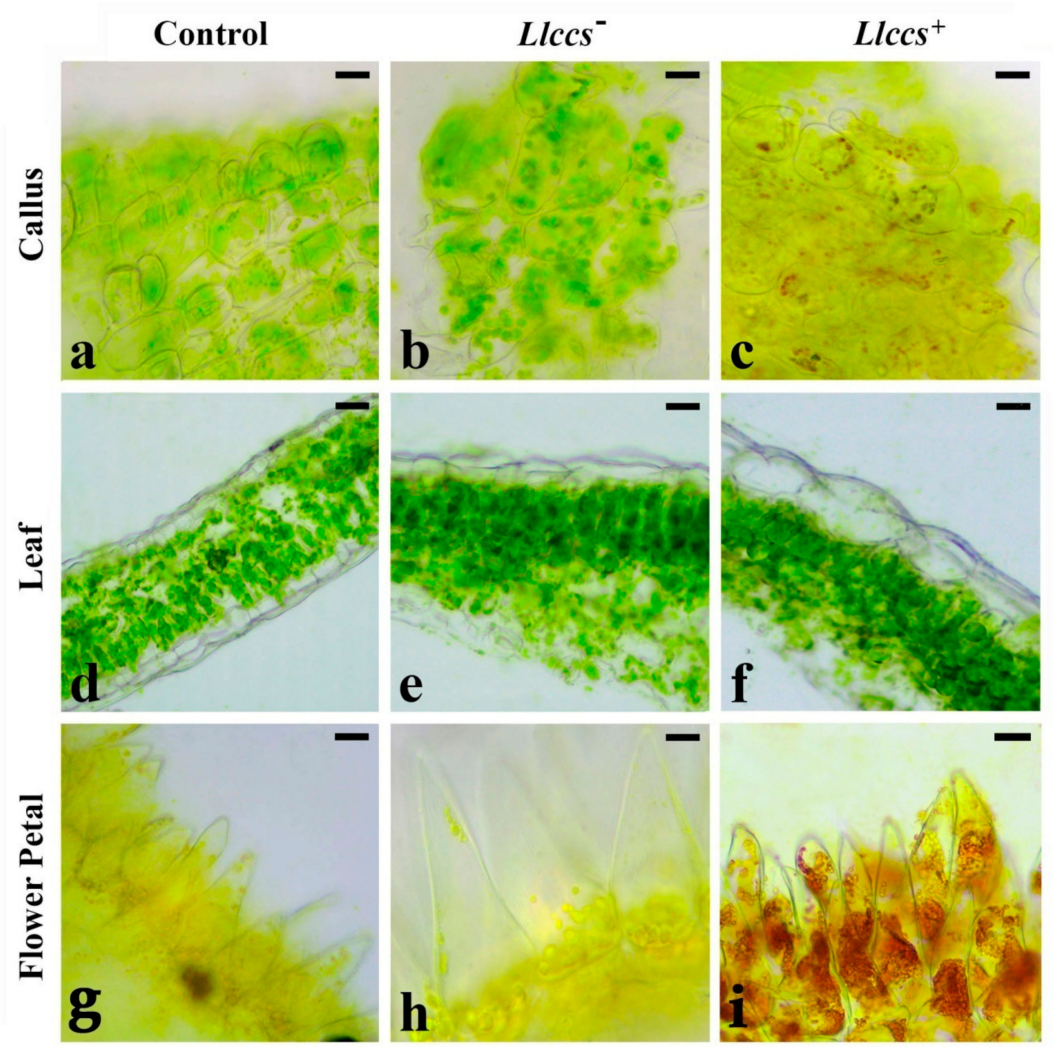

Figure 9. Histological analysis ofcalli, leaves and flower petals of control and transgenic *Llccs*⁻ and *Llccs*⁺ *V. cornuta* plants. (**a**–**c**) Cells of control (**a**), *Llccs*⁻ (**b**) and *Llccs*⁺ (**c**) Callus tissue; (**d**–**f**) Cross sections of control (**d**), *Llccs*⁻ (**e**) and *Llccs*⁺ (**f**) leaves; (**g**–**i**) Epidermal cells on the adaxial side of control (**d**), *Llccs*⁻ (**e**) and *Llccs*⁺ (**f**) flower petals. Note the accumulation of newly synthesized pigment in the chromoplasts of *Llccs*⁺ callus cells (**c**) and in the epidermal cells in adaxial side of fully opened flowers (**i**). Bars: 200 μm.

