

# Green corolla segments in a wild *Petunia* species caused by a mutation in *FBP2*, a *SEPALLATA*-like MADS box gene

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**Abstract** A *Petunia inflata* isolate with a novel phenotype of a purple corolla limb with green corolla segments (GCS) was characterized. The GCS have stomata and trichomes on the adaxial side, and resemble calyx segments in epidermal morphology. The GCS phenotype was inherited in a recessive manner. In the GCS plant, a novel *inhibitor*/defective *spm*-like transposable element (*dPifTp1*) was inserted in the second intron of the *Floral Binding Protein 2 (FBP2)* gene. The sequence of the resulting transcript contained five silent mutations as compared the corresponding open reading frame of *P. × hybrida FBP2* mRNA. The GCS phenotype co-segregated with an *FBP2* fragment containing a *dPifTp1* insertion. The transcript level of the *FBP2* gene in GCS flowers was markedly lower than that in wild-type (WT) flowers, suggesting that par-

tially inhibited *FBP2* gene expression caused the morphogenesis of calyx-like tissue in the corolla segments of GCS flowers. Gene expression pattern analysis using a full-length *Petunia* floral cDNA microarray indicated that some photosynthesis-related genes were expressed at significantly higher levels in the GCS of GCS flowers, but the mRNA levels of most other genes in the GCS were similar to those in the WT corolla. Taken together, these data suggest that the partial loss of *FBP2* expression does not shift global gene expression in the corolla segments of the GCS flower toward that of calyx, even though calyx-like morphogenesis was established in the corolla segments.

**Keywords** cDNA microarray · *FBP2* gene · Floral morphology · MADS box · *Petunia* · Transposon

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## Abbreviations

FBP2 Floral Binding Protein 2  
GCS Green corolla segments  
spm Suppressor mutator  
WT Wild type

## Introduction

In order to satisfy consumer demand for floricultural crops, it is necessary for breeders to control various floral phenotypes, such as flower colour, depth, size, and shape. If a bi-coloured version of a flower that was previously available as only a single colour becomes available, such a floral phenotype is welcomed by breeders because it might be highly attractive to consumers. *Petunia* (*Petunia × hybrida* Vilm., Solanaceae) is a major floricultural crop in gardens in temperate regions. It has had a long history of breeding since the 1830s (Paxton 1836), and today's garden petunias

comprise almost all possible flower colours. Cultivars that produce bi-coloured flowers are also available. Such floral phenotypes are called Picotee (Fig. 1a, flowers with a whitish margin and a coloured centre), Morn (flowers with a coloured margin and whitish centre), or Star (coloured flowers with five radial lines of a different colour).

In the present study, we introduce a wild *Petunia* with a floral phenotype that may be classified as a Picotee, but the colour of the margin is green instead of white. This phenotype resembles that of a lost cultivar described in 1838 by Joseph Harrison (quoted in Bailey 1896), with “a red body colour with grass-green border”. It also resembles a phenotype of a mutant in the *Floral Binding Protein 2* (*FBP2*) gene that was described as having “petals with an overall diffuse green hue, the effect being strongest in the areas surrounding the main veins and at edges of the petals” (Vandenbussche et al. 2003). Since this phenotype can be described in various ways, it seems useful to first discuss the terminology of the floral organs of this plant. *Petunia* has gamopetalous flowers, so that the coloured part of the flower could be called the “corolla” which is a collective term for the petals of a flower. In gamopetalous flowers, individual petals are fused at their base to form corolla tube (Esau 1977). The most showy part in the frontal view of a *Petunia* flower is a continuation of the fused corolla tube which is patulous, not tubular, in this plant. In the description of floral morphology of *Petunia* species, accordingly, the fused and patulous part of the corolla is customarily called corolla limb in distinguishing from non-fused more outer parts

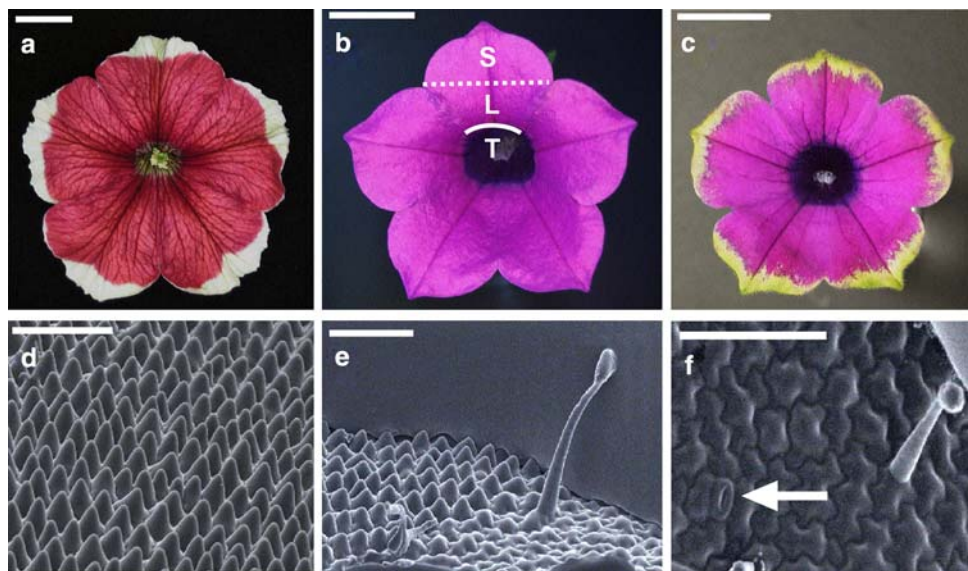
which is called corolla segments (Fig. 1b; e.g. Ando and Hashimoto 1993). The WT *P. inflata* R. E. Fr. has five evident purple segments on the margin of a corolla limb of the same colour (Fig. 1b). Therefore, the phenotype under consideration can be described as a purple corolla limb with green corolla segments (GCS), which will be referred to simply as green segments in the present study (Fig. 1c). Strictly speaking, the green part does not cover the whole of corolla segments but is restricted to the outer margin of segments. The green part was never observed in the corolla limb. These terminologies will be valuable as an aid in recognition of the compartment existing within a single petal. As for sepals, we used a term, calyx, (a collective term for the sepals of a flower, Esau 1977), to make terms uniform.

