

# Transforming the Snapdragon Aurone Biosynthetic Genes into Petunia Alters Coloration Patterns in Transgenic Flowers

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## Abstract

Aurones belong to a small class of flavonoids that provide yellow color in some floricultural plants including snapdragon. To explore novel flower coloration, two full-length cDNAs encoding chalcone 4'-O-glucosyltransferase (designated as *SRY4'CGT*) and aureusidin synthase (designated as *SRYAS1*) in the aurone biosynthetic pathway were cloned from yellow flowers of snapdragon (*Antirrhinum majus* cv. Ribbon Yellow). Binary vectors were constructed and transformed separately into *Petunia hybrida* harboring blue flowers. Only a few flowers in 4 out of 9 transgenic *SRY4'CGT* plants showed variegated blue-white sectors; as time passed, amounts of variegated flowers and proportion of white sectors in the background blue color of the new-born flowers gradually increased, until finally, the petal color was completely white in all late-born flowers. In contrast, a few flowers in 3 out of 13 transgenic *SRYAS1* plants showed variegated blue-white sectors; but, the amounts of variegated flowers did not increase over the whole flowering stage, and no complete white flowers were observed. RNA samples isolated from blue and white sectors of T<sub>1</sub> transgenic *SRY4'CGT* plants were analyzed by reverse transcription-PCR, transgenic *SRY4'CGT* transcripts were detected in both sectors; however, transcripts of an upstream gene, chalcone synthase (*CHS*), were abundantly detected in the blue sectors but largely reduced in the white sectors, suggesting that the expression of *CHS* gene was suppressed in white sectors of transgenic plants. Furthermore, HPLC coupled with mass spectrometry demonstrated cyanidin, malvidin and their derivatives were absent in white sectors, causing the white phenotype. Our findings may be attractive to molecular breeders.

## Keywords

*Agrobacterium*-Mediated Transformation, Aurone, Compound Identification, Flower Color, Genetic Engineering, HPLC Profiling, Suppression, Transgene-Induced Gene Silencing

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## 1. Introduction

Flower color is an important feature in floricultural plants, contributing to the major value in the flower market. In general, flower color is determined by three classes of pigments: flavonoids, carotenoids and betalains. Flavonoids are the major contributors in the determination of a range of colors such as yellow, orange, red and purple; and the carotenoids are the red, orange and yellow lipid-soluble pigments found embedded in the membranes of chloroplasts and chromoplasts [1] [2]. The third class of pigments, betalains, can be found only in certain plant species of the Carophyllales and in some higher fungi such as fly agaric [2] [3]. Over the past few decades, almost all the key genes in the main biosynthesis pathways of flavonoids and carotenoids have been cloned from a range of plant species, and genetic engineering has been carried out to breed new cultivars with novel flower colors in many floricultural plants [2]-[8]. Some branch/modifying enzymes are limited to certain plant species; however, most of these genes are not yet known.

Flavonoids, starting from the general phenylpropanoid pathway, can be subdivided into several major groups according to their structures: chalcones, aurones, isoflavonoids, flavones, flavonols, flavan-3-ols, anthocyanins, condensed tannins (proanthocyanins) and phlobaphenes [3] [9]. Among these groups, anthocyanins, flavonols, chalcones and aurones contribute color to plants. Aurones are the bright yellow pigments found in some ornamental flowers such as snapdragon (*Antirrhinum majus*), cosmos and coreopsis [10]. Biochemical and flavonoid mutant studies from *A. majus* clearly revealed that aurones were produced from chalcones [4] [10]. The first common flavonoid compound is 2',4',6',4'-tetrahydroxychalcone (THC) or also known as naringenin chalcone, which is the catalytic product of the key enzyme chalcone synthase (CHS). In many floricultural plants, THC is converted into naringenin by chalcone isomerase (CHI), and naringenin is further converted into different kinds of anthocyanins, flavonols and flavones by several enzymes in the flavonoid biosynthesis pathway. In yellow-flowering plants such as snapdragon, THC is converted into THC 4'-*O*-glucoside by UDP-glucose: chalcone 4'-*O*-glucosyltransferase (4'CGT), and THC 4'-*O*-glucoside is further converted into aureusidin 6'-*O*-glucoside (yellow pigment) by aureusidin synthase (AS) [11]. In addition, *in vitro* activity of AS, catalyzing THC into aureusidin (yellow pigment), has also been reported [10]. Both genes, designated as *AmAS1* and *Am4'CGT*, have been cloned from yellow snapdragon (*A. majus* cv. Yellow Butterfly) and it has been demonstrated that both genes are essential for aurone biosynthesis and yellow coloration [10] [11]. Subsequently, coexpression of the *AmAS1* and *Am4'CGT* transgenes together with suppression of endogenous anthocyanin biosynthetic gene [flavanone 3-hydroxylase (*F3H*) or dihydroflavonol 4-reductase (*DFR*)] produced yellow flowers in transgenic lines of *Torenia hybrid* cv. Summer Wave Blue, a cultivar has blue flowers [11].

We are interested in molecular breeding of flower color since flower color is one of the most attractive characteristics in garden ornamentals and contributes to the major value in the floricultural markets [2] [12]. *Petunia* is one of the most popular ornamentals worldwide, and has been used as one of the model plants for various studies including flavonoid biosynthesis, floral development, male sterility, self-incompatibility, retroelement activity, transposons, epigenetics, transformation, senescence, and so on [13] [14]. In this study, we try to generate bright yellow flowers in *petunia*. As a first step to achieve our goal, here we report the cloning and construction into expression vectors of *AS1* and *4'CGT* cDNAs from yellow flowers in another snapdragon cultivar, *Antirrhinum majus* cv. Ribbon Yellow. These constructs were individually introduced into *Petunia hybrida* harboring blue flowers by using *Agrobacterium*-mediated method. Although yellow flowers were not observed; however, a few flowers in several transgenic plants showed variegated blue-white sectors and attractive coloration patterns. Here we just summarize what we have done and observed in single gene transformation. Breeding transgenic plants coexpressing both transgenes are in progress. The findings reported herein will be useful for breeding new cultivars with attractive coloration patterns.

## 2. Materials and Methods

### 2.1. Plant Materials

Seeds of *Antirrhinum majus* cv. Ribbon Yellow and *Petunia hybrida* cv. Extra Blue were purchased from Known-You Seed Company, Taiwan. For gene cloning, seeds of *A. majus* were germinated, and young seedlings were transferred into pots and grown in a greenhouse until maturity. For plant transformation, seeds of *P. hybrida* were sterilized with 1% sodium hypochlorite for 20 min, washed thoroughly with sterile water, and germinated on MS basal medium containing MS salts [15], 2% sucrose, 0.8% bacto-agar, pH 5.7. The cultures were then

incubated in a 26°C growth chamber under a 16-h illumination (100  $\mu\text{mol}/\text{m}^2/\text{sec}$ ) and 8-h dark cycle. *In vitro* explants grown for 2 to 3 weeks were used to establish the plant transformation system.

## 2.2. Gene Cloning and Construction of Plant Expression Vector

To clone the aurone biosynthetic genes, RNA from different developmental stages of yellow flowers of *A. majus* was isolated by TRIzol protocol as previously described [16]. Primers SRY4'CGT-F1 (5'-ATGGGAGAAGAA-TACAAGAAA-3') and SRY4'CGT-R1 (5'-TTAACGAGTGACCGAGTTGAT-3') specific for *A. majus* chalcone 4'-*O*-glucosyltransferase (4'CGT), and primers SRYAS1-F1 (5'-ATGTTCAAAAATCCTAATATC-3') and SRYAS1-R1 (5'-TTAGCCATCAAGCTCAATCTT-3') specific for *A. majus* aureusidin synthase (AS1) were synthesized to amplify full-length reading frames of SRY4'CGT (1374 bp) and SRYAS1 (1689 bp), respectively. RT-PCR was carried out as previously described [16]. PCR products were analyzed by 1% agarose gel electrophoresis. After purification, PCR products were cloned into pGEM-T Easy vector (Promega), and then sequenced by an automatic DNA sequencer (ABI PRISM 3730 DNA sequencer, Perkin-Elmer). To construct the expression vectors, PCR products of snapdragon 4'CGT and AS1 were cloned into Gateway binary vectors pH2GW7 (carrying the hygromycin selection marker) and pK2WG7 (carrying the kanamycin selection marker), respectively, by using the pCR8/GW/TOPO TA cloning kit (Invitrogen). The resulting vectors pSRY4'CGT and pSRYAS1 were isolated, screened by PCR analysis, and confirmed by DNA sequencing analysis.

## 2.3. Agrobacterium-Mediated Transformation

To introduce the vector DNA into *Agrobacterium*, 50  $\mu\text{l}$  competent cells of *Agrobacterium tumefaciens* strain GV3101 were mixed with 100 ng plasmid DNA (pSRY4'CGT or pSRYAS1) and incubated on ice for 1 to 2 min. The mixture was then transferred into an ice-cold 0.1-cm electroporation cuvette. Electroporation was carried out in a Bio-Rad Gene Pulser II (USA) using the following parameters: 25  $\mu\text{F}$ , 200  $\Omega$ , and 1.25 kV. After electroporation, 1 ml of YEP medium (10 g/l peptone; 10 g/l yeast extract; 5 g/l NaCl; pH 7.0) was added immediately into the mixture, incubated at 28°C for 2 h, and 200  $\mu\text{l}$  of bacterial mixture was transferred onto selective YEP plates containing 100 mg/l spectinomycin. The plates were incubated at 28°C for 2 days. A single colony of plasmid-carrying *Agrobacterium* was picked up and incubated in 4 ml YEP medium containing 100 mg/l spectinomycin at 28°C overnight. The value of OD<sub>600</sub> value should be 0.1 - 0.3. One ml of overnight culture was added to 50 ml AB medium (5 g/l glucose; 1 g/l NH<sub>4</sub>Cl; 0.3 g/l MgSO<sub>4</sub>·7H<sub>2</sub>O; 0.15 g/l KCl; 10 mg/l CaCl<sub>2</sub>; 2.5 mg/l FeSO<sub>4</sub>·7H<sub>2</sub>O; 3 g/l K<sub>2</sub>HPO<sub>4</sub>; 1.15 g/l NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O) supplemented with 100 mg/l spectinomycin, and incubated for 1 day at 28°C. Acetosyringone (Fluka Chemika, Switzerland) and glucose were added to the AB/spectinomycin medium to yield a final concentration of 100  $\mu\text{M}$  and 5%, respectively, and the culture was further incubated 4 h at 28°C. The bacterial culture was then centrifuged at 5000 rpm for 5 min, and the pellet was dissolved in KCMS medium (MS salts; 0.9 mg/l thiamine; 0.2 mg/l 2,4-dichlorophenylacetic acid (2,4-D); 200 mg/l KH<sub>2</sub>PO<sub>4</sub>; pH 5.6) supplemented with 100  $\mu\text{M}$  acetosyringone and 5% glucose. The suspension was then diluted such that the final OD<sub>600</sub> reading was 0.8 - 1.0.

