Original Paper

Flower color modification of *Petunia hybrida* commercial varieties by metabolic engineering

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Abstract   Petunia flower colors are mainly due to flavonoids. The flower color of commercial varieties of *Petunia hybrida* was successfully modified by the suppression of endogenous flavonoid biosynthetic genes, the expression of a hetelorogous flavonoid biosynthetic gene, and the combination of both. Flower color changed from purple to almost white or from purple to red by the suppression of the endogenous gene expression, from red to orange by the down-regulation of the flavonoid 3′-hydroxylase gene and the expression of the rose dihydroflavonol 4-reductase gene, and from violet to pale violet by the expression of the flavonol synthase or flavone synthase gene. These results clearly indicate the usefulness of metabolic engineering of the flavonoid biosynthetic pathway to modify flower color. Only a few of the transgenic petunia exhibited phenotypic stability. For commercialisation, it is necessary to generate many independent transgenic lines, select elite lines with stable phenotypes and maintain them in tissue culture.

Key words: Anthocyanin, flavonoid, *Petunia hybrida*.

Flower color is mainly derived from anthocyanin, a colored class of flavonoids. They are usually localized in the vacuoles of petal epidermal cells (Goto and Kondo 1991; Tanaka et al. 2004). The structures of anthocyanin, in particular, the number of the hydroxyl groups on the B-ring and the modification of anthocyanins with aromatic acyl groups, affect their color. Anthocyanins become bluer when the number of hydroxyl groups on the B-ring and attached aromatic acyl groups increase. The presence of copigments, such as flavonols and flavones, contributes to a bathochromic shift of anthocyanins and, thus, flower color (Goto 1987). The structural genes encoding the enzymes involved in the hydroxylation, glycosylation, and acylation reactions have been obtained (Tanaka et al. 2004). The genes encoding flavonol and flavone synthases have also been cloned as reviewed (Tanaka et al. 2004). Vacuolar pH

and coexisting metal ions are also important in the determination of flower color, but their manipulation is still not feasible because vacuolar pH regulation and metal uptake are not well understood in terms of their biochemistry and molecular biology. The value of metabolic engineering to modify flower color has been reviewed (Tanaka et al. 1998; Mol et al. 1999; Forkmann and Martern 2001; Tanaka et al. 2004).

Petunia flower colors, except yellow, are derived from flavonoid and anthocyanins. Petunia is a good model for the study of flavonoid/anthocyanin biosynthesis because of its genetics and molecular biology (Holton and Cornish 1995). Many of the genes involved in the pathway have been cloned from petunia for the first time, such as, for example, flavonoid 3′,5′-hydroxylase (F3′5′H, Holton et al. 1993a), flavonol synthase (FLS, Holton et al. 1993b), anthocyanidin 3-glucoside

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rhamnosyltransferase (3RT, Brugliera et al. 1994), and flavonoid 3′-hydroxylase (F3′H, Brugliera et al. 1999). Petunia has two F3′5′H loci, and the *Hf1* locus plays major role (Holton et al. 1993a). *Hf1* and *Hf2* encode very similar F3′5′H in terms of their amino acid and DNA sequences (about 95% identity, Holton et al. 1993a). Wild-type petunia flowers contain acylated delphinidin-type anthocyanins and flavonols (Figure 1), and their color is reddish purple (low pH) or violet (high pH). The flower color becomes redder when the flowers produce cyanidin-type anthocyanins and/or non-acylated anthocyanins.

Petunia DFR cannot reduce dihydrokaempferol, which explains the lack of pelargonidin-type anthocyanins and, thus, the brick red orange flower color in petunia (Forkmann and Ruhau 1987). The pelargonidin-accumulating brick red/orange petunia was generated by expression of a maize *DFR* gene in a petunia that is deficient in F3′5′H, F3′H and FLS activities and

accumulates dihydrokaempferol (Meyer et al. 1987). The pelargonidin-accumulating brick-red/orange petunia was obtained by expression of a gerbera and a rose *DFR* gene (Helariutta et al. 1993; Tanaka et al. 1995).

Petunia hybrida is one of the most popular potted-plant species. Cascade/creeping types of *P. hybrida* derived from a wild species have been commanding high market values for last 15 years. Although petunia flower color was modified by genetic transformation, the results have been mainly obtained by over- or down-expression of a single gene in experimental petunia lines whose genotypes are characterized. In this study, we aimed to modify flower color of the commercial petunias including creeping/cascade varieties by genetic engineering focusing on commercial values of phenotypes. The changes are the result of: 1. Gene suppression; 2. Suppression of an endogenous gene and expression of a heterologous gene; and 3. Modification of copigments. The results should be also significant to engineer plant secondary metabolic pathway.