3.3.2. UHPLC Analysis of Carotenoid Pigments in Flower Petals

UHPLC-DAD analysis confirmed the presence of an additional peak in flowers of all tested *Llccs*$^{+}$ plants which was absent in control and *Llccs*$^{-}$ plants (Figure 10). This peak (RT 6.05, Figure 10) was identified as capsanthin after comparison to the corresponding standard RT and absorption spectra. The pigment capsorubin was not detected in any of the tested samples of transgenic flowers, possibly because of overlapping RT with matrix pigments.

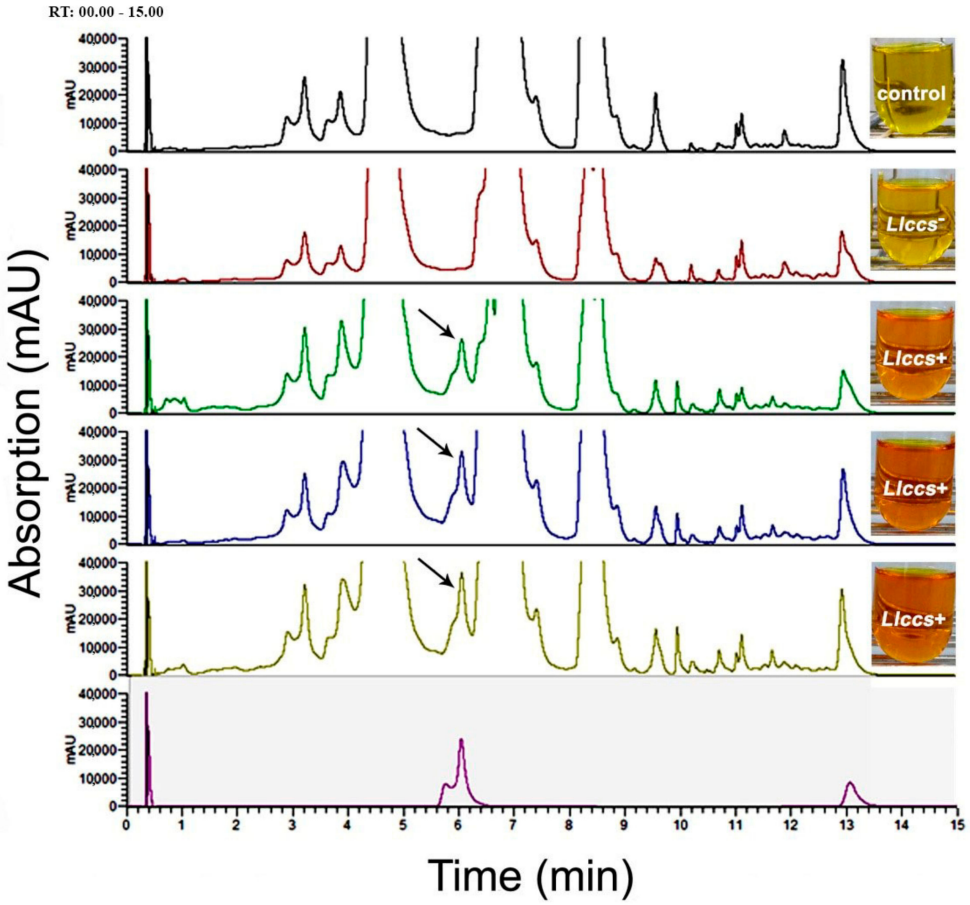

Figure 10. UHPLC chromatograms of saponified extracts isolated from petals of untransformed (control), *Llccs*$^{-}$ and *Llccs*$^{+}$ flowers (lines 2, 3 and 7, respectively) of *V. cornuta* and capsanthin standard (1.0 µg). The arrows indicate the capsanthin peak in *Llccs*$^{+}$, 1.31, 1.76 and 1.62 µg/g, respectively). Actual appearance of the analyzed extracts is indicated right of chromatograms.

The content of capsanthin in *Llccs*$^{+}$ flower petals varied from 1.31 to 1.76 µg/g fresh weight in different regenerants (Figure 8c). According to the obtained data, it can be concluded that the alteration of flower color in *Llccs*$^{+}$ plants was due to the synthesis of new pigment capsanthin, which was stored in chromoplasts of epidermal cells of the petals. The capsanthin content in flower petals correlates with the copy number of *Llccs* transcripts in petals of *Llccs*$^{+}$ regenerants (Figure 8b,c).