*Agrobacterium*-mediated transformation was carried out as previously described [17] with modifications. For pre-culture prior to *Agrobacterium* infection, cotyledons or leaves were excised from 2- to 3-week-old *in vitro* plantlets of *P. hybrida* one day before infection, and incubated in KCMS solid medium supplemented with 100  $\mu\text{M}$  acetosyringone for one day. Infection was carried out by adding 15 ml of diluted *Agrobacterium* suspension to the pre-cultured explants for 1 h. Excess *Agrobacterium* was blotted from the explants on sterile filter papers, then the infected explants were transferred onto KCMS solid medium containing 100  $\mu\text{M}$  acetosyringone and 5% glucose, and left to grow for 2 days in a dark growth chamber at 26°C. Explants were then washed 2-3 times with shaking (the first shaking was at 220 rpm, and subsequent shakings were at 100 rpm, 30 min each time) in KCMS liquid medium supplemented with 200 mg/l timentin. Explants were dried with sterile filter papers, and transferred to MSZ medium (MS salts; Nitsch vitamins; 1% sucrose; 2% mg/l zeatin; 0.7% bacto-agar; pH 6.0) supplemented with 200 mg/l timentin to inhibit *Agrobacterium* growth and 10 mg/l hygromycin (for selection in transforming vector pSRY4'CGT) or 100 mg/l kanamycin (for selection in transforming vector pSRYAS1), and incubated at 26°C in a growth chamber with a 16-h light (100 - 200  $\mu\text{mol}/\text{m}^2/\text{sec}$ ) and 8-h dark cycle. Explants were transferred to a new MSZ medium supplemented with 200 mg/l timentin and suitable antibiotic (10 mg/l hygromycin or 100 mg/l kanamycin) every two weeks. Formation of the initial calli could be seen on the wound

site of explants after 3 weeks of transformation, and shoots were regenerated after 6 weeks. Single shoot was excised from the callus and transferred onto the MS basal medium supplemented with 100 mg/l timentin. The rooted plantlets were transferred into pots and grown in a greenhouse.

#### 2.4. Reverse Transcription-PCR Analysis

Total RNA was extracted from transgenic flowers, treated with DNase I, and 100 ng of treated RNA was used to perform reverse transcription-PCR (RT-PCR) analysis as previously described [12]. For amplification of gene-specific region, primers SRYAS1-F1 (5'-ATGTTCAAAAATCCTAATATC-3') and SRYAS1-R2 (5'-ACCAGGTGAATCATAGTTCCA-3') were employed to amplify the 624-bp-long DNA fragment of snapdragon AS1 (GenBank accession no. EF650014), primers SRY4'CGT-F2 (5'-ATGGGAGAAGAATACAAGAAAAC-3') and SRY4'CGT-R3 (5'-CATGCTGGTGGATACTTC-3') were employed to amplify the 390-bp-long DNA fragment of snapdragon 4'CGT (GenBank accession no. EF650015), and primers CHS-C1 (5'-ACAACAAGGGCGCTC-GAG-3') and CHS-C2 (5'-CAAGCCCTTACCAGTAG-3') were employed to amplify the 558-bp-long DNA fragment of *Petunia* chalcone synthase (GenBank accession No. AF233638).

#### 2.5. Seedling Assay for Kanamycin or Hygromycin Resistance

T<sub>1</sub> seeds from self-fertilized transgenic plants were sterilized with 1% sodium hypochlorite as mentioned above. They were germinated in MS basal medium supplemented with 10 mg/l hygromycin (for transgenic SRY4'CGT lines) or 100 mg/l kanamycin (for transgenic SRYAS1 lines) for 3 weeks. Seedlings with white or pale green cotyledons, no true leaf development, inhibition of root extension and plant development were considered to be hygromycin or kanamycin sensitive, while seedlings with green cotyledons and healthy development of leaves and roots were considered to be hygromycin or kanamycin resistant.

#### 2.6. Sample Preparation

Freeze-dried petals from transgenic *Petunia* flowers carrying *SRY4'CGT* or *SRYAS1* transgene were homogenized by using liquid nitrogen with a pestle and mortar, transferred into a tube and dissolved in 50% acetonitrile containing 0.1% (vol/vol) trifluoroacetic acid (TFA) to a final concentration of 0.1 g/ml (fresh weight/vol). The mixture was rotated for 3 h at room temperature, and then briefly centrifuged. The supernatant was transferred into a clean tube, and then vacuum-dried. The extracts were resuspended in 50% acetonitrile containing 0.1% (vol/vol) formic acid (FA), the final concentration was adjusted to 0.1 g/ml (fresh weight), and used for HPLC-DAD-MS/MS analysis.

#### 2.7. HPLC-DAD-MS/MS Analysis

The Agilent 1100 series liquid chromatograph system (Agilent, Waldbronn, Germany) coupled with a diode-array detector (DAD) and a Thermo Finnigan LCQ Advantage ion-trap mass spectrometer (San Jose, CA, USA) was employed for analysis at the Metabolomics Core Facility of the Agricultural Biotechnology Research Center, Academia Sinica, Taiwan. The extracts were separated by HPLC with a RP-C18 column (Phenomenex Luna 3  $\mu$ m C18, 150 mm  $\times$  2.0 mm) at a flow rate of 0.2 ml/min. The mobile phase consisted of water containing 0.1% (v/v) FA (mobile phase A) and acetonitrile containing 0.1% (v/v) FA (mobile phase B). The following conditions were used for the gradient elution: 10% - 23% B for 3 min, next with 23% B for 17 min, 23% - 80% B for 2 min and then 80% B for 3 min. The sample injection volume was 10  $\mu$ l. Eluting peaks were monitored by DAD under the following conditions: Chalcones, aurones, anthocyanidins, and flavones were monitored respectively at 360 nm, at 400 nm, at 520 nm, and at 360 nm [11]. Both positive and negative ion polarity modes were used for compound ionization. The MS optimized parameters in positive ion mode were as follows: source voltage, 4.5 kV; sheath gas (N<sub>2</sub>), 50 arbitrary units; capillary temperature, 250°C; capillary voltage, 3 V; tube lens offset voltage, 30 V; in the negative ion mode, source voltage, -4 kV; capillary voltage, -47 V; tube lens offset voltage, -45 V. For full scan MS analysis, spectra were recorded in the range of  $m/z$  100 - 2000. The data-dependent program was set so that the three most intense ions in each survey scan were selected and subjected to tandem mass spectrometry. The HPLC-DAD-MS/MS system was controlled by Xcalibur 2.0 SR2 software (Thermo Finnigan, San Jose, CA, USA).

### 3. Results

#### 3.1. Cloning and Sequence Analysis of Full-Length cDNAs in the Aurone Biosynthetic Pathway from Yellow Snapdragon Flowers

Yellow flowers of snapdragon (*Antirrhinum majus* cv. Ribbon Yellow) at different stages from tiny flower bud to mature flower (Figure 1) were harvested, total RNA was isolated, and RT-PCR was carried out to clone the full-length cDNAs encoding chalcone 4'-*O*-glucosyltransferase (designated as SRY4'CGT) and aureusidin synthase (designated as SRYAS1) in aurone biosynthetic pathway. The SRY4'CGT clone is 1374 bp in length and contains a reading frame of 457 amino acids. Our clone is the same length as the published *A. majus* Am4'CGT nucleotide sequence ([11]; GenBank accession No. AB198665); however, it differed from the published clone by 16 nucleotides and 8 amino acids (Figure 2). To examine the accuracy of the sequence of our SRY4'CGT cDNA clone, the corresponding genomic DNA fragment was amplified by PCR analysis using genomic DNA isolated from *A. majus* cultivar Ribbon Yellow and primer set (SRY4'CGT-F1 and SRY4'CGT-R1) and then sequenced. As shown in Figure 2, nucleotide sequences of our cDNA and the genomic clones were identical, indicating that the genomic *SRY4'CGT* gene has no intron. This result is consistent with the finding that there is no intron in the *Am4'CGT* gene of *A. majus* [11]. The differences between the published clone and our clone reflect the two different cultivars (Ribbon Yellow *versus* Yellow Butterfly) examined. The full-length cDNA and genomic DNA encoding SRY4'CGT in cultivar Ribbon Yellow were submitted to GenBank database with accession numbers EF650015 and JQ234673, respectively. It was reported that chalcone 4'-*O*-glucosyltransferase catalyzes 2',4',6', 4-tetrahydroxychalcone (THC) to THC 4'-*O*-glucoside (colorless) or 2',4',6',3,4-pentahydroxychalcone (PHC) to PHC 4'-*O*-glucoside (colorless) in aurone biosynthetic pathway [11].

The other clone, SRYAS1, is 1689 bp in length and contains a reading frame of 562 amino acids. Our SRYAS1 differed from the published snapdragon AmAS1 nucleotide sequence ([10]; GenBank accession no. AB044884) by two bases in the coding region at nucleotide positions 285 and 892; however, the amino acids were identical to the corresponding published AmAS1 protein sequence (Figure 3). The full-length cDNA encoding SRYAS1 in snapdragon cultivar Ribbon Yellow was submitted to GenBank database with accession number EF650014. It was reported that aureusidin synthase catalyzes THC 4'-*O*-glucoside (colorless) or PHC 4'-*O*-glucoside (colorless) to produce aureusidin 6-*O*-glucoside (yellow pigment) or bracteatin 6-*O*-glucoside (yellow pigment), respectively, in the aurone biosynthetic pathway [11]. The deduced amino acid sequence of SRYAS1 protein showed high sequence similarity to the plant polyphenol oxidase family [10], which has been demonstrated to be responsible for flower coloration [10] [11] [18].

The relevant cDNA fragments were cloned separately into Gateway vectors, obtaining the expression vectors pSRY4'CGT and pSRYAS1 (Figure 4), which were confirmed by restriction enzyme digestion and DNA sequencing.

#### 3.2. Isolation and Phenotypic Observation of Transgenic *Petunia* Plants Carrying SRY4'CGT or SRYAS1 Transgene

*Agrobacterium*-mediated transformation was carried out to transform pSRY4'CGT and pSRYAS1 separately into *P. hybrida*. Finally, 9 putative transgenic plants were obtained from a total of 186 cotyledon explants by



**Figure 1.** Flower phenotype of snapdragon. Seeds of snapdragon (*Antirrhinum majus* cv. Ribbon Yellow) were germinated, and young seedlings were transferred into pots and grown in greenhouse until maturity. RNA was isolated from different stages of tiny flower bud to mature flower.