Materials and methods

Plant materials

The *P. hybrida* cultivar Surfinia Purple, Surfinia Purple Mini, Surfinia Hot Pink, Surfinia Violet Mini, Surfinia Violet (Suntory Flowers Ltd., Japan), and Baccarat Red (Sakata Seed Co., Japan) are grown in contained glasshouses in Osaka, Japan. Surfinias have cascade/creeping characters. These petunia cultivars were maintained *in vitro* and subject to *Agrobacterium* (Lazo et al. 1991) mediated transformation as described previously (Fukuchi-Mizutani et al. 2003). The transgenic petunia plants were also grown in the glasshouse. The lines exhibiting stable phenotypes were selected, from which tissue culture plants were established. Tissue culture plants were vegitatively maintained on MS hormone free media (Murashige and Skoog 1962) containing 3% sucrose and 0.5% Agargel (Sigma) at 15°C and renewed every 3 months.

Vector construction

The cDNAs encoding petunia F3′,5′H (Holton et al. 1993a), FLS (Holton et al. 1993b), F3′H (Brugliera et al. 1999), anthocyanidin 3-glucoside rhamnosyltransferase (3RT, Brugliera et al. 1994), anthocyanidin 3-rutinoside acyltransferase (AR–AT, Brugliera and Koes, unpublished results), and flavanone 3-hydroxylase (F3H, unpublished results) were kindly provided by Florigene Ltd. Rose *DFR* and *FLS* cDNAs and torenia flavone synthase (*FNS*) cDNA were described previously (Tanaka et al. 1995; Tanaka et al. 2003; Akashi et al. 1999, respectively).

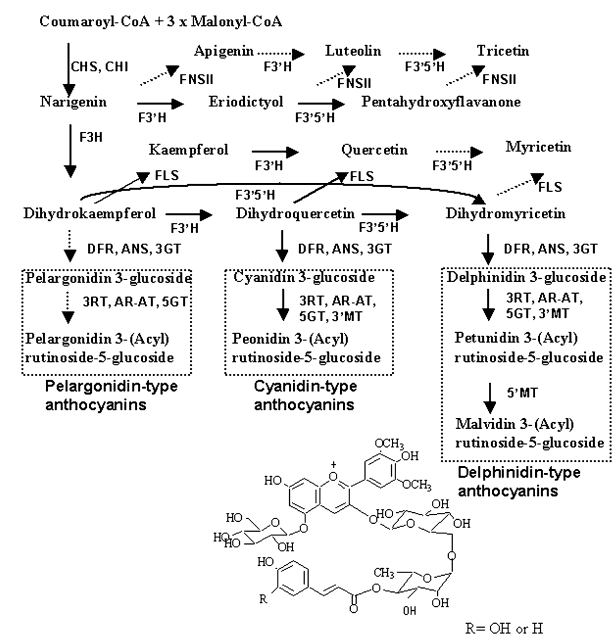Binary vectors for color modification were constructed as reported previously (Fukuchi-Mizutani et al. 2003). In

Figure 1. Simplified flavonoid biosynthetic pathway of Petunia hybrida. (Holton and Cornish 1995; Ando et al. 2004). Modification by 3RT and AR–AT does not change the anthocyanidin class but allows the modification pathway to synthesize methylated anthocyanidins (malvidin, petunidin and peonidin) in petunia. Modification by aromatic alyl group (caffeic acid or coumaric acid) and methyl groups give the flower colour to bluer hue (Ando et al. 2004). The structure shown in the figure is malvidin 3-(coumaroyl or caffeoyl) rutinoside-5-glucoside that is a major anthocyanin in petunia. Reactions that petunia does not have are shown by dotted arrows. Pelargonidin and myricetin are not synthesized due to the substrate specificities of DFR and FLS, respectively. Petunia does not have FNS. CHS; chalcone synthase, CHI; chalcone isomerase, F3H; flavanone 3-hydroxylase, F3′H; flavonoid 3′-hydroxylase, F3′5′H; flavonoid 3′5′-hydroxylase, FLS; flavonol synthase, FNS; flavone synthase, DFR; dihydroflavonol 4-reductase, ANS; anthocyanidin synthase, 3GT; anthocyanidin 3-glucosyl-transferase, 3RT; anthocyanidin 3-glucoside rhamnosyltransferase, AR–AT; anthocyanidin 3-rutinoside acyltransferase, MT; anthocyanin methyltransferase.

principal, functional full length cDNAs were inserted between constitutive promoters and terminators in sense or antisense orientation (Figure 2). Binary vectors containing an enhanced cauliflower mosaic virus (CaMV) 35S promoter were derived from Mitsuhara et al. (1996). The *Mac1* promoter was from Comai et al. (1990). Binary vector backbones were pBin19 or pBinPlus having neomycine phosphotransferase II (*nptII*) gene as the selectable marker (van Engelen et al. 1995).

Flavonoid and molecular analysis

Experimental procedures for quantification of flavonoids were previously described in detail (Fukui et al. 1998; Murakami et al. 2004). In short, flavonoids were extracted from 500 mg (fresh weight) of petals. Anthocyanins were hydrolyzed with HCl and quantified with high-pressure liquid chromatography (HPLC, LC-10ADvp, Shimadzu Co., Kyoto, Japan) using an ODS-A312 column (YMC Co., Kyoto, Japan) monitored with a photodiode array (A400–600 nm, SPD M10A, Shimadzu Co.). For other flavonoids (flavonols, flavones and dihydroflavonols), they were hydrolyzed with β-glucosidase (Shin Nihon Chemical Co., Anjo, Japan) and nariginase (Sigma-Aldrich Co.) and quantified with an HPLC system (Model 305, Gilson Inc.) using a Develosil G30-UG5 column (Nomura Chemicals Co., Seto, Japan) monitored with the photodiode array (A250–400).