3.3.3. Chromaticity Changes in the CIE L*a*b* System in Transgenic Flowers

We evaluated the degree of changes in flower color of *Llccs*$^{+}$ plants by comparing the chromaticity of the fresh flower petals of greenhouse-grown control, *Llccs*$^{-}$ and *Llccs*$^{+}$ plants, measured by colorimeter and expressed by *CIE L*a*b** system (Table 5). The flower color of *Llccs*$^{-}$ plants did not differ significantly from the control plants. In flowers of *Llccs*$^{+}$ transformants, the proportion of yellow and red color in the flowers was changed in comparison to control and *Llccs*$^{-}$ transgenic *V. cornuta* plants (Table 5). The values of

color lightness (L*), the yellowness (b*) and color hue (h*) were significantly lower in *Llccs*$^{+}$ flowers as compared to control and *Llccs*$^{-}$ flowers.

The most significant contribution in color alteration in *Llccs*$^{+}$ transgenic plants is the proportion of red color (a*), which was significantly higher in the flowers of *Llccs*$^{+}$ flowers as compared to the flowers of control and *Llccs*$^{-}$ plants (Table 5). The values of color purity (C*) did not differ significantly among the examined flowers.

Table 5. Chromaticity changes in the downward directed petal of control, *Llccs*$^{-}$ and *Llccs*$^{+}$ transgenic greenhouse-grown *V. cornuta* plants expressed by CIE L*a*b* system.

Sample	L*	a*	b*	C*	h*
Control	78.32 ± 0.51 ^{b}	−4.02 ± 0.83 ^{a}	71.06 ± 2.73 ^{b}	71.38 ± 2.71 ^{a}	93.83 ± 0.74 ^{b}
Llccs^{-}	78.54 ± 0.51 ^{b}	−4.15 ± 1.07 ^{a}	70.88 ± 2.40 ^{b}	71.35 ± 2.34 ^{a}	94.26 ± 1.03 ^{b}
Llccs^{+}	69.26 ± 0.29 ^{a}	13.22 ± 0.98 ^{b}	64.66 ± 0.65 ^{a}	66.40 ± 0.58 ^{a}	78.34 ± 0.89 ^{a}

Control, *Llccs*$^{-}$ and *Llccs*$^{+}$—fresh flowers of respective plants; L*—lightness; a*—redness and greenness (negative values indicate green and positive values indicate red); b*—yellowness and blueness (negative values indicate blue and positive values indicate yellow); C*—purity of color and h*—hues of color. The data in the table represent mean values ± standard error. Mean values within the same column marked with different letters differ significantly based on the Fisher's LSD test ($p \leq 0.05$).

4. Discussion

In horticulture, there is a constant need for new ornamental cultivars with altered characteristics [63]. Genetic engineering created opportunities for improving existing or introducing new desired traits in plants [64]. Flower color is one of the most studied traits, besides different types of stress and pest resistance, improved floral morphology and longevity, induced early flowering and enhanced fragrance [64]. At least 50 ornamental species were transformed by engineered *Agrobacteria* followed by shoot regeneration from different explant types [7,64,65] but data about *Viola* transformations are rare [59]. This work is the first report on *A. tumefaciens*-mediated transformation of *V. cornuta* and the successful introduction of foreign genes for flower color change.

Up to date, the main strategy to modify plant flower color by metabolic engineering of the carotenoid pathway was based on overexpression of single genes from bacteria, such as *Agrobacterium aurantiacum* [28,30,31,66], *Pantoea agglomerans* [26,27], cyanobacterium *Synechocistis* [67], sea bacterium *Paracoccus* [66], *Brevundimonas* sp. SD-212 [31] or green algae, *Hematococcus pluvialis* [68]. In most cases flower color alterations in whole flower or some flower parts in derived transgenic plants were observed, although in some cases no visible change in flower color occurred, despite the expression of the transgene [67,69]. In this work, we demonstrate the introduction of the capsanthin-capsorubin synthase gene from the ornamental plant, *Lilium lancifolium*, and its expression in another ornamental plant, *V. cornuta*. Stable integration of transgene allows synthesis and accumulation of red pigment, which accumulated in petals of flowers altering their color.