AB198665	ATGGGAGAAG	AATACAAGAA	AACACACACA	ATAGTCTTTC	ACACTTCAGA	AGAACACCTC	AACTCTCAA	TAGCCCTTGC	AAAGTTCATA	ACCAAACACC
EF650015	ATGGGAGAAG	AATACAAGAA	AACACACACA	ATAGTCTTTC	ACACTTCAGA	AGAACACCTC	AACTCTCAA	TAGCCCTTGC	AAAGTTCATA	ACCAAACACC
JQ234673	ATGGGAGAAG	AATACAAGAA	AACACACACA	ATAGTCTTTC	ACACTTCAGA	AGAACACCTC	AACTCTCAA	TAGCCCTTGC	AAAGTTCATA	ACCAAACACC
AB198665	ACTCTTCAAT	CTCCATCACT	ATCATCAGCA	CTGCCCCCGC	CGAATCTTCT	GAAGTGGCCA	AAATTATTAA	TAATCCGTC	ATAACTTACC	GCGGCCCTCAC
EF650015	ACTCTTCAAT	CTCCATCACT	ATCATCAGCA	CTGCCCCCGC	CGAATCTTCT	GAAGTGGCCA	AAATTATTAA	TAATCCGTC	ATAACTTACC	GCGGCCCTCAC
JQ234673	ACTCTTCAAT	CTCCATCACT	ATCATCAGCA	CTGCCCCCGC	CGAATCTTCT	GAAGTGGCCA	AAATTATTAA	TAATCCGTC	ATAACTTACC	GCGGCCCTCAC
AB198665	CGCGGTAGCG	CTCCCTGAAA	ATCTCACCAG	TAACATTAAT	AAAAACCCCG	TCGAACTTTT	CTTGGAAATC	CCTCGTCTAC	AAAAAGCCAA	CCTTGGAGAG
EF650015	CGCGGTAGCG	CTCCCTGAAA	ATCTCACCAG	CAACATTAAT	AAAAACCCCG	TCGAACTTTT	CTTGGAAATC	CCTCGTCTAC	AAAAAGCCAA	CCTTGGAGAG
JQ234673	CGCGGTAGCG	CTCCCTGAAA	ATCTCACCAG	CAACATTAAT	AAAAACCCCG	TCGAACTTTT	CTTGGAAATC	CCTCGTCTAC	AAAAAGCCAA	CCTTGGAGAG
AB198665	GCTTTACTAG	ATATTTTCGG	AAAATCCGAT	ATCAAAGCAT	TAATCATCGA	TTTCTTCTGC	AATGGCGCAT	TTGAAGTATC	CACCAGCATG	AACATAACCA
EF650015	GCTTTACTAG	ATATTTTCGG	AAAATCCGAT	ATCAAAGCAT	TAATCATCGA	TTTCTTCTGC	AATGGCGCAT	TTGAAGTATC	CACCAGCATG	AACATAACCA
JQ234673	GCTTTACTAG	ATATTTTCGG	AAAATCCGAT	ATCAAAGCAT	TAATCATCGA	TTTCTTCTGC	AATGGCGCAT	TTGAAGTATC	CACCAGCATG	AACATAACCA
AB198665	CTTACTTCGA	CGTCAGTGGC	GGCGCTTTTC	TCCTCTGCAC	GTTTCTCCAC	CACCCGACAC	TACACCAAAC	TGTTCTGTTG	GACATTGCGG	ATTTGAACGA
EF650015	CTTACTTCGA	CGTCAGTGGC	GGCGCTTTTC	TCCTCTGCAC	GTTTCTCCAC	CACCCGACAC	TACACCAAAC	TGTTCTGTTG	GACATTGCGG	ATTTGAACGA
JQ234673	CTTACTTCGA	CGTCAGTGGC	GGCGCTTTTC	TCCTCTGCAC	GTTTCTCCAC	CACCCGACAC	TACACCAAAC	TGTTCTGTTG	GACATTGCGG	ATTTGAACGA
AB198665	TTCTGTTGAG	ATGCCCGGGT	TCCCATTAAT	TCACTCCTCT	GATTTACCAA	TGAGTTTGTG	TTATCGTAAG	ACTAATGTTT	ACAAACACTT	TCTAGACACT
EF650015	TTCTGTTGAG	ATGCCCGGGT	TCCCATTAAT	TCACTCCTCT	GATTTACCAA	TGAGTTTGTG	TTATCGTAAG	ACTAATGTTT	ACAAACACTT	TCTAGACACT
JQ234673	TTCTGTTGAG	ATGCCCGGGT	TCCCATTAAT	TCACTCCTCT	GATTTACCAA	TGAGTTTGTG	TTATCGTAAG	ACTAATGTTT	ACAAACACTT	TCTAGACACT
AB198665	TCCTTAAACA	TGCGCAAATC	GAGTGGGATA	CTCGTGAACA	CGTTTGTTCG	GCTCGAGTTT	CGAGCTAAGG	AAGCCTTGTG	CAACGGTTTG	TACGGTCCAA
EF650015	TCCTTAAACA	TGCGCAAATC	GAGTGGGATA	CTCGTGAACA	CGTTTGTTCG	GCTCGAGTTT	CGAGCTAAGG	AAGCCTTGTG	CAACGGTTTG	TACGGTCCAA
JQ234673	TCCTTAAACA	TGCGCAAATC	GAGTGGGATA	CTCGTGAACA	CGTTTGTTCG	GCTCGAGTTT	CGAGCTAAGG	AAGCCTTGTG	CAACGGTTTG	TACGGTCCAA
AB198665	CTCCGCCTGT	TTATTTACTT	TCACATACAA	TTGCGAACC	CCACGACACT	AAAGTGTGG	TAAACCAACA	CGATGCGCTA	TCATGGCTTG	ATTTGCAGCC
EF650015	CTCCGCCTGT	TTATTTACTT	TCACATACAA	TTGCGAACC	CCACGACACT	AAAGTGTGG	TAAACCAACA	CGATGCGCTA	TCATGGCTTG	ATTTGCAGCC
JQ234673	CTCCGCCTGT	TTATTTACTT	TCACATACAA	TTGCGAACC	CCACGACACT	AAAGTGTGG	TAAACCAACA	CGATGCGCTA	TCATGGCTTG	ATTTGCAGCC
AB198665	TAGTAAAAGC	GTGATTTTCC	TTTGTTTCCG	AAGAAGAGGA	GCGTTCCTCAG	CACAACAGTT	GAAAGAAATT	GCGATAGGGT	TGGAGAAGAG	TGGATGTCGA
EF650015	TAGTAAAAGC	GTGATTTTCC	TTTGTTTCCG	AAGAAGAGGA	GCGTTCCTCAG	CACAACAGTT	GAAAGAAATT	GCGATAGGGT	TGGAGAAGAG	TGGATGTCGA
JQ234673	TAGTAAAAGC	GTGATTTTCC	TTTGTTTCCG	AAGAAGAGGA	GCGTTCCTCAG	CACAACAGTT	GAAAGAAATT	GCGATAGGGT	TGGAGAAGAG	TGGATGTCGA
AB198665	TTTCTTTGGT	TGGCCCGCAT	TTACCCGGAG	ATGGACTTAA	ATGCGTCTCT	GCCGGAGGGT	TTTCTATCGA	GAACTAAAGG	AGTAGGGTTT	GTGACAAACA
EF650015	TTTCTTTGGT	TGGCCCGCAT	TTACCCGGAG	ATGGACTTAA	ATGCGTCTCT	GCCGGAGGGT	TTTCTATCGA	GAACTAAAGG	AGTAGGGTTT	GTGACAAACA
JQ234673	TTTCTTTGGT	TGGCCCGCAT	TTACCCGGAG	ATGGACTTAA	ATGCGTCTCT	GCCGGAGGGT	TTTCTATCGA	GAACTAAAGG	AGTAGGGTTT	GTGACAAACA
AB198665	CATGGGTGCC	GCAGAAAAGG	GTGTTGAGTC	ATGATGCAGG	GGGGGGGTTT	GTGACTCATT	GGGGTGGAG	TTGCGTCTT	GAAGCGCTGT	CGTTCGGTGT
EF650015	CATGGGTGCC	GCAGAAAAGG	GTGTTGAGTC	ATGATGCAGG	GGGGGGGTTT	GTGACTCATT	GGGGTGGAG	TTGCGTCTT	GAAGCGCTGT	CGTTCGGTGT
JQ234673	CATGGGTGCC	GCAGAAAAGG	GTGTTGAGTC	ATGATGCAGG	GGGGGGGTTT	GTGACTCATT	GGGGTGGAG	TTGCGTCTT	GAAGCGCTGT	CGTTCGGTGT
AB198665	CCCGATGATT	GGTTGGCCGT	TGTACGCAGA	GCAGAGGATC	AATAGGGTGT	TCATGGTGGG	GAAATAAAG	GTGGCCTGCTG	CATTGGATGA	GGAAGATGGA
EF650015	CCCGATGATT	GGTTGGCCGT	TGTACGCAGA	GCAGAGGATC	AATAGGGTGT	TCATGGTGGG	GAAATAAAG	GTGGCCTGCTG	CATTGGATGA	GGAAGATGGA
JQ234673	CCCGATGATT	GGTTGGCCGT	TGTACGCAGA	GCAGAGGATC	AATAGGGTGT	TCATGGTGGG	GAAATAAAG	GTGGCCTGCTG	CATTGGATGA	GGAAGATGGA
AB198665	TTTGTGACCG	CGATGGAGTT	GGAGAAGCGC	GTCAGGGAGT	TGATGGAGTC	GGTAAAGGGG	AAAGAAGTGA	AGGCCCGTGT	GGCGGAATTG	AAAATCTCTA
EF650015	TTTGTGACCG	CGATGGAGTT	GGAGAAGCGC	GTCAGGGAGT	TGATGGAGTC	GGTAAAGGGG	AAAGAAGTGA	AGGCCCGTGT	GGCGGAATTG	AAAATCTCTA
JQ234673	TTTGTGACCG	CGATGGAGTT	GGAGAAGCGC	GTCAGGGAGT	TGATGGAGTC	GGTAAAGGGG	AAAGAAGTGA	AGGCCCGTGT	GGCGGAATTG	AAAATCTCTA
AB198665	CAAAGGCAGC	CGTGAGTAAA	GGTGGATCGT	CCTTGGTTTC	TTTGGAGAAG	TTTCATCACT	CGGTCACTCG	TTAA		
EF650015	CAAAGGCAGC	CGTGAGTAAA	GGTGGATCGT	CCTTGGTTTC	TTTGGAGAAG	TTTCATCACT	CGGTCACTCG	TTAA		
JQ234673	CAAAGGCAGC	CGTGAGTAAA	GGTGGATCGT	CCTTGGTTTC	TTTGGAGAAG	TTTCATCACT	CGGTCACTCG	TTAA		
AB198665	MGEYKKTHT	IVFHTSEHL	NSSIALAKFI	TKHSSISIT	IISTAPAESS	EVAKIINNPS	ITYRGLTAVA	LPENLTSNIN	KNPVLEFEEI	PRLQANLRE
EF650015	MGEYKKTHT	IVFHTSEHL	NSSIALAKFI	TKHSSISIT	IISTAPAESS	EVAKIINNPS	ITYRGLTAVA	LPENLTSNIN	KNPVLEFEEI	PRLQANLRE
JQ234673	MGEYKKTHT	IVFHTSEHL	NSSIALAKFI	TKHSSISIT	IISTAPAESS	EVAKIINNPS	ITYRGLTAVA	LPENLTSNIN	KNPVLEFEEI	PRLQANLRE
AB198665	ALLDISRKSD	IKALIIDFFC	NAAFEVSTSM	NIPTYFDVSG	GAPLLCTFLH	HPTLHQTVRG	DIADLNDVSE	MPGFPLIHS	DLPMSLFYRK	SNVYKHLDT
EF650015	ALLDISRKSD	IKALIIDFFC	NAAFEVSTSM	NIPTYFDVSG	GAPLLCTFLH	HPTLHQTVRG	DIADLNDVSE	MPGFPLIHS	DLPMSLFYRK	SNVYKHLDT
JQ234673	ALLDISRKSD	IKALIIDFFC	NAAFEVSTSM	NIPTYFDVSG	GAPLLCTFLH	HPTLHQTVRG	DIADLNDVSE	MPGFPLIHS	DLPMSLFYRK	SNVYKHLDT
AB198665	SLNMRKSSGI	LVNTFVALEF	RAKEALSNGL	YGTPPPVYLL	SHTIAEPHDT	KVLVNHQDCL	SWLDLQPSKS	VIFLCFRRG	AFSAQQLKEI	AIGLEKSGCR
EF650015	SLNMRKSSGI	LVNTFVALEF	RAKEALSNGL	YGTPPPVYLL	SHTIAEPHDT	KVLVNHQDCL	SWLDLQPSKS	VIFLCFRRG	AFSAQQLKEI	AIGLEKSGCR
JQ234673	SLNMRKSSGI	LVNTFVALEF	RAKEALSNGL	YGTPPPVYLL	SHTIAEPHDT	KVLVNHQDCL	SWLDLQPSKS	VIFLCFRRG	AFSAQQLKEI	AIGLEKSGCR
AB198665	FLWLARISPE	MDLNALLPEG	FLSRTKGVGF	VTNTWVPQKE	VLSHDAVGGF	VTHCGWNSVL	EALSFVPMI	GWPLYAQRRI	NRVFMVBEIK	VALPLDEEDG
EF650015	FLWLARISPE	MDLNALLPEG	FLSRTKGVGF	VTNTWVPQKE	VLSHDAVGGF	VTHCGWNSVL	EALSFVPMI	GWPLYAQRRI	NRVFMVBEIK	VALPLDEEDG
JQ234673	FLWLARISPE	MDLNALLPEG	FLSRTKGVGF	VTNTWVPQKE	VLSHDAVGGF	VTHCGWNSVL	EALSFVPMI	GWPLYAQRRI	NRVFMVBEIK	VALPLDEEDG
AB198665	FVTAMELEKR	VRELMESVKG	KEVKRRVAEL	KISTKAAVSK	GGSSLVALEK	FINSVTR				
EF650015	FVTAMELEKR	VRELMESVKG	KEVKRRVAEL	KISTKAAVSK	GGSSLVALEK	FINSVTR				
JQ234673	FVTAMELEKR	VRELMESVKG	KEVKRRVAEL	KISTKAAVSK	GGSSLVALEK	FINSVTR				