Molecular analysis of transgenic plants such as Southern and Northern analysis was also previously described (Fukuchi-Mizutani et al. 2003). Quantitative
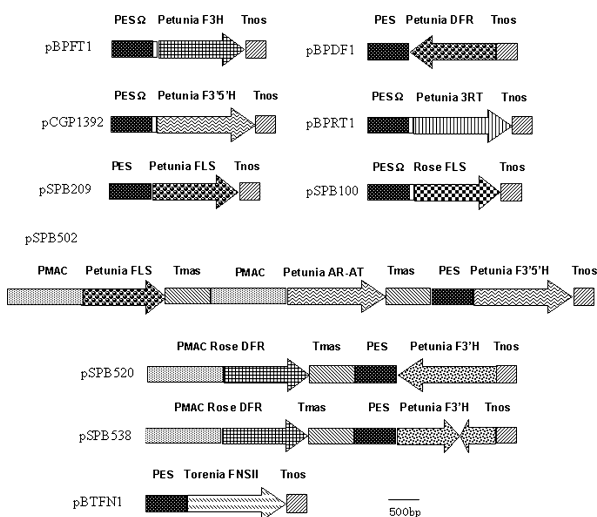

Figure 2. Schematic representation of the expression cassettes in T-DNA of binary vectors constructed for color modification. Directions of + strand of cDNA were shown by arrows. Both Mac 1 (Pmac, Comai et al. 1990) and enhanced CaMV 35S (PES, Mitsuhara et al. 1996) promoters are constitutive. *Ω* sequence is a translational enhancer derived from the binary vector (Mitsuhara et al. 1996). All of them have *npt II* gene as the selectable marker for plant transformation.

Pmac: Mac 1 promoter, PES: an enhanced CaMV 35S promoter, Tmas: manopine synthase terminator, Tnos: nopaline synthase terminator.

RT-PCR analysis was carried out using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems) and Super Script First-Strand Synthesis System for RT-PCR (Invitrogen) following the manufacturer's protocols.

Results and discussion

Down-regulation of endogenous gene toward acyanic petunias

Consumers usually appreciate pure while varieties. Although a pure white petunia was obtained by antisense suppression of *CHS-A* from *P. hybrida* Surfinia Purple (Figure 3B, Tanaka et al. 1998), it was not as vigorous as the host and was prone to insect attack. Most likely, the complete loss of flavonoids resulted in less protection to various kinds of stress. The white color became unstable after a few years of cultivation in the greenhouse (Figure 3C). *F3H* and *DFR* genes were alternate targets of gene suppression to obtain white or pale shades of flower color, and the binary vector pBPFT1 (sense suppression of *F3H* gene) and pBPDF1 (antisense suppression of *DFR* gene) were constructed (Figure 2) and subjected to the transformation of the Surfinia Purple Mini. Forty-one of 65 and 63 of 165 transgenic plants exhibited modified flower color.

F3H and *DFR* gene suppression in this study yielded star-shaped phenotypes (Figure 3D and E). Although complete suppression of *F3H* and *DFR* should yielded acyanic flowers, only a very pale flower (Figure 3E) was obtained. *DFR* gene suppression also yielded a star shape with spots (Figure 3E). Antisense and sense suppression of the chalcone synthase-A (*CHS-A*) gene in petunia yielded white and variegated flower color (van der Krol et al. 1988; Napoli et al. 1990). Phenotypic varieties may depend on the gene of target of suppression and petunia host varieties.

Down-regulation of endogenous genes toward red

In order to convert delphinidin-type anthocyanins to cyanidin-type anthocyanins, *F3'5'H* gene expression was down-regulated using a binary vector, pCGP1392 (sense suppression of the *F3'5'H* gene encoded by *Hf1* locus), in Surfinia Purple. Thirteen of 74 transgenic plants showed altered flower color that closely resembled the flowers of Surfinia Hot Pink (Figure 4A). Cyanidin-type anthocyanins dominated in these transgenic plants, indicating that the *F3'5'H* gene was down-regulated. The flavonoid data for one of the transgenic lines, PT13-4, is shown in Table 1. The flower color resembled to that of Surfinia Hot Pink that is a sport of Surfinia Purple. Surfinia Hot Pink had peonidin as the major anthocyanidin and seems to have a mutation at the *Hf1* locus (Table 1).