In various ornamental species, plant regeneration and transformation efficiency are commonly cultivar-dependent and attempts to develop an efficient protocol are often time-consuming [7]. The fact that cells of younger material often have greater regeneration capacity directs the choice of explants, for example, in carnations, the cells around the shoot tip meristem were the most suitable for transformation [8,70], as well as hypocotyl explants in tomatoes [71]. Successful genetic transformation of *V. cornuta* "Lutea Splendens" was achieved only when initial explants were hypocotyl explants. Other explants such as petiole and leaf parts of in vitro grown shoots were not suitable for transformation of *V. cornuta* or for its micropropagation [72], as it was shown for *V. diffusa* [73]. However, the high portion of *V. cornuta* hypocotyl explants become necrotic after cocultivation with *Agrobacterium* (data not shown), and an additional step of hypocotyl preculture before inoculation was necessary to increase the explants survival, as was also shown for other plant species [74–76]. Namely, the preculture is a common step in a number of transformation protocols, which allows for some time for the explants to recover from wounding (cutting) and to adjust to the new media prior to exposure to agrobacterial attack,

thus preventing explant necrosis. In this work, we found that two days were sufficient to prevent massive necrosis of inoculated hypocotyls. Longer preculture of seven days, however, allowed for significant regeneration of uninoculated explants at 2.5 and even 5 mg/L hygromycin (Figure 2c), and so this preculture duration was not further considered because it would interfere with efficient selection, resulting in false-positive regenerants.

One of the crucial steps in plant transformation protocol is the use of selectable marker genes for the screening of transgenic material. We used the pWBVec10a transformation vector which carries the *hpt* gene for hygromycin resistance and hygromycin was used as the selection agent. This selective agent is an aminoglycoside antibiotic which, besides kanamycin, is the second most often used antibiotic for the screening of transgenic plant material in a wide range of plants species [77]. In general, the presence of antibiotics in selection media negatively affect plant regeneration to different degrees in non-transformed tissue due to inhibition of the protein synthesis in chloroplasts. Plant species, genotypes, as well as different explants of the same plant, may differ in their sensitivity/tolerance to certain antibiotics [78–80]. Our results indicated that hypocotyl explants of *V. cornuta* are very sensitive to hygromycin. The selection of potentially transformed shoots of *V. cornuta* started with a lower concentration of hygromycin (1 mg/L) to allow survival and regeneration of shoots from potentially transformed cells. The concentration of hygromycin was then gradually increased to a concentration that was found to inhibit shoot regeneration. It seems that such a procedure can increase the efficiency of transformation, allowing transformed cells to divide, grow and develop [78,79]. The efficiency of *V. cornuta* genetic transformation was evaluated after PCR confirmation of successful integration of all genes and ranged from 0.33% to 2%, depending on the vector used for transformation. The fact that all three genes, *hpt*, *uid A* and *Llccs* were successfully integrated into the plant genome to a high degree confirms the stability of the constructed vector. There are several reasons for the relatively low efficiency of genetic transformations of *V. cornuta*. The efficiency of genetic transformation and plant regeneration in *Viola cornuta* was comparable with the efficiency of genetic transformation of *V. diffusa*, where low efficiency of *Agrobacterium*-mediated genetic transformation (0.98%) was also recorded [73], which reflects the ability of the *Viola* species for genetic transformation by this method. The low percentage of callus formations and shoot regenerations from inoculated explants as compared to the controls is expected since these explants were cultivated on media with both hygromycin and cefotaxime, whereas the control explants were grown on antibiotic-free media. While hygromycin is indeed a selectable agent, cefotaxime may also affect the regeneration and growth of plant tissues in culture [80]. High percentages of callus formation and shoot regeneration (100% and 32%) from control explants indicate that the previously developed regeneration protocol from hypocotyl explants [56] is well optimized for *V. cornuta* cv. "Lutea Splendens". On the other hand, a low percentage of regeneration after transformation and subsequent selection on hygromycin suggests that this species may be too sensitive to this antibiotic, as was shown in *V. diffusa* [73] and that for future experiments constructs with other selectable markers should be considered. Additionally, we found that *V. cornuta* transgenic plants show lower ability for shoot multiplication and that the rooting ability of transformed shoots was lower in comparison to control non-transformed shoots. It is well known that the introduction and expression of a transgene, in general, can cause numerous, unexpected changes, either at the level of gene expression or metabolism of the host plant [81–83]. This pleiotropic effect of the transgene may also reflect on the efficiency of shoot regeneration from transformed tissue [83]. There are some literature data that show genetic transformation with bacterial *crtW* or crtZ genes controlled by the CaMV35S promotor have no influence on regeneration and growth of transgenic plants [30,67]. The manipulation of the carotenoid biosynthetic pathway may cause negative effects on plant growth and regeneration especially in photosynthetic tissue, because they are involved in protection against chlorophyll bleaching [18]. Furthermore, manipulation of carotenoid synthesis may change the level of endogenous plant growth regulators, such as abscisic acid and gibberellins since they are precursors of these plant growth regulators. In addition,