(a)

(b)

**Figure 2.** Nucleotide sequence (panel (a)) and deduced amino acid sequence (panel (b)) of snapdragon cDNA encoding chalcone 4'-O-glucosyltransferase (4'CGT). Accession number AB198665 is the published *Am4'CGT* sequence from *Antirrhinum majus* cv. Yellow Butterfly [11], EF650015 is our cDNA sequence from *A. majus* cv. Ribbon Yellow (*SRY4'CGT*), and JQ234673 is our genomic sequence. Nucleotides or amino acids that differed from the published sequence [11] are indicated by highlights.

transforming the vector pSRY4'CGT and using 10 mg/l hygromycin as selection agent (Table 1); and 13 putative transgenic plants were obtained from a total of 205 cotyledon explants by transforming the vector pSR-YAS1 and using 100 mg/l kanamycin as selection agent (Table 2). Transformation efficiency for *SRY4'CGT* and *SRYAS1* transgenes was 4.8% and 6.3%, respectively. All transgenic plants were grown in a greenhouse until

AB044884	ATGTTCAAAA	ATCCTAATAT	CGCTATCAC	AAACATCTT	CCAAATCCAA	TGACAACGAT	CAAGAATCCT	CCCATCGTTG	TAAGCACATT	CTATTATTTA
EF650014	ATGTTCAAAA	ATCCTAATAT	CGCTATCAC	AAACATCTT	CCAAATCCAA	TGACAACGAT	CAAGAATCCT	CCCATCGTTG	TAAGCACATT	CTATTATTTA
AB044884	TAATAACCTT	ATTCTACTT	ATAGTTGGC	TGTACATCG	CAACTCTCTC	GCCTATGCC	GGTTTGCCCT	GACCTCAACC	GGCCCTATCG	CGGCCCTGA
EF650014	TAATAACCTT	ATTCTACTT	ATAGTTGGC	TGTACATCG	CAACTCTCTC	GCCTATGCC	GGTTTGCCCT	GACCTCAACC	GGCCCTATCG	CGGCCCTGA
AB044884	TGTACACAAA	TGTGGTCAG	CAGACTTGCC	ACCTGGCACA	GCCCCAATAA	ACTGTTGTCC	CCCAATCCCC	GCTAAAATCA	TCGAATTTCGA	GCTACCACCT
EF650014	TGTACACAAA	TGTGGTCAG	CAGACTTGCC	ACCTGGCACA	GCCCCAATAA	ACTGTTGTCC	CCCAATCCCC	GCTAAAATCA	TCGAATTTCGA	GCTACCACCT
AB044884	CCCTCCACTA	CCATGAGGGT	TGCGCGTGG	GCTCATTTAG	TTGATGATGC	ATACATTGCC	AAATTCAGA	AAGCCGTTGA	GCTTATGCGA	GCTCTACCTG
EF650014	CCCTCCACTA	CCATGAGGGT	TGCGCGTGG	GCTCATTTAG	TTGATGATGC	ATACATTGCC	AAATTCAGA	AAGCCGTTGA	GCTTATGCGA	GCTCTACCTG
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EF650014	TTTTGGAATC	ATGATTCACC	TGTTGGAATC	ACAAATCCCA	CAATGTTTAT	TGATACATAT	TCTTCGCTGT	ACGATAGTTT	ACGGGACAGT	AATCATCACG
AB044884	CACCAACCTA	CGTAGACTTG	AACTACGCC	TTTCGATTC	CGACATPACC	ACTACTCCTG	AAGAGCAAA	GATTTAAAC	CTTAAAATG	TGTACAGACA
EF650014	CACCAACCTA	CGTAGACTTG	AACTACGCC	TTTCGATTC	CGACATPACC	ACTACTCCTG	AAGAGCAAA	GATTTAAAC	CTTAAAATG	TGTACAGACA
AB044884	AATGGTGTCTG	AGCGCTAAGA	CTCCACAGCT	TTCTCTCGG	CGCCCATACC	GACGTGGGG	CCAGAATTT	CCCGGGTGTG	GTCGATTTGA	GTTAGTCCCT
EF650014	AATGGTGTCTG	AGCGCTAAGA	CTCCACAGCT	TTCTCTCGG	CGCCCATACC	GACGTGGGG	CCAGAATTT	CCCGGGTGTG	GTCGATTTGA	GTTAGTCCCT
AB044884	CATGCGATGA	TACATTTATG	GACCGGTTCT	GAGAACACGC	CCATAGGCGA	GAACATGGGG	GCTTCTTACT	CAACGGCTAG	AGACCCGATA	TTTTTGGCTC
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AB044884	ATCATTGCAA	CGTCGATAGA	ATGTGGTCCA	TATGGAAGAC	CCTAGGAGGG	CCGCGGAGGA	CGGACTTAAC	AGATCCAGAT	TTTCTTGATG	CCTCTTTCTG
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AB044884	TTTTTATGAC	GAAAACGCA	AGATGGTTC	GGTCAAGGTT	CGGGATTGCT	TAGATGAAA	GAAACTAGGG	TACGTTTATC	AAGATGTTGA	GATTCCGTTG
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AB044884	ACAGAGCTTT	AAAAGTTATC	GTGACGAGG	CGAAGAAAAC	TAGAAGTAGG	AAAGAAAAG	ACGAGTTAGA	AGAGATTTTA	GTGATTGAAG	GGATTGAAC
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AB044884	GGAAGAGAG	CACGGGCAG	TAAAATCGA	CGTTTATAT	AATGCTGACG	AAGATGACCT	TGCGGTGATT	TCGCGGAGA	ATGCTGAGTT	CGCCGGGAGT
EF650014	GGAAGAGAG	CACGGGCAG	TAAAATCGA	CGTTTATAT	AATGCTGACG	AAGATGACCT	TGCGGTGATT	TCGCGGAGA	ATGCTGAGTT	CGCCGGGAGT
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AB044884	AAGATGATTA	TGTGTGGTC	ACTTTGGTTC	CGAAGAACGC	CGGAGATGCG	ATCAAGATTC	ATAATGTCAA	GATTGAGCTT	GATGGCTAA	
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(a)

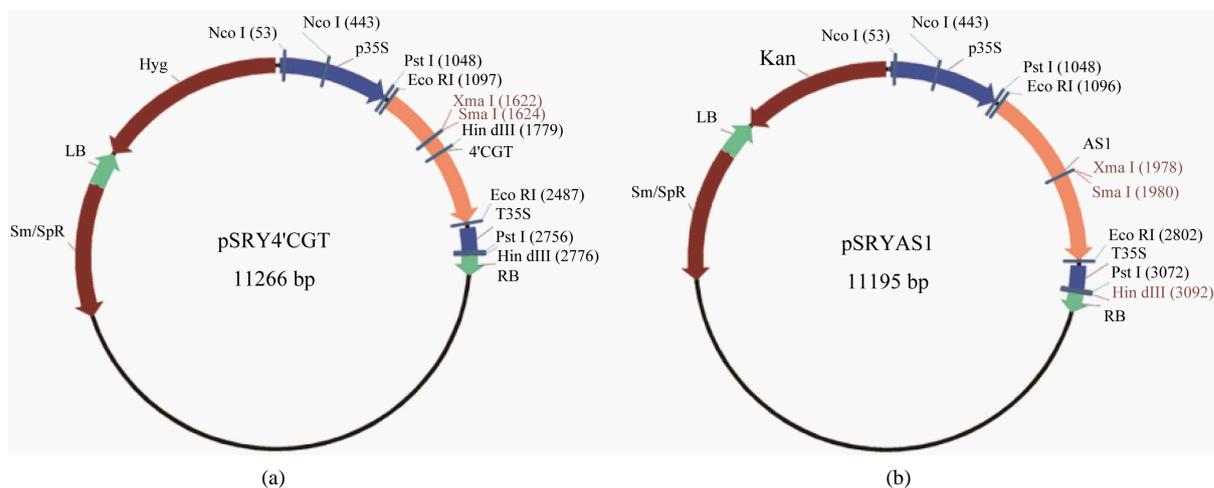
AB044884	MFKNPNIRYH	KLSSKSNNDN	QESSHRCKHI	LLFIITLFL	IVGLYIANS	AYARFASTST	GPIAAPDVTK	CGQDLPFGT	APINCCPPI	AKIIDFELFP
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AB044884	PS*TRVRR	AHLVDDAYIA	KFKKAVELMR	ALPEDDPRSF	KQANVHCAY	CAGAYNQAGF	TNLKLIHRS	WLFPPHRY	IYFFERILGK	LINDTTFALP
EF650014	PS*TRVRR	AHLVDDAYIA	KFKKAVELMR	ALPEDDPRSF	KQANVHCAY	CAGAYNQAGF	TNLKLIHRS	WLFPPHRY	IYFFERILGK	LINDTTFALP
AB044884	FWNYDSPGM	TIPSMFIDTN	SSLYDSLDRS	NHQPTIVDL	NYAFSDSDNT	TPEEQMILN	LKIVVRQMS	SAKTPQLFFG	RPYRRQDEF	FGVGSIELVF
EF650014	FWNYDSPGM	TIPSMFIDTN	SSLYDSLDRS	NHQPTIVDL	NYAFSDSDNT	TPEEQMILN	LKIVVRQMS	SAKTPQLFFG	RPYRRQDEF	FGVGSIELVF
AB044884	HGMHILWTS	ENTFYGENMG	AFYSTARDPI	FFAHHSNVDR	MWSINKVLGG	FRRLDLDPD	FLDASFVYD	ENAEVRVKV	RCDLDEKLG	VYVDVEIFW
EF650014	HGMHILWTS	ENTFYGENMG	AFYSTARDPI	FFAHHSNVDR	MWSINKVLGG	FRRLDLDPD	FLDASFVYD	ENAEVRVKV	RCDLDEKLG	VYVDVEIFW
AB044884	LNTRTPPKVS	PSLLKKFHRT	NTANPRQVFP	AILLRVLKVI	VTRPKKTRSR	KENDELEEL	VIEGIELERD	HGHVKFDVYI	NADEDDLAVI	SPENAEFAGS
EF650014	LNTRTPPKVS	PSLLKKFHRT	NTANPRQVFP	AILLRVLKVI	VTRPKKTRSR	KENDELEEL	VIEGIELERD	HGHVKFDVYI	NADEDDLAVI	SPENAEFAGS
AB044884	FVSLWHKPIK	GKRTKQLLT	LSICDILEDL	DADEDDIVLV	TLVPRNAGDA	IKIHNVKIEL	DG			
EF650014	FVSLWHKPIK	GKRTKQLLT	LSICDILEDL	DADEDDIVLV	TLVPRNAGDA	IKIHNVKIEL	DG			

(b)

**Figure 3.** Nucleotide sequence (panel (a)) and deduced amino acid sequence (panel (b)) of snapdragon cDNA encoding aureusidin synthase (AS1). Accession number AB044884 is the published *AmAS1* sequence from *An-tirrhinum majus* cv. Yellow Butterfly [10], EF650014 is our cDNA sequence from *A.majus* cv. Ribbon Yellow (*SRYAS1*). Nucleotides that differed from the published sequence [10] are indicated by highlights.

maturity.

Of the 9 transgenic *Petunia* plants carrying the *SRY4'CGT* transgene, only a few flowers (from transformants *SRY4'CGT*-1, -6, -7, -10) showed variegated blue-white sectors (Table 1). Those transformants that produced variegated blue-white flowers, at the late stage of flowering, more and more new-born flowers produced variegated blue-white flowers, and the areas of white sectors increased in the blue background of the petals. As time passed, most of the old flowers without alteration in pigmentation faded, and the number of new-born flowers carrying more white sectors/regions in the background blue color increased. Finally, petal color was changed to completely white in all the late-born flowers (Figure 5). In contrast, of the 13 transgenic *Petunia* plants carrying *SRYAS1* transgene, three (*SRYAS1*-4, -6, -12) showed variegated blue-white flowers at the late stage of flowering (Table 2): a few variegated flowers carrying small portions of white sectors in the background blue petals were observed in these 3 transgenic lines. However, complete white flowers were not observed. The growth



**Figure 4.** Restriction maps of transformation vectors pSRY4'CGT (a) and pSRYAS1 (b).

**Table 1.** Alteration in flower color and inheritance in T<sub>1</sub> progeny in transgenic *Petunia* plants carrying *SRY4'CGT* transgene.