Rhamnosylaion of delphinidin 3-glucoside itself does

not alter the color much. However, the rhamnosylaion allows further acylation and methylation (Figure 1) and thus alter flower color (Ando et al. 2004). *3RT* gene expression was suppressed using pBPRT1 (sense suppression of the *3RT* gene) in Surfinia Purple. Six of 69 transgenic plants exhibited pink flowers (Figure 4B and C). One of the lines, PT14-10, was subjected to anthocyanin analysis. While acylated anthocyanins occupied 99% in total anthocyanins in Surfinia Purple flowers, they occupied only 23% in PT14-10 flowers. PT14-10 flowers contained delphinidin 3-glucoside (45% of total anthocyanins), which the host did not contain. The amount of delphinidin also increased in PT14-10 (Table 1). These results indicated that the *3RT* gene was successfully down-regulated. Curiously, the flowers were puckered or crumpled as observed previously in the non-transgenic petunias that are deficient in the *3RT* gene and thus accumulate delphinidin 3-glucoside (Brugliera 1994; Ando et al. 2004). It is interesting that the similar morphological phenotype was reproduced by genetic engineering in this study. Ando et al. concluded that the accumulation of delphinidin 3-glucoside in flowers is associated the inferior floral traits and thus breeders have removed accumulation of delphinidin 3-glucoside in commercial petunias (Ando et al. 2004). Similar changes in flower color and anthocyanin composition were reported for transgenic petunia harboring antisense *3RT* gene (Brugliera et al. 1994), which indicates that down-regulation of the target gene can be achieved with either sense or antisense suppression.

Intense red petunia flowers, such as the cultivar Baccarat Red, accumulate large amounts of cyanidin (Table 1). The binary vector pSPB502 (Figure 2) was constructed to redirect metabolic pathway of Surfinia Purple Mini from malvidin to cyanidin by suppressing three genes (*Hf1 F3′5′H*, *AR–AT* and *FLS* genes) simultaneously. Fifty-five of 109 transgenic plants showed various degrees of change in flower color, and four lines exhibited an appealing red flower color (Figure 4D and E). The flavonoid analysis of the most stable line, PT84-73 (Figure 4D and E), is shown in Table 1. The results of the flavonoid analysis of PT84-73 indicated that the all three gene expressions had been successfully down-regulated because the amount of cyanidin and peonidin increased (due to suppression of *F3′5′H* genes), the ratio of (malvidin and petunidin)/delphinidin decreased (due to suppression of *AR–AT* gene, Figure 1), and the amount of flavonols decreased (due to suppression of *FLS* gene). The accumulation of dihydroquercetin implies that the metabolic flux toward anthocyanidin and/or flavonols was not efficient.

Quantitative RT-PCR revealed that *F3′5′H* (both *Hf1* and *Hf2* genes) and *AR–AT* transcript had decreased to 1–4% of those of the host. No apparent decrease in the *FLS* transcripts was observed while the amount of

flavonols decreased. We speculate that *FLS* transcripts might be down-regulated in petal epidermal cells where flavonoids accumulated while there were the transcripts in the other cells in the petals.

Some plants obtained from the same experiments showed unstable phenotypes and one plant generated various phenotypic flowers (Figure 4F).

The flower color of PT84-73 was generally stable for four years in the glasshouse but the color eventually lost its stability (Figure 4G). Tissue culture plants of PT84-73 were established when the line was selected, and they were maintained *in vitro* during the same period. When they were transferred to soil after four years, the original flower color of PT84-73 was obtained (Figure 4H).

Surfinia Purple was transformed with the same binary vector, pSPB502. Seventeen of 38 had altered phenotype (PT103-26, Figure 4I). The amount of cyanidin and peonidin increased, delphinidin derivatives decreased, and the ratio of methylated anthocyanidin decreased, which indicates that *F3′5′H* and AR–AT gene expression had been down-regulated. The results were confirmed by quantitative RT-PCR analysis: both *Hf1* and *Hf2* genes were down-regulated to about one tenth, and AR–AT genes were to less than one tenth in PT103-26 petals. Although the amount of *FLS* transcripts was down-regulated by about half, the amount of flavonols increased, which indicates that the suppression of the *FLS* gene was not achieved. Blockage of the *F3′5′H* gene might provide more substrate for *FLS* to result in more flavonols in PT103-26 petals. The flower color destabilized after three years in the greenhouse (Figure 4J), but a plant that was revived from the maintained tissue culture gave the same color as PT103-26 (Figure 4K).

These results suggest that the phenotypes of the transgenic plants are not always stable but those maintained in tissue culture maintain their altered phenotypes. In other words, transgenic plants should be maintained in tissue culture so that they can keep their modified characteristics. Since these Surfinia petunias are vegitatively propagated from tissue culture plants every year for commercial propagation, maintaining tissue culture plants should be able to supply the altered color plants. It would be also interesting to grow these transgenic petunias in field to examine their phenotypic stability. The transgenic petunias expressing maize *DFR* lost their original orange flower color in a field test (Meyer and Heidman 1994).