the influence of the newly synthesized pigment capsanthin in *Llccs*$^{+}$ plants on the reduction of the regenerative ability, shoot multiplication and rooting of the transformed callus tissue of *V. cornuta* should not be excluded and needs to be analyzed in the future. The observed differences in morphometric parameters among the three groups of plants (control, *Llccs*$^{-}$ and *Llccs*$^{+}$) in our study, cannot be directly related to the expression of any of the introduced genes—*Llccs*, *uidA* or *hpt*. Rather, the influence of culture conditions, particularly the duration of the presence of different antibiotics in selective media where the transgenic plants were cultivated, or differential response to acclimatization to greenhouse conditions can be the reasons for the observed differences. Overall, the resulting differences in morphological and phenotypic characteristics reported in this work for genetic transformation of *V. cornuta* may be explained as a consequence of physiological, genetic or epigenetic changes induced by culture in vitro as well as in the regeneration process during and after genetic transformation and should be investigated in future work.

The most evident change directly correlated with *Llccs* gene expression was the color alteration in *V. cornuta* transgenic flowers. Vegetative parts of the *V. cornuta* transgenic plants were green in color and no visible change in color was observed. We found *Llccs* gene expression also in leaves of *Llccs*$^{+}$ transgenic plants but a difference in leaf color was not recorded probably due to the fact that LlCCS was targeted by the chromoplasts which are usually absent in chloroplast-containing tissues as was shown in *Llccs*-transgenic iris tissue [48]. UHPLC analysis revealed that in the petals of the *Llccs*$^{+}$ transgenic plants a novel red carotenoid is produced. This pigment was identified as capsanthin based on comparison with an authentic standard, while we could not confirm capsorubin production with the used UHPLC-DAD system. To date, only a red-orange *Llccs*-transgenic calli of *I. germanica* both capsanthin and capsorubin accumulation was confirmed after ultraperformance liquid chromatography-tandem mass (UHPLC-MS/MS) analysis [48]. Besides iris calli, to date, capsanthin production by CCS expression was demonstrated in transgenic plants of *Nicotiana benthamiana* [84], *Solanum lycopersicum* [54] and *Oryza sativa* [85]. Recently the production of this pigment was achieved also in *Escherichia coli* strains [86]. Contrary to our findings, in leaves of *Nicotiana benthamiana* plants transfected with a viral RNA vector that harbored pepper *ccs* gene, capsanthin is accumulated also in leaves which gave orange color to the whole plant [52]. The formation and accumulation of orange-red pigment in chromoplasts were also recorded in cells in *Llccs*$^{+}$ callus tissue of *V. cornuta*. This observation may be very useful for further research on factors affecting the regulation of biogenesis, synthesis and accumulation of these pigments in callus tissue. The accumulation of newly synthesized pigment was observed in epidermal cells of *Llccs*$^{+}$ transformed flower petals of *V. cornuta* plants in numerous globular chromoplasts. There were no differences in the shape of epidermal cells between transformed and untransformed flowers. A similar observation was reported in *Lotus japonicus* flowers obtained after genetic transformation with *crtW* gene from marine bacteria controlled by the CaMV35S promoter [30].