Transgenic plant	Flower color	No. of resistant (R) and sensitive (S) seedlings from T <sub>0</sub> selfed seeds		Test ratio (R:S)	Remarks
		R	S		
SRY4'CGT-1	Blue-white				No seeds
SRY4'CGT-2	Blue	178	172	1:1	
SRY4'CGT-3	Blue				No seeds
SRY4'CGT-4	Blue	282	170	2:1	
SRY4'CGT-5	Blue				No seeds
SRY4'CGT-6	Blue-white	265	308	1:1	
SRY4'CGT-7	Blue-white	577	191	3:1	
SRY4'CGT-10	Blue-white	138	43	3:1	
SRY4'CGT-12	Blue	189	98	2:1	

Seeds were germinated on MS medium containing 10 mg/l hygromycin for 3 weeks.

pattern of a typical transgenic *SRYAS1* plant carrying variegated flowers is shown in **Figure 6**. In addition, the amount and appearance of variegated blue-white flowers during the late stage of flowering (or plant senescence) in transgenic *SRYAS1* plants were less than and later than, respectively, those of transgenic *SRY4'CGT* plants.

### 3.3. Gene Expression in T<sub>0</sub> Transgenic *SRYAS1* and *SRY4'CGT* Plants Carrying Variegated Blue-White Flowers

To examine the presence and expression of transgenes in transgenic plants, total RNA was isolated from variegated blue-white flowers in three transgenic *SRYAS1* plants (SRYAS1-4, -6, -12) and four transgenic *SRY4'CGT* plants (SRY4'CGT-1, -6, -7, -10). In addition, total RNA was also isolated from blue flowers in wild-type *P. hybrida*. RT-PCR analysis was carried out to investigate the expression of transgene in transgenic plants (**Figure 7**). As expected, no transcripts were detected in wild-type *Petunia* flowers when gene-specific *SRYAS1* or *SRY4'CGT* primers were employed. Transgenic *SRYAS1* transcripts were detected in all three transgenic *SRYAS1* plants, and transgenic *SRY4'CGT* transcripts were detected in all four transgenic *SRY4'CGT* plants (**Figure 7**). Thus, these results demonstrate clearly the presence and expression of transgenes in transgenic plants we examined.

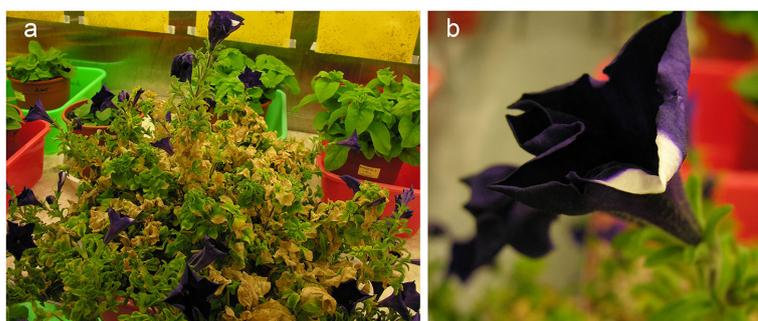


**Figure 5.** Alternation of flower coloration in transgenic *Petunia* plants carrying *SRY4/CGT* transgene. (a) At the beginning of flowering, only a few flowers (indicated by arrow) showed white sectors at the edge of the blue petals. (b) and (c) Over time, flowers with regular blue color faded, and at the middle and late stages of flowering variegated flowers carrying white sectors in a blue background, or complete white flowers, appeared. (d) During the late stage of flowering, variegated blue-white flowers gradually faded and new flowers emerging at that stage were almost or all white in color. As a result, all the flowers at the late-flowering stage in transgenic plants looked white in color. (e) to (j) Flower phenotypes from a spot of white color in the blue background to complete white color observed in the same transgenic plant.

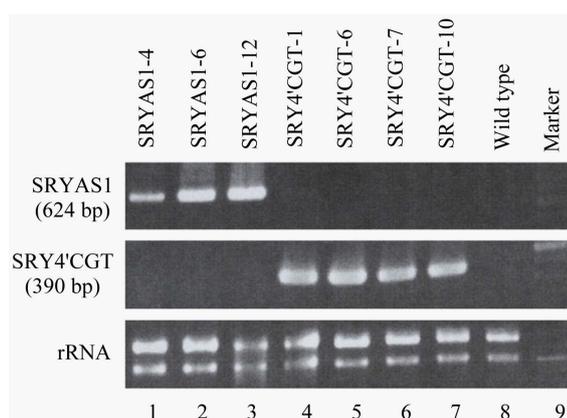
**Table 2.** Alteration in flower color and inheritance in T<sub>1</sub> progeny in transgenic *Petunia* plants carrying *SRYAS1* transgene.

Transgenic plant	Flower color	No. of resistant (R) and sensitive (S) seedlings from T <sub>0</sub> selfed seeds		Test ratio (R:S)	Remarks
		R	S		
SRYAS1-1	Blue	123	121	1:1	
SRYAS1-2	Blue	55	16	3:1	
SRYAS1-3	Blue	437	188	2:1	
SRYAS1-4	Blue-white	150	56	3:1	
SRYAS1-5	Blue	196	85	2:1	
SRYAS1-6	Blue-white	134	59	2:1	
SRYAS1-7	Blue	0	340		
SRYAS1-8	Blue				No seeds
SRYAS1-9	Blue	110	109	1:1	
SRYAS1-10	Blue	394	181	2:1	
SRYAS1-11	Blue	0	466		
SRYAS1-12	Blue-white	299	110	3:1	
SRYAS1-13	Blue				No seeds

Seeds were germinated on MS medium containing 100 mg/l kanamycin for 3 weeks.



**Figure 6.** Flower phenotype in transgenic *Petunia* plants carrying *SRYAS1* transgene. (a) Most transgenic flowers were blue in color, similar to wild-type flowers. (b) A small number of transgenic flowers had white sectors on the edge of a blue petal.



**Figure 7.** Gene expression in  $T_0$  transgenic *SRYAS1* and *SRY4'CGT* plants carrying variegated blue-white flowers. Total RNA was isolated from variegated blue-white flowers in three transgenic *SRYAS1* plants (*SRYAS1*-4, -6, -12) and four transgenic *SRY4'CGT* plants (*SRY4'CGT*-1, -6, -7, -10). “Wild type” represented RNA sample isolated from wild-type *Petunia* flowers (blue color). RT-PCR was conducted for 25 cycles using F1 and R2 for amplification of *SRYAS1* gene (expected size of this fragment was 624 bp), and F2 and R3 primers for amplification of *SRY4'CGT* gene (expected size of this fragment was 390 bp). The rRNA visualized by ethidium bromide staining is indicated as a loading control.

### 3.4. Inheritance in $T_1$ Progeny of Transgenic Plants

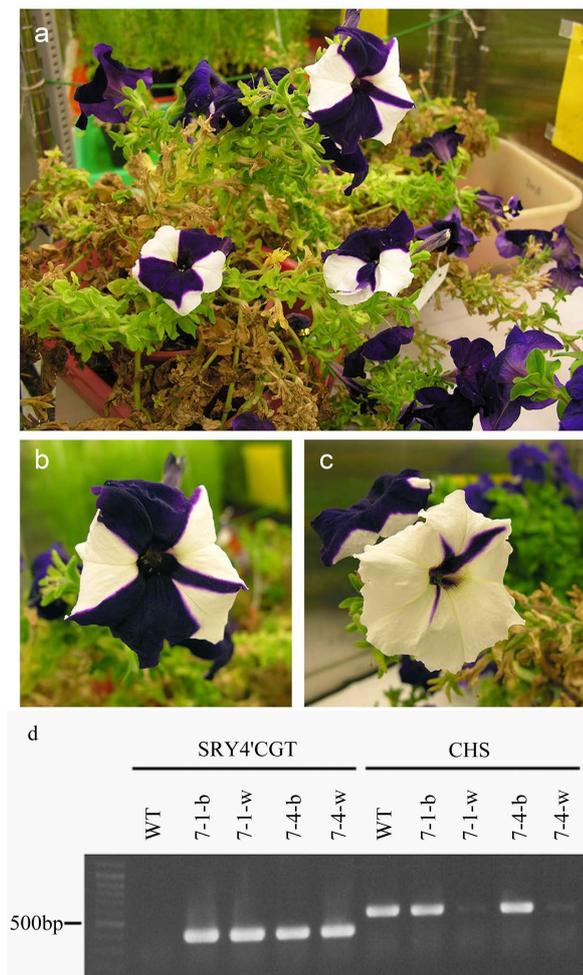
The stable integration of the T-DNA region in two transformation vectors (**Figure 4**) into the petunia chromosomal genome was investigated by germinating  $T_1$  seeds from self-fertilized  $T_0$  transgenic plants on MS medium containing 10 mg/l hygromycin (**Table 1**) or 100 mg/l kanamycin sulfate (**Table 2**). In transgenic plants carrying *SRY4'CGT* transgene (**Table 1**),  $T_1$  seeds were not available from three transformants (*SRY4'CGT*-1, -3, -5). Hygromycin-resistant and hygromycin-sensitive  $T_1$  seedlings segregated in a 3:1 ratio in 2 transformants (*SRY4'CGT*-7 and *SRY4'CGT*-10), indicating the presence of single copy of the hygromycin-resistant transgene in the nuclear genomes of the 2 lines (**Table 1**). Since the transformation vector p*SRY4'CGT* contains expression cassettes for the selection marker gene (hygromycin resistant gene) and snapdragon *SRY4'CGT* transgene within the T-DNA region (**Figure 4(a)**), it is reasonable to predict that the same copy number of the hygromycin resistant transgene and *SRY4'CGT* transgene will be found in each transformant.

All transgenic plants carrying *SRYAS1* transgene are shown in **Table 2**. No seeds were obtained from two transformants, *SRYAS1*-8 and *SRYAS1*-13. All  $T_1$  seedlings from the two transformants *SRYAS1*-7 and *SRYAS1*-11 were sensitive to the selection agent kanamycin, suggesting that these two lines may not contain the foreign T-DNA fragment and can be considered to be false-positive clones. Thus, transformation efficiency in transgenic *SRYAS1* plants was recalculated as 5.4%. Single insertion of the *nptII* transgene was determined in

three transgenic plants SRYAS1-2, SRYAS1-4 and SRYAS1-12, as the segregation ratio of resistant and sensitive seedlings in these 3 transgenic plants was 3:1 (**Table 2**). Similarly, as transformation vector pSRYAS1 contains expression cassettes for the selection marker gene (*nptII*) and snapdragon *SRYAS1* transgene within the T-DNA region (**Figure 4(b)**), it is reasonable to predict the same copy number of the *nptII* transgene and *SRYAS1* transgene will be found in each transformant.