Generation of orange petunia

Although orange petunias have been made by expressing maize, gerbera and rose *DFR* genes in petunia accumulating dihydrokaempferol, when the rose *DFR* gene was expressed in a petunia that was dominant in the *F3′5′H* or *F3′H* gene, pelargonidin was not synthesized
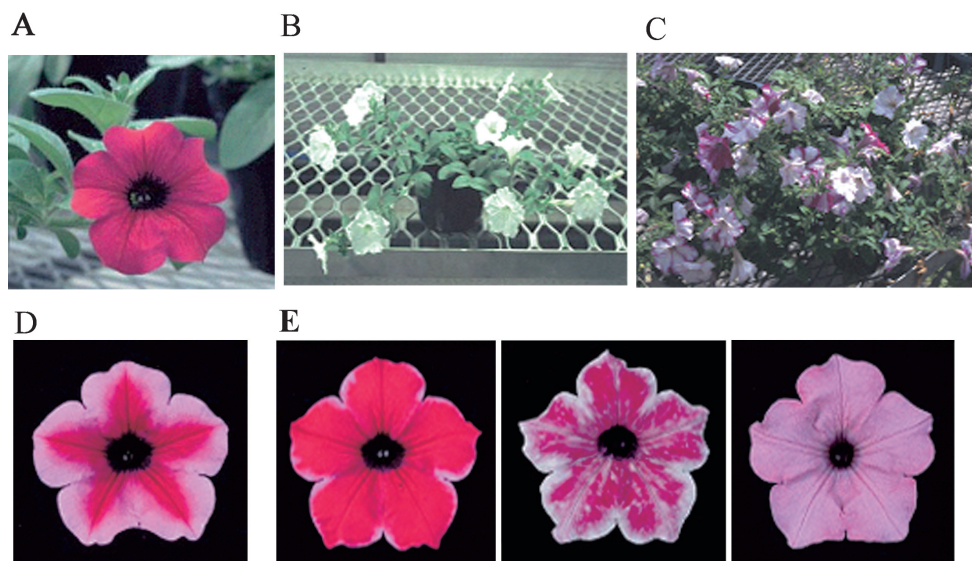
Figure 3. Phenotypes generated by gene suppression. (A) A *Petunia hybrida* Surfinia Purple Mini flower, (B) A transgenic Surfinia Purple Mini plant harboring the antisense *CHS-A* gene (Tanaka et al. 1998). The flowers are pure white, (C) After a few years in the contained glasshouse, the flower color became unstable and showed variegated patterns, (D) A flower of a transgenic Surfinia Purple Mini plant harboring the sense F3H gene (pBPFT1), (E) Flower of transgenic Surfinia Purple Mini plant harboring antisense DFR gene plants (pBPDF1). They showed various phenotypes. Some plants occasionally had different phenotypes depending on the flowers.
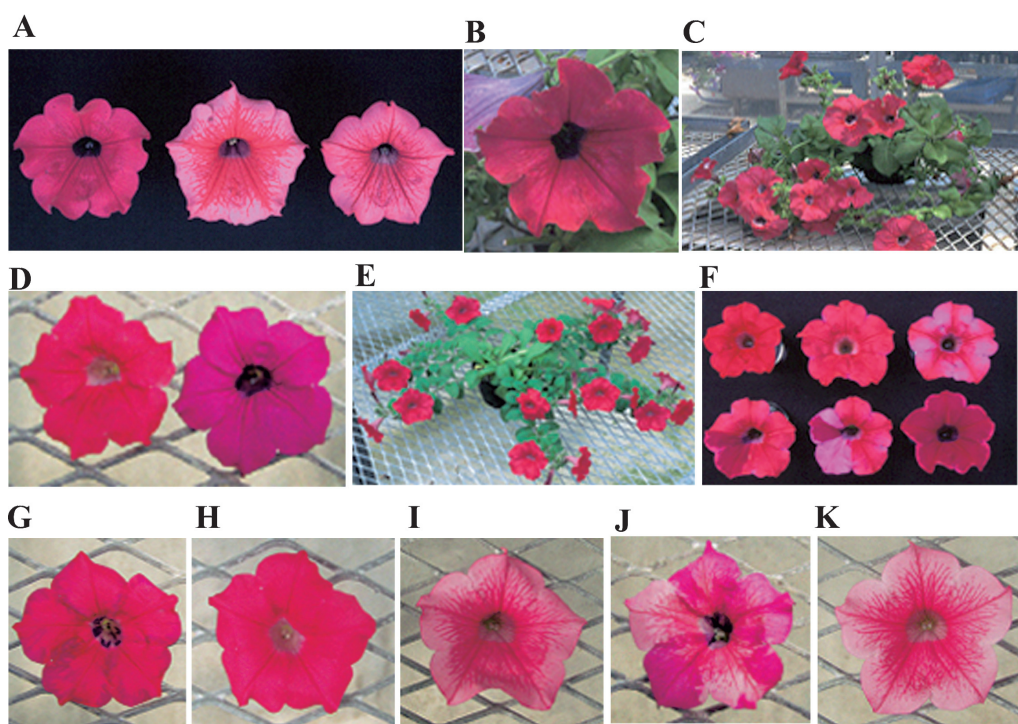