In our work, we observed differences in *Llccs* gene expression in flower petals and leaves between the *Llccs*$^{+}$ transformed plants. In general, the level of transgene expression regulated by the 35S promoter, used in our study, can vary from tissue to tissue, mainly as a consequence of the positional effect [87,88]. There is a lot of evidence that CaMV 35S activity may vary in different plant species, among and within tissues [83], as well as under different environmental conditions [89]. In addition, the activity of this promoter is developmentally regulated [90]. T-DNA is randomly incorporated into the plant genome, integrating at different sites on the chromosome and in a different number of copies [89,91]. A large number of transgene copies under the control of 35S can be inserted at one site on the chromosome. Additionally, multiple copies of T-DNA can be inserted as direct or inverted replicates. Insertion of T-DNA in inverted form (head-to-head or tail-tail) can lead to reduced transgene expression [90,92]. On the other hand, high expression under the CaMV 35S promoter may lead to toxicity effects in the plant or over-expression of some genes may trigger mechanisms of homology gene silencing by the plant [93]. It should be

noted that the amount of newly synthesized pigment, capsanthin, roughly corresponds to the expression level of the *Llccs* gene in petals of the three analyzed transformants (Figure 8); however, the sample size of three lines is too small for statistical validation of the apparent correlation.

The amount of carotenoids in flowers and fruits is determined by the rate of biosynthesis and degradation, as well as the storage capacities in the cells [94]. The plastids are the primary site of the biosynthesis and accumulation of carotenoids in flower petals and fruits. Histological observations revealed that pigments in *V. cornuta* flowers have subcellular locations in chromoplasts located in the upper epidermal cells of petals. In the early stage of development, fruits and flowers display a pale-green color and carotenoids are accumulated in chloroplasts along with chlorophylls [22]. During flower development, changes in pigment composition occurred with the transition of chloroplasts to chromoplasts [95]. The same phenomenon was observed during the development of flowers of *V. cornuta* (Figure 7). Flower petals were light yellow-green at the beginning of the process of flower development and subsequently, flowers gained yellow color in *V. cornuta* non-transformed control plants. In *Llccs*$^{+}$ transgenic plants, significant accumulation of the pigment was observed even at the onset of the flowering. It seems that the flower color in *V. cornuta Llccs*+ transgenic plants is a consequence of both syntheses of the new protein and increased number of developed globular-type chromoplasts which serve as storage compartments in the cells (Figure 9).

Capsanthin, as xanthophyll carotenoids, may be accumulated in free form, as well as in esterified form in chromoplasts of fruits and flowers. The esterification may facilitate carotenoid sequestration, stability and accumulation in chromoplasts. This is well documented in fruits of pepper where the final carotenoid accumulation is highly correlated with the esterification of xanthophylls [95]. In leaves of *N. benthamiana* plants transfected with a viral RNA vector that harbored pepper ccs gene, capsanthin is accumulated mainly in free form. On the other side, in *Llccs* iris transgenic calli ~80–85% of capsanthin was in esterified form due to the fact that LlCCS was directed to the chromoplasts. In our work, we evaluated the total capsanthin content in flower petals while further separate quantification of free and esterified forms may explain the mechanism of capsanthin storage in chromoplasts of *V. cornuta* petal cell of *Llccs*+ transgenic plants.

Color is a trait that can be affected by subjective sensation due to each individual eyes differences, as well as environmental factors [96,97]. In most studies about the alteration of the flower color by manipulation of carotenoid genes, where changes in color were recorded, quantification of the degree of the changes was omitted [27,30,66]. To objectively express the degree and direction of the color alteration in *V. cornuta* flower petals, we additionally quantified color change by a colorimeter. A positive parameter a* indicated a significant move of a flower's color from yellow to an orange-red component in *Llccs*$^{+}$ flower petals of *V. cornuta,* confirming that ectopic expression of *Llccs* gene for capsanthin-capsorubin synthase from tiger lily to another ornamental plant may significantly alter flower color toward red hues (of the color).

5. Conclusions

Flower color alteration in *V. cornuta* "Lutea Splendens" *Llccs*$^{+}$ transgenic plants occurred due to metabolic engineering of carotenoid genes. The synthesis and accumulation of new carotenoid pigment, capsanthin, which does not naturally exist in these plants, significantly contributed to color alteration in flower petals from yellow to different shades of orange. Further enhancement of capsanthin and/or capsorubin syntheses, or the improved sequestration of these newly synthesized κ-xanthophylls, as well as usage of flower-specific promoters, may be the next strategy that could lead to the development of deeper shades of orange and red flowers in *Llccs*$^{+}$ transgenic plants.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/horticulturae7090324/s1, Figure S1: PCR analysis of genomic DNA of *Llccs*$^{+}$ transgenic shoots (a–d).

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