### 3.5. Gene Expression in T<sub>1</sub> Transgenic *SRY4'CGT* Plants

Five T<sub>1</sub> hygromycin-resistant seedlings were randomly selected in hygromycin-containing medium from T<sub>0</sub> selfed seeds of transgenic plant *SRY4'CGT*-7 that showed significant alteration in flower coloration, and grown in a greenhouse until maturity. Instead of no phenotypic change in 3 T<sub>1</sub> transgenic plants, obvious variegated blue-white flowers were still observed in 2 T<sub>1</sub> transgenic plants (**Figure 8**). However, no complete white flowers



**Figure 8.** Flower phenotype and gene expression patterns in T<sub>1</sub> transgenic *SRY4'CGT*-7 plants. (a) Mature plant of T<sub>1</sub> transgenic *SRY4'CGT*-7-T<sub>1</sub>-1. Five hygromycin-resistant T<sub>1</sub> seedlings from transgenic *SRY4'CGT*-7 T<sub>0</sub> plant were randomly-selected and grown until maturation. Among them, 2 T<sub>1</sub> progenies, designed as *SRY4'CGT*-7-T<sub>1</sub>-1 and *SRY4'CGT*-7-T<sub>1</sub>-4, showed variegated blue-white flowers as parental *SRY4'CGT*-7 T<sub>0</sub> plant, whereas no alteration in flower color was observed in the rest of the three T<sub>1</sub> progenies. (b) An enlarged flower in transgenic *SRY4'CGT*-7-T<sub>1</sub>-1 plant. (c) An enlarged flower in transgenic *SRY4'CGT*-7-T<sub>1</sub>-4 plant. (d) Gene expression patterns in T<sub>1</sub> transgenic *SRY4'CGT*-7 flowers. RNA was isolated from blue sectors of T<sub>1</sub> transgenic flowers of *SRY4'CGT*-7-T<sub>1</sub>-1 (abbreviated as 7-1-b) or white sectors of the same plant (abbreviated as 7-1-w), or from blue sectors of T<sub>1</sub> transgenic flowers of *SRY4'CGT*-7-T<sub>1</sub>-4 (abbreviated as 7-4-b) or white sectors of the same plant (abbreviated as 7-4-w). RT-PCR was conducted for 25 cycles using F2 and R3 primers for amplification of *SRY4'CGT* gene (expected size of this fragment was 390 bp), and using C1 and C2 primers for amplification of *CHS* gene (expected size of this fragment was 558 bp).

were found even at the late stage of flowering in any lines. To examine the gene expression in these variegated flowers, white sectors and blue sectors were dissected from variegated flowers in 2 T<sub>1</sub> transgenic lines, RNA was isolated, and RT-PCR was performed using gene-specific primers (**Figure 8(d)**). As predicted, no *SRY4'CGT* transcripts were detected in wild-type *Petunia* flowers. Transgenic *SRY4'CGT* transcripts were detected in both blue and white sectors from these two transgenic lines. When specific primers were employed for an upstream gene chalcone synthase (*CHS*) in the flavonoid/aurone biosynthetic pathway, *CHS* transcripts were only detected in blue sectors but not in white sectors of the transgenic plants examined (**Figure 8(d)**), suggesting that the expression of *CHS* gene was suppressed in the white sectors of transgenic plants.

### 3.6. High-Performance Liquid Chromatography (HPLC) Profile in T<sub>1</sub> Transgenic Flowers

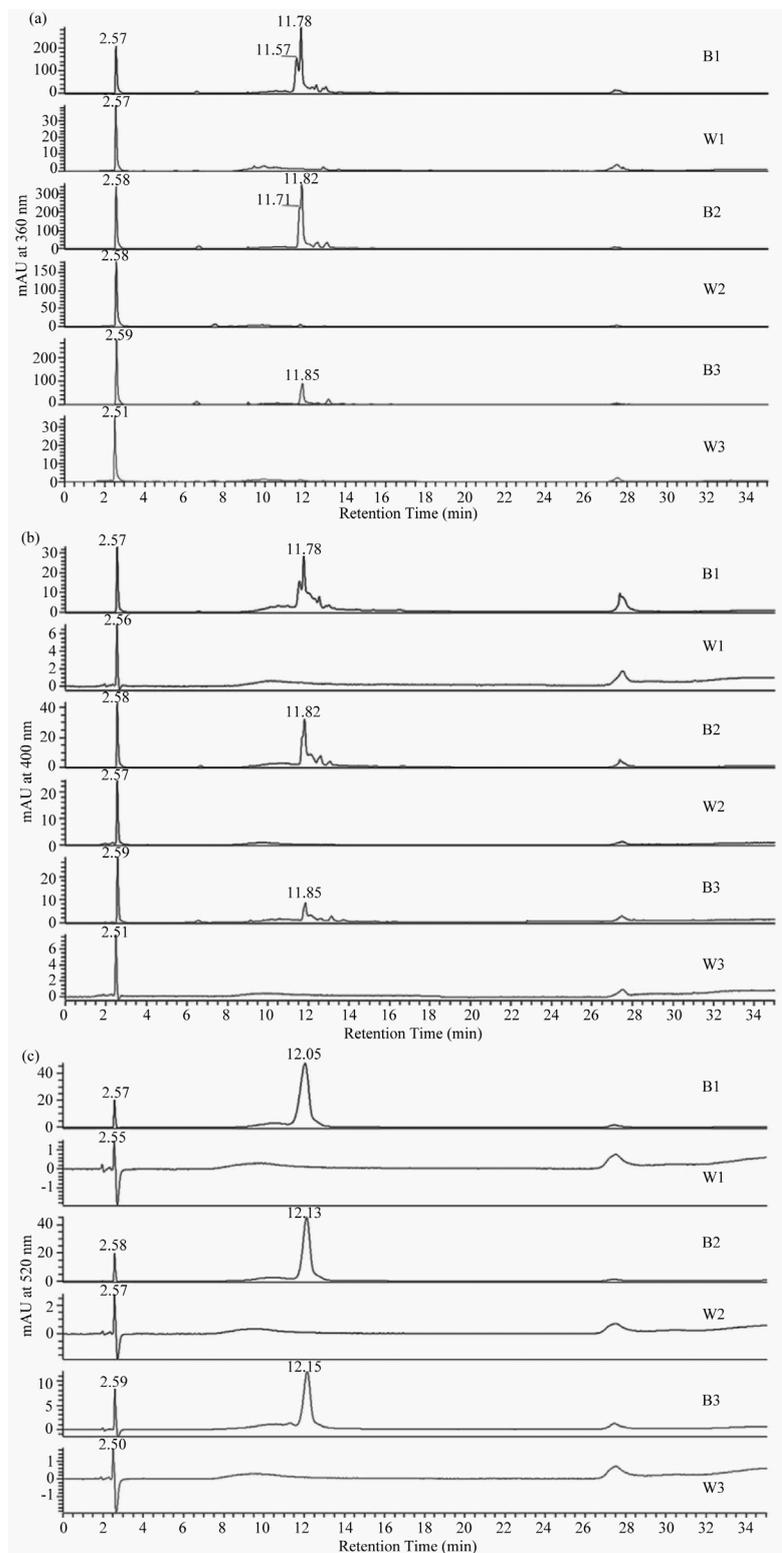
To examine the pigment profile in transgenic flowers, and more importantly, to investigate whether the appearance of white sectors in blue background petals is due to the loss of anthocyanins, white sectors and blue sectors were dissected from variegated flowers in T<sub>1</sub> transgenic *SRY4'CGT* and *SRYAS1* lines, and ground into a powder by liquid nitrogen. After extraction, crude extracts were analyzed by HPLC. For each sample, similar HPLC patterns were obtained when monitoring was performed at 360 nm (for measurement of chalcones, flavones), 400 nm (for measurement of aurones) or 520 nm (for measurement of anthocyanidins). As shown in **Figure 9**, an absorption peak was detected in all samples when retention time (RT) approached 2.51 to 2.59 min. However, two peaks at RT = 11.57 min and RT = 11.78 min were detected in blue sectors from transgenic *SRY4'CGT*-7-T<sub>1</sub>-1 flowers (designed as B1) but not in white sectors from transgenic *SRY4'CGT*-7-T<sub>1</sub>-1 flowers (designed as W1). Again, two peaks at RT = 11.71 min and RT = 11.82 min were detected in blue sectors from transgenic *SRY4'CGT*-7-T<sub>1</sub>-4 flowers (designed as B2) but not in white sectors from transgenic *SRY4'CGT*-7-T<sub>1</sub>-4 flowers (designed as W2). Only one peak at RT = 11.85 min was detected in blue sectors from transgenic *SRYAS1*-3-T<sub>1</sub>-5 flowers (designed as B3) but not in white sectors from transgenic *SRYAS1*-3-T<sub>1</sub>-5 flowers (designed as W3); however, the HPLC pattern in B3 was distinguishable from the HPLC patterns in B1 and B2, which represented two individual T<sub>1</sub> plants from the same T<sub>0</sub> parent *SRY4'CGT*-7 plant. Besides the constitutive peak at RT = 2.51 - 2.59 min in all blue and white samples, almost no significant peaks were detected from white sectors (W1, W2, W3), suggesting that colored pigments may not exist or may accumulate at very low level in these white sectors. HPLC patterns monitored at 360 nm, 400 nm and 520 nm are shown in **Figures 9(a)-(c)**, respectively.

### 3.7. Compound Identification by Tandem Mass Spectrometry (MS/MS)

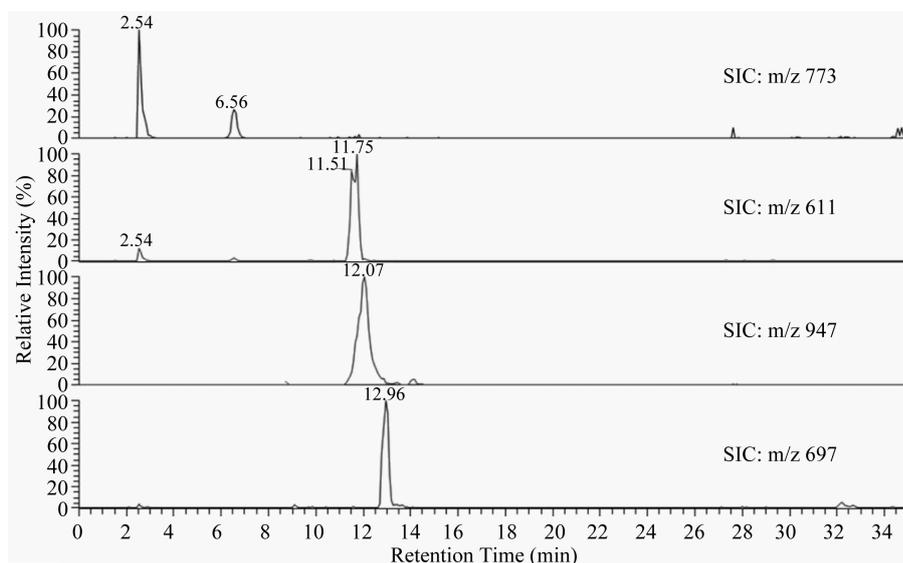
To further identify the major compounds in these peaks, tandem mass spectrometry (MS/MS) was employed to identify fragmentation patterns of different anthocyanins. As shown in **Figure 10** and **Figure 11**, several major peaks obtained from different retention times were chosen and then analyzed. The molecular weight of each fragment was measured as mass-to-charge ratio ( $m/z$ ) in the ionization process, and the most abundant fragment in each RT was considered as 100%. Compound identification, mainly based on molecular ions and product ions, was performed with the aid of existing literatures spectral analysis regarding flavonoids/anthocyanins [19]-[22]. Cyanidin triglucoside was found as major compound in the RT = 2.62 min fraction (**Figure 10**; **Figure 11**). Fragmentation pattern of cyanidin 3,5,5'-triglucoside is shown in **Figure 12(a)**.

Next, major compounds were identified in peaks around RT = 11.51 - 12.96 min (**Figure 10**; **Figure 11**). It is worth noting that these peaks can be detected only in the blue sectors of transgenic flowers but not in white sectors. The red color of cyanidin and its glucoside derivatives (cyanidin glucoside, cyanidin diglucoside, cyanidin malonyldiglucoside), and mauve (pale purple) color of malvidin and its derivative malvidin-3-(*p*-coumaroyl)-rutinoside-5-glucoside were identified in these tissues. Fragmentation patterns of these anthocyanins are shown in **Figure 12(b)**, **Figure 13(a)** and **Figure 13(b)**. These data demonstrated clearly that the major compounds of anthocyanins extracted from the blue sectors of transgenic *SRY4'CGT* and *SRYAS1* flowers are cyanidin, malvidin and their derivatives. In contrast, cyanidin or malvidin was not detected or was accumulated at a very low level in the white sectors of transgenic flowers, as a result, white color was observed.