Figure 4. Down-regulation of endogenous genes toward red. (A) Flowers of Surfinia Purple (right), a transgenic Surfinia Purple plant (PT13-4) harboring the sense *Hf1 F3′5′H* gene (pCGP1392, middle), and Surfinia Hot Pink, (B) A flower of a transgenic Surfinia Purple plant (PT14-10) harboring the sense *3RT* gene, (C) A PT14-10 plant, The plant maintains creeping morphology. (D) Flowers of a transgenic Surfinia Purple Mini (PT84-73, left) harboring the sense *Hf1 F3′5′H*, *AR–AT*, and *FLS* genes (pSPB502) and Surfinia Purple Mini (right), (E) A PT84-73 plant, The plant maintains creeping morphology. (F) Various flowers from one transgenic Surfinia Purple Mini plant pSBP502. One plant sometimes had flowers with various phenotypes, (G) A flower of PT84-73 that was maintained in the glasshouse for four years. The flower lost phenotypic stability and partly regained the flower colour of the host, Surfinia Purple Mini. This is not desirable for commercialisation. (H) A flower of a PT84-73 plant that was maintained in tissue culture and then revived in a pot. It has the same phenotype as the original PT84-73 (D, left), (I) A transgenic Surfinia Purple plant (PT103-26) harboring the sense *Hf1 F3′5′H*, *AR–AT*, and *FLS* genes (pSPB502), (J) A flower of PT103-26 that was kept in the glasshouse for three years. The flower lost phenotypic stability and partly regained the flower colour of the host, Surfinia Purple, (K) A flower of a PT103-26 plant that has been maintained in tissue culture and revived in a pot. The flower was as same as the original altered phenotype of PT103-26 (I).

Table 1.   Amount of flavonoid aglycons in the petals of the hosts and the transgenic petunias (mg/g wet weight).

Variety	Vector	line	Pel	Cya	Peo	Del	Pet	Mal	K	Q	M	DHK	DHQ	A	L	T
Surfinia Purple	-	Host	n.d.	n.d.	n.d.	0.029	0.049	0.804	0.055	0.032	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Surfinia Purple	pCGP1392	PT13-4	n.d.	0.008	0.277	n.d.	0.011	0.0510	0.050	0.148	n.d.	0.183	n.d.	n.d.	n.d.	n.d.
Surfinia Hot Pink	-	Host	n.d.	0.014	0.438	n.d.	0.034	0.153	0.103	0.091	n.d.	0.274	n.d.	n.d.	n.d.	n.d.
Surfinia Purple	pBPRT1	PT14-10	n.d.	n.d.	n.d.	0.154	0.203	0.267	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
Surfinia Purple	pSPB502	PT103-26	0.003	0.220	0.177	0.003	0.004	0.013	0.152	0.149	n.d.	n.d.	0.467	n.d.	n.d.	n.d.
Surfinia Purple Mini	-	Host	n.d.	0.002	0.006	0.022	0.41	2.291	0.46	0.562	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Surfinia Purple Mini	pSPB502	PT84-73	n.d.	0.499	0.274	0.007	0.016	0.023	0.035	0.149	n.d.	n.d.	0.453	n.d.	n.d.	n.d.
Baccara Red	-	Host	n.d.	0.661	0.073	0.031	0.046	0.031	0.079	0.988	n.d.	n.d.	0.279	n.d.	n.d.	n.d.
Baccara Red	pSPB520	PT94-17	0.966	0.179	0.013	0.107	0.063	0.022	0.968	0.096	n.d.	1.884	n.d.	n.d.	n.d.	n.d.
Baccara Red	pSPB538	PT102-16	0.601	0.107	0.007	0.035	0.018	n.d.	0.648	0.037	n.d.	1.445	0.354	n.d.	n.d.	n.d.
Surfinia Violet	-	Host	n.d.	n.d.	n.d.	n.d.	0.113	1.470	1.899	0.169	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Surfinia Violet	pSPB100	PT17-28	n.d.	n.d.	n.d.	n.d.	0.106	0.747	1.848	0.194	0.147	n.d.	n.d.	n.d.	n.d.	n.d.
Surfinia Violet	pSPB209	PT47-10	n.d.	n.d.	0.024	n.d.	0.205	2.165	0.278	0.183	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Surfinia Violet Mini	-	Host	n.d.	n.d.	n.d.	0.025	0.354	3.834	0.945	0.157	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Surfinia Violet Mini	pBTFN1	PT92-65	n.d.	n.d.	n.d.	0.018	0.160	2.060	0.787	0.140	n.d.	n.d.	n.d.	0.012	0.012	0.245

Pel; pelargonidin, Cya; cyanidin, Peo; peonidin, Del; delphinidin, Pet; petunidin, Mal; malvidin, K; kaempferol, Q; quercetin, M; myricetin, DHK; dihydrokaempferol, DHQ; dihydroquercetin, A; apigenin, L; luteolin, T; tricetin, n.d.; not detected, N. A.; not analyzed.

(unpublished results). It is not easy to obtain dihydrokaempferol-accumulating commercial varieties (recessive in *F3′5′H*, *F3′H* and *FLS*), and extensive breeding efforts are necessary to make a pelargonidin-producing orange commercial variety from a transgenic plant accumulating pelargonidin (Oud et al. 1995).

In order to suppress an endogenous *F3′H* gene and overexpress the rose *DFR* gene, pSPB520 (antisense suppression of *F3′H* gene) and pSPB538 (RNAi suppression of *F3′H* gene) were constructed (Figure 2) and used to transform Baccarat Red. About 900 bp and 500 bp of the *F3′H* cDNA formed an inverted repeat and a loop, respectively, in pSPB538. Twenty-nine of 108 and 65 of 84 transgenic plants had altered brick-red flower color (Figure 5A, B, and C). The RNAi construct (pSPB538) yielded higher frequency of flower color changes, which indicates that RNAi is superior to antisense to achieve the suppression of the gene in prupose. The transcription of double-strand RNA (RNAi) has been reported to be useful to suppress endogenous gene expression (Wang and Waterhouse 2001) and its efficacy is confirmed in petunia in this study.

Since the obtained flower color phenotype is commercially useful, the plants were kept in the glasshouse to select stable lines, and four lines (one from pSPB520 (PT94-17) and three from pSPB538 (PT102-7, PT102-16, and PT102-58) were selected. An unstable flower phenotype is also shown in figure 5D.

A decrease of quercetin and an increase of kaempferol show that *F3′H* activity was successfully down-regulated (PT94-17 and PT102-16 in Table 1). Pelargonidin, which native petunia does not produce, was the most abundant anthocyanidin (Table 1). The results revealed that the *F3′H* gene was down-regulated and the rose *DFR* gene was over-expressed; furthermore, they indicate that it is feasible to down regulate an endogenous gene and over-express a heterologous gene at the same time to modify the metabolic pathway in transgenic plants. Quantitative RT-PCR analysis of PT102-16 and PT102-58 flowers confirmed that *F3′H* gene expression was down-regulated to 3–5%, the rose *DFR* gene was expressed, and the petunia anthocyanidin synthase gene expression level was unaffected.

The accumulation of dihydrokaempferol indicates that the metabolic flux to anthocyanidin was inefficient and that further expression of the rose *DFR* gene or blockage of the *FLS* gene may produce a more intense orange petunia.

Thirty lines derived from pSPB538 were subjected to genomic Southern analysis using the *nptII* gene as a probe. The copy numbers varied from one copy to multicopies (Figure 5E). PT102-16 and PT102-58 had one copy of the transgene, and PT102-7 had two copies. The results may indicate that transgenic plants containing a smaller T-DNA copy number tend to give more stable phenotypes.

Modification of copigment biosynthesis using flavonol and flavone synthase cDNAs

Copigment (flavonols and flavones) biosynthesis was modified to examine if it is feasible to engineer flower color their amounts. The petunia *FLS* gene under the control of the constitutive promoter (pSPB209, Figure 2) was introduced into Surfinia Violet. Four of 39 transgenic plants had slightly redder flowers (Figure 6A), and a selected line (PT47-10) had fewer flavonols and more anthocyanidins (Table 1). The results indicate that an endogenous *FLS* gene was suppressed rather than over-expressed. A decrease of petunia *FLS* transcripts in PT47-10 was confirmed by Northern blot analysis (data not shown).

In order to achieve elevated amount of flavonols, a heterologous *FLS* gene was used, with which sense-suppression could be avoided. The binary vector containing rose *FLS* (pSPB100, Figure 2) was introduced to Surfinia Violet, and 47 transgenic plants were obtained. Sixteen of them exhibited paler flowers. Flavonoid analysis of PT17-28 revealed that the petals produced a smaller amount of anthocyanins. The total amount of flavonols did not increase, and bluing by copigmentation was not evident. More flavonol may be necessary to produce significant bluing. The enhanced CaMV 35S promoter used in this study may not be suitable to elevate amount of flavonols in petunia.

However, myricetin, which petunia does not accumulate due to the substrate specificity of petunia FLS (Figure 1, Forkmann et al. 1985), was detected. This result indicates that rose FLS works in the transgenic petunia because rose FLS is able to catalyze dihydromyricetin to myricetin when the rose *FLS* gene is expressed in yeast (data not shown). The transcripts of rose *FLS* were detected by Northern blot analysis, and the level of petunia *FLS* transcripts were unchanged (data not shown).

The torenia *FNSII* gene in pBTFN1 was introduced to Surfinia Violet to synthesize flavones that petunias do not produce. Six of 65 transgenic plants had paler flowers and contained flavones, such as tricetin, which do not accumulate in petunia, and a smaller amount of anthocyanin. The data for PT92-65 is shown in Table 1. The results confirmed that the torenia *FNS* gene functioned in a heterologous plant. The transcripts of the torenia *FNS* gene were confirmed by Northern blot analysis (data not shown). Higher expression of the gene and/or suppression of the *DFR* gene may be necessary in order to achieve a higher flavone/anthocyanin ratio and obtain a bluer petunia by copigmentation.

The results obtained here clearly confirm that flower color modification by metabolic engineering is useful to breed novel floricultural crops, as reviewed (Tanaka et al.