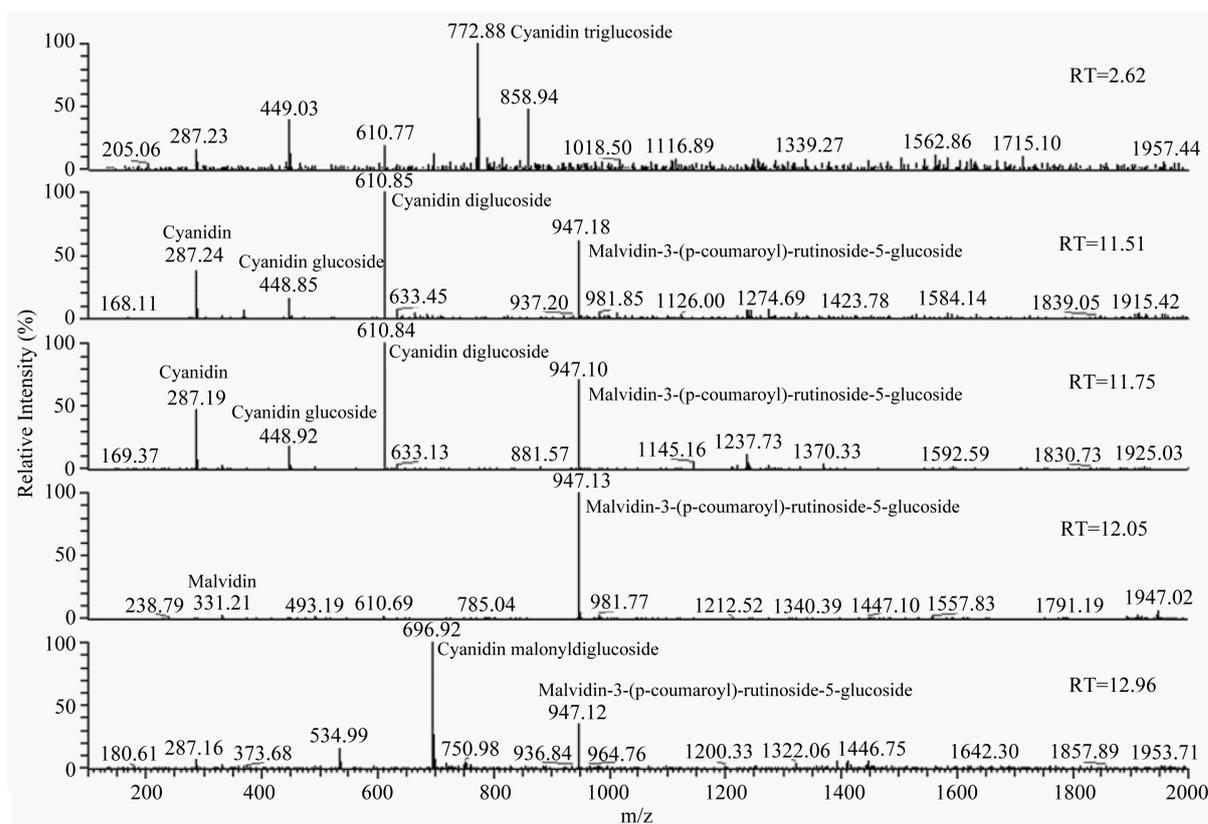
Finally, to evaluate whether aurones (the target yellow pigment) can be detected in our analysis system, yellow petals from snapdragon were excised, and sample preparation and HPLC-DAD-MS/MS were performed as previously described. As shown in **Figure 14(a)**, several peaks including RT = 2.51 min were detected in HPLC analysis. Compound identification in the fraction at RT = 11.25 min revealed that aureusidin glucoside and its



**Figure 9.** HPLC chromatograms of transgenic *Petunia* flowers detected at different absorption spectra. (a) 360 nm; (b) 400 nm; (c) 520 nm. Abbreviations: B1, Blue sectors from transgenic SRY4'CGT-7-T<sub>1</sub>-1 flowers; W1, White sectors from transgenic SRY4'CGT-7-T<sub>1</sub>-1 flowers; B2, Blue sectors from transgenic SRY4'CGT-7-T<sub>1</sub>-4 flowers; W2, white sectors from transgenic SRY4'CGT-7-T<sub>1</sub>-4 flowers; B3, Blue sectors from transgenic SRYAS1-3-T<sub>1</sub>-5 flowers; W3, white sectors transgenic SRYAS1-3-T<sub>1</sub>-5 flowers.

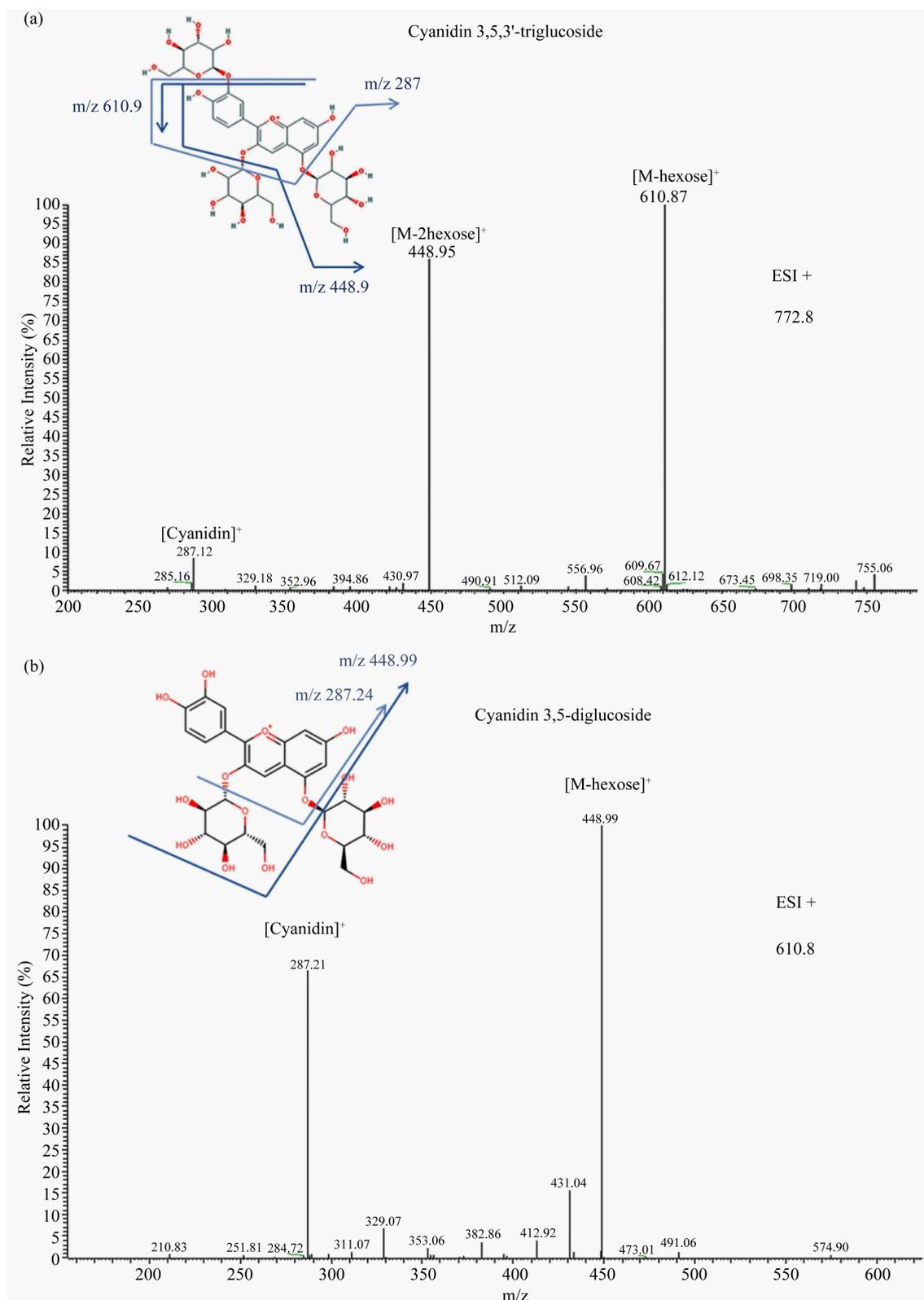


**Figure 10.** Selected ion chromatograms (SIC) of Electrospray Ionization (ESI) mass spectra detecting precursor ions from cyanidin 3,5,3'-triglucoside ( $m/z$  773), cyanidin 3,5-diglucoside ( $m/z$  611), malvidin-3-(*p*-coumaroyl)-rutinoside-5-glucoside ( $m/z$  947), and cyanidin malonyl-diglucoside ( $m/z$  697).

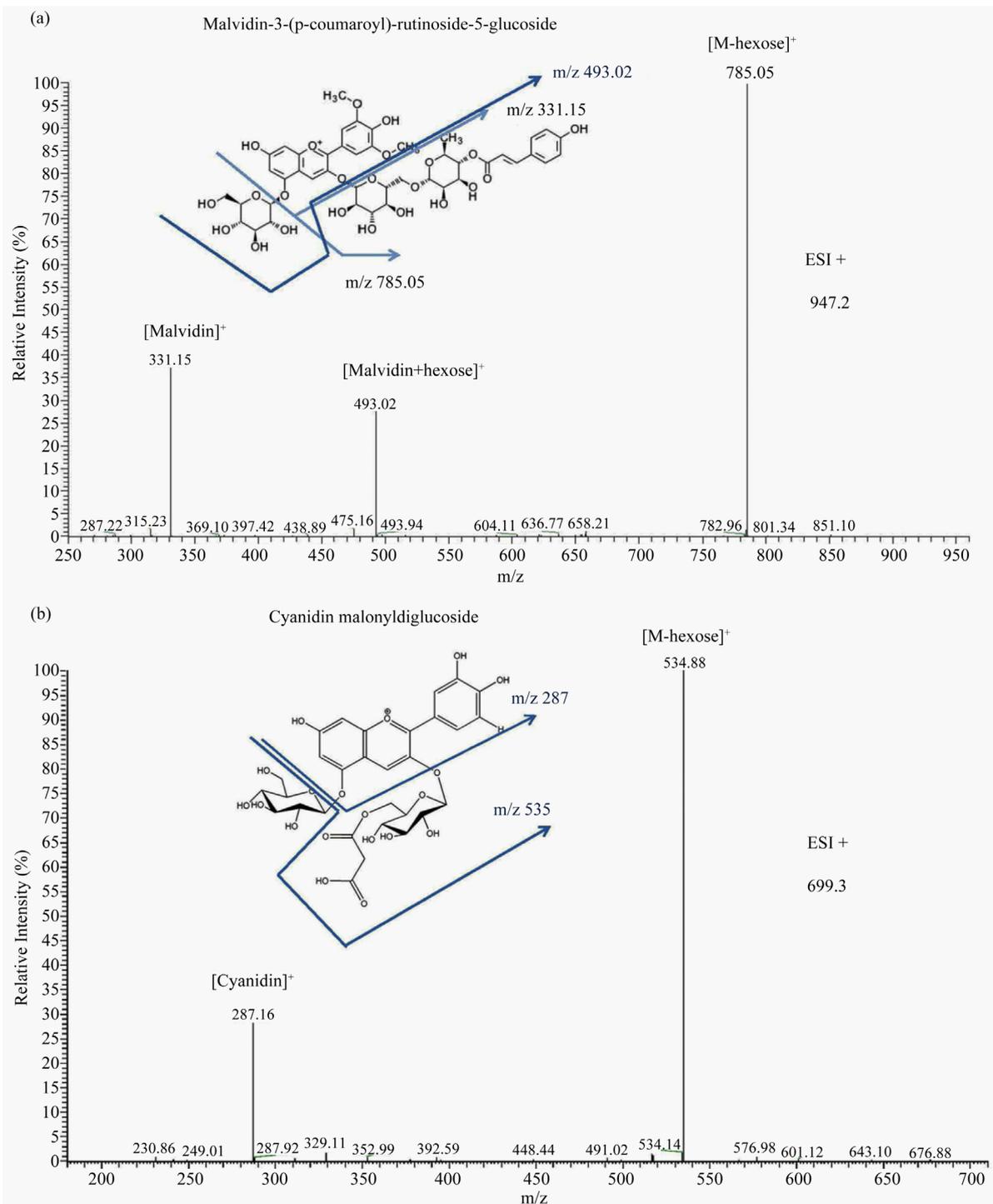


**Figure 11.** Mass spectra at different retention times.

dimer are the major compounds in that fraction (**Figure 14(b)**). It is worth pointing out that aureusidin is a yellow pigment belonging to the aurone, and aureusidin 6-*O*-glucoside (yellow pigment) occurs in the yellow petals of snapdragon. In addition, aureusidin 6-*O*-glucoside is the product of aureusidin synthase, one of our target

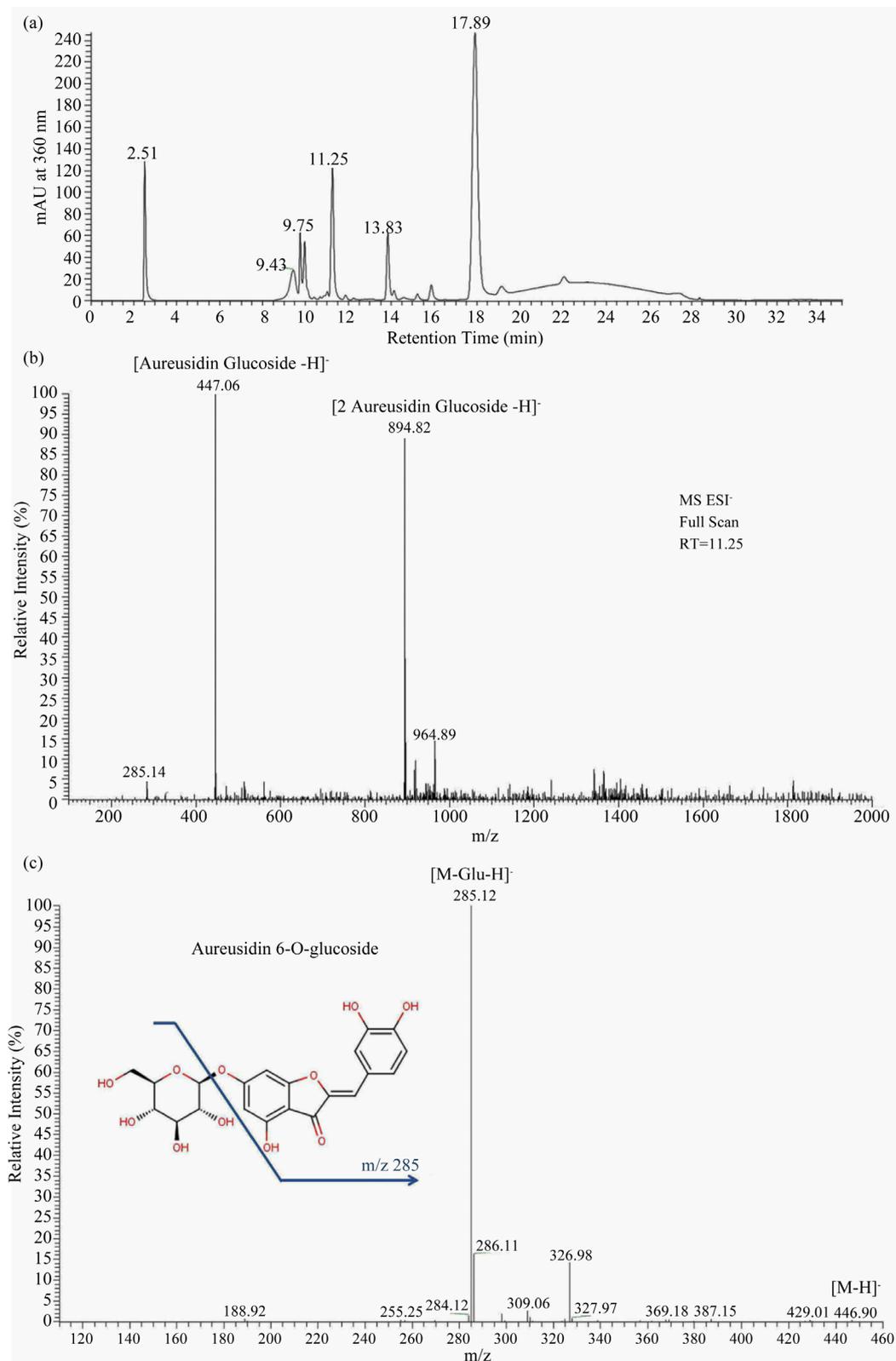


**Figure 12.** (a) MS/MS production ion spectrum of  $m/z$  772.8 ion observed in the MS spectrum for cyanidin 3,5,3'-triglucoside. (b) MS/MS production ion spectrum of  $m/z$  610.8 ion observed in the MS spectrum for cyanidin 3,5-diglucoside.



**Figure 13.** (a) MS/MS production ion spectrum of  $m/z$  947.2 ion observed in the MS spectrum for malvidin-3-(*p*-coumaroyl)-rutinoside-5-glucoside. (b) MS/MS production ion spectrum of  $m/z$  699.3 ion observed in the MS spectrum for cyanidin malonyldiglucoside.

genes (*SRYAS1*) in this study. The fragmentation pattern aureusidin glucoside is shown in **Figure 14(c)**. In conclusion, yellow pigment of aureusidin glucoside can be detected in our analysis system. However, aureusidin or aureusidin glucoside was not found in our transgenic *Petunia* flowers. In addition, genistin 7-*O*-glucuronide (an isoflavone derivative) was identified as a major compound in fraction at RT = 17.89 min (data not shown).



**Figure 14.** (a) HPLC chromatogram of yellow flowers from snapdragon detected at 360 nm; (b) Aureusidin glycoside and its dimer are the major compounds in the fraction at RT = 11.25 min. (c) MS/MS product ion spectrum of  $m/z$  446.9 ion observed in the MS spectrum for aureusidin 6-*O*-glucoside.

HPLC coupled with tandem mass spectrometry was employed to analyze the blue and white sectors from variegated flowers of transgenic *SRY4'CGT* and *SRYAS1* plants, and the results clearly demonstrated that cyanidin, malvidin, and their derivatives are present in the blue sectors, but none of anthocyanins are present in white sectors, causing the white phenotype.

#### 4. Discussion

Flavonoids/anthocyanins are major contributors to flower coloration. As a result, most studies on genetic engineering of flower color have been carried out via the flavonoid biosynthetic pathway [3] [6] [7] [23] [24]. Introduction of an additional *CHS* gene into different varieties of *Petunia hybrida* resulted in variegated flowers with white sectors in some transgenic lines and complete white flowers in other transgenic lines [25] [26]. Molecular dissection of these transformants showed that both the introduced and endogenous *CHS* genes were “turned down” or silenced to different degrees, and as a result, loss of pigmentation in transgenic petals was observed [25] [26]. *CHS* is a key enzyme catalyzing one molecule of 4-coumaroyl-CoA and three molecules of malonyl-CoA into one molecule of naringenin chalcone, which is the central intermediate in the biosynthesis of different classes of flavonoids including aurones. In this study, two key genes of snapdragon (*SRY4'CGT* and *SRYAS1*) in the aurone biosynthetic pathway were cloned, and transformed separately into *P. hybrida* by an *Agrobacterium*-mediated method. Some transformants carrying the *SRY4'CGT* or *SRYAS1* transgene showed variegated blue-white flowers (Table 1; Table 2), which were similar to the previous transformants carrying the *CHS* transgene [25] [26]. A dramatic change in flower color from almost complete blue to variegated blue-white sectors and then complete white in certain transformants carrying *SRY4'CGT* transgene during plant senescence was also observed (Figure 5). This pattern of alteration in flower coloration has not been previously reported.

Transgene-induced gene silencing is a quite common phenomenon in plants, for example, host genes can be silenced as a consequence of the presence of a homologous transgene [27] [28]. However, in this study, no sequence similarity was found between the host *CHS* gene and the *SRY4'CGT* transgene (or between the host *CHS* gene and the *SRYAS1* transgene). It is interesting to find that expression of *SRY4'CGT* transgene can also suppress the expression of the non-homologous host *CHS* gene in the white sectors (Figure 8(d)). On the other hand, suppression of endogenous *CHS* expression is related to the transgene-induced gene silencing, which is considered to be sequence homology-dependent event [27] [28]. For example, an expression vector p*CHS* carrying *Petunia CHS* transgene was transformed into tobacco, nucleotide sequence analysis revealed that both *Petunia* and tobacco *CHS* cDNAs contained 1170 bp in length and sequence identity were 91%; as a result, four out of seven transgenic plants produced completely white flowers as compared to the pink flowers in wild-type tobacco, and it was further demonstrated that cosuppression of *CHS* expression resulted the loss of cyanidin pigments in those transgenic white flowers [12]. It has been reported that overexpression of apple anthocyanidin reductase (*ANR*) genes in tobacco caused inhibition of expression of both chalcone isomerase (*CHI*) and *DFR* genes in transgenic flowers, leading to loss of anthocyanin and resulting in white, pale pink and white/red variegated flowers as compared to wild-type red flowers [29]. Since low sequence identity between apple *ANS* gene and tobacco *DFR* gene, the authors concluded that cosuppression of tobacco *DFR* gene might not be related to RNA silencing, and might be due to competition or interaction among different enzymes involved in the flavonoid biosynthetic pathway [29]. Therefore, our transgenic plants should be good material for studying gene expression and regulation in the aurone/flavonoid biosynthetic pathway.

In T<sub>1</sub> progeny assay, a number of abnormal segregation ratios (*i.e.*, R:S = 1:1 or R:S = 2:1) or no seed production was observed (Table 1; Table 2). We noticed that pollen was extremely rare in transgenic *SRY4'CGT*-1 and *SRYAS1*-13 plants; this may explain why no seeds were produced in these plants (Table 1; Table 2) (Figure 15). In brief, some of the transgenic plants we obtained in this study seem abnormal. The genus *Petunia*, belonging to the family *Solanaceae*, comprises more than 30 species. Most petunias are diploid ( $2n = 14$ ) and are interfertile with different petunia species. The origin of *P. hybrida* is unknown, but there is general agreement that it was derived from interspecific crossing between a white-flowered species (probably *P. axillaris*) and a pigmented species (probably *P. integrifolia*) near 190 years ago [30]. The complicated genetic background of *P. hybrid* commercial varieties may cause those abnormal phenotypes we observed in this study.

From the point of view of biotechnology application, as the loss of the newly acquired traits in subsequent generations or under certain environmental conditions has been reported elsewhere [6] [31] [32], the next important task is to ensure the reliability and stability of phenotypes reported here after several generations of



**Figure 15.** Abnormal flower phenotype showing an extra petal-like structure in two anthers out of five anthers in some transgenic *Petunia* flowers. (a) Dissection of an abnormal flower. Arrows indicated an extra petal-like structure in two out of five anthers. The fifth anther harboring normal phenotype is not shown in this panel. (b) An enlarged photo of one abnormal anther. The extra petal-like structure appear at the top of the anther. (c) and (d) The abnormal petal-like structure is still presented and even the pollen sac is broken at the late stage of flower development.

propagation. Furthermore, it is very important to breed transgenic petunia plant carrying both *SRYAS1* and *SRY4'*-*CGT* by crossing experiment, which is in progress. In addition, it will be very interesting to examine whether the same phenotypes can be achieved by transforming p*SRYAS1* or p*SRY4'*CGT vector into other floricultural plants, such as *Cleome spinosa*. *Agrobacterium*-mediated transformation has been established in this value plant [33].

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