1998; Mol et al. 1999; Forkmann and Martern 2001;   Tanaka et al. 2004). It is not always easy to obtain
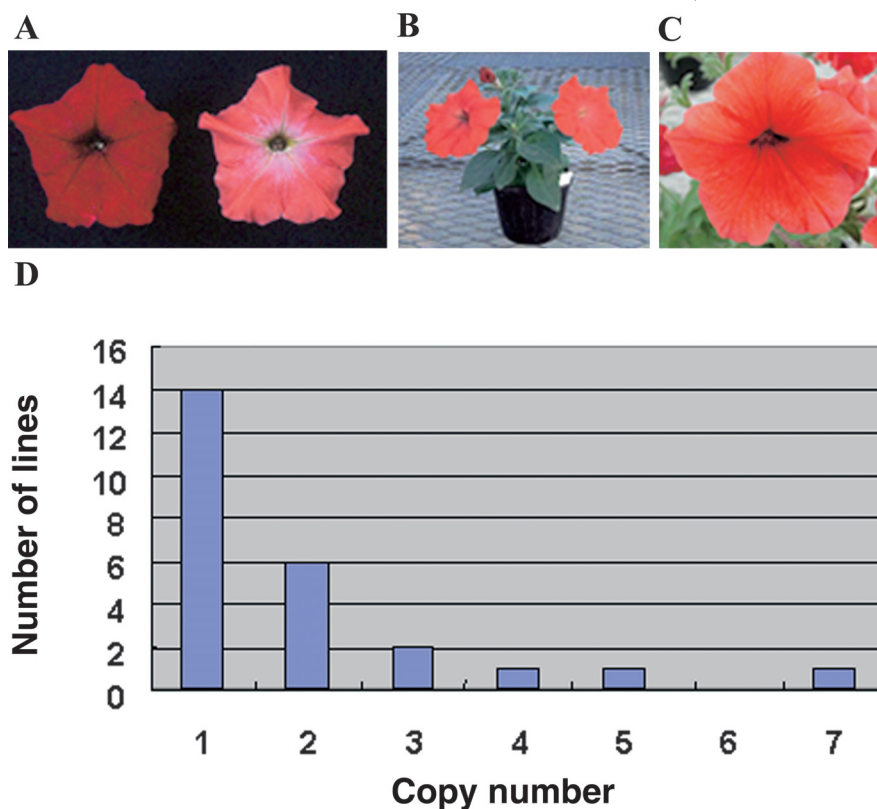

Figure 5.   Generation of orange petunia. Suppression of the F3′H gene by antisense (pSPB520) or the RNAi method (pSPB538) and expression of the rose DFR gene in cyanidin-producing red petunia (A, left) yielded pelargonidin-producing orange petunia (A, right), (B) A transgenic plant (PT94-17) in a pot, (C) A flower that lost the phenotypic stability and partially regained the host red color, (D) T-DNA copy number analysis of the transgenic plants harboring pSPB520. Among the three most stable lines, two plants had a single copy insert, and the other one had two.
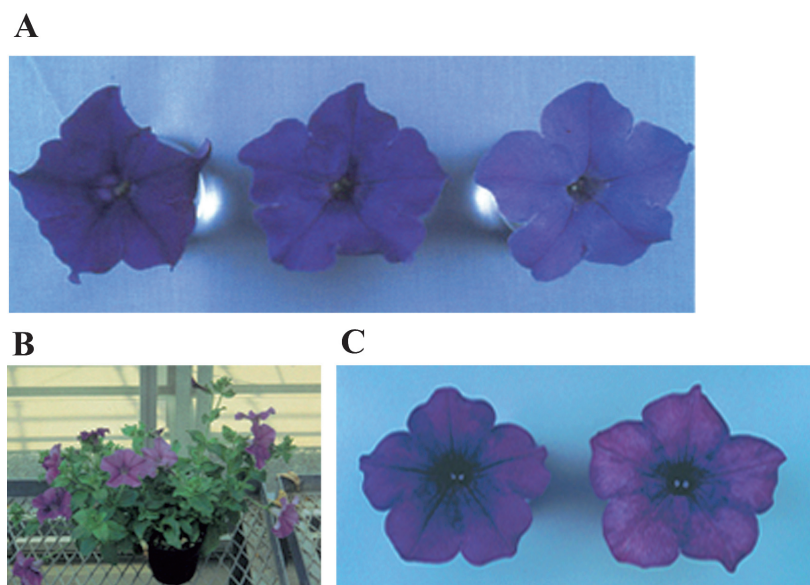

Figure 6.   Modification of copigment biosynthesis. (A) Flowers of Surfinia Violet (middle) and one of transgenic plants with pSPB209 (PT47-10, left). Suppression of flavonol biosynthesis resulted in darker and slightly redder color. A flower of a transgenic plant expressing rose *FLS* gene has a paler flower (PT17-28, right), (B) A plant of PT17-28. Only the flower color was modified, and the creeping character did not change. (C) Surfinia Violet Mini (left) and a transgenic petunia expressing torenia *FNSII* gene (right).

transgenic plants with stable phenotypes. The stability of gene suppression may be an obstacle to achieve constantly modified phenotypes. Irreversible gene knockout is ideal but is unfeasible except in rare cases (Terada et al. 2002). More practically, it is necessary to generate many transgenic plants and select lines with stable phenotypes. In other words, an efficient transformation system is essential for product development. It is also important to maintain the selected lines in tissue culture.

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