Here, we provide genetic and molecular information on the GCS phenotype caused by a mutated *FBP2* allele, including a novel inhibitor/defective *spm* (*Idspm*)-like transposable element. A thorough analysis was also conducted for the mutated *FBP2* gene in terms of its interaction with other genes, using a full-length cDNA microarray constructed from *Petunia* flowers (Shimamura et al. 2007).

## Materials and methods

### Plant material

An individual of *Petunia inflata* R. E. Fr. (Solanaceae) with the GCS phenotype was found among the progeny of plants



**Fig. 1** Phenotypes of *Petunia inflata* and *P. × hybrida* used in the present study: wild type (WT) and the green corolla segment type (GCS). **a** ‘Baccara Red Picotee’. **b** WT flower: S (corolla segment), L (corolla limb), T (corolla tube). **c** GCS flower with a green hue on the corolla segments. **d–f** Scanning electron microscopic images of the corolla surface. **d** Adaxial surface of WT corolla segments covered with conical epidermal cells. **e** Adaxial surface of GCS corolla segments

covered with irregularly shaped epidermal cells. The green site of the corolla segments (at right) is covered with flat epidermal cells, trichomes, and stomata, and the adjacent inside purple site (at left) is covered with conical epidermal cells without trichomes or stomata. **f** Close-up of the epidermal tissues (adaxial side) of GCS corolla segments showing the presence of trichomes and stomata, indicated by the arrow. Bars 10 mm (**a, c**) and 100  $\mu$ m (**d–f**)

from the seeds collected in a wild population in Paraguay. The WT *P. inflata* used in the present study was obtained from the same progeny, and was confirmed to not segregate the GCS phenotype in progeny produced by bud self-pollination. GCS and WT *P. inflata* plants, as well as commercial cultivars of *P. × hybrida* Vilm., ‘Baccara Light Salmon’, ‘Baccara Blue Picotee’, ‘Baccara Red Picotee’, and ‘Baccara Rose Picotee’ (Sakata Seeds Co., Ltd., Yokohama, Japan), were grown in a greenhouse following standard practices for garden petunias.

#### Microscopic analysis

For scanning electron microscopy observations, the mould-cast technique (Jernstedt et al. 1992) was used with modifications. Plant surface samples were moulded with dental silicone Exadenture (GC Co., Tokyo, Japan), and replicas were obtained by pouring 2 Ton clear epoxy resin (ITW Industry Co. Osaka, Japan) into the silicon moulds and incubating them at 60°C for 1 h. The corolla surface replica was observed using a Hitachi S-2400 scanning electron microscope at 10 kV.

#### Isolation of the *FBP2* gene

Total DNA was extracted from ~5 g of young leaves using a modified CTAB method (Lassner et al. 1989). The *FBP2* genomic sequence, corresponding to almost the full-length cDNA (35–755 of GenBank accession number M91666), was amplified using the primers fbp2-f1 (5'-TATGGGAAGAGGTAGAGTTGAGCTT-3') and fbp2-r5 (5'-CAACCAGCCAGCCATGTAGTT-3'). The PCR product was gel-purified using a QIAEX II Gel Extraction kit (Qiagen, Tokyo, Japan) and then ligated into pGEM-T Easy according to the manufacturer's instructions (Promega, Tokyo, Japan). DNA sequencing was carried out using an ABI PRISM Dye Terminator Cycle Sequencing FS Core kit (PE Applied Biosystems, Foster City, CA, USA). The sequencing products were analyzed using an ABI 3100 DNA Sequencer (PE Applied Biosystems). Homology and sequence identity were confirmed using the BLAST program (Altschul et al. 1997). The sequences determined in this paper were registered in GenBank under accession number AB375307, AB375308 and AB375309.

#### Isolation of *FBP2* cDNAs

Total RNA was extracted from flower buds of developmental stage III (see below) using Trizol reagent (Invitrogen, Carlsbad, CA, USA). A measure of 1 µg of total RNA was used for the amplification of *FBP2* cDNAs with reverse-transcription PCR, using a TaKaRa One Step RNA PCR kit (TaKaRa, Shiga, Japan) with the primers fbp2-f1 and fbp2-

r5. The resulting *FBP2* cDNA fragments were inserted into a plasmid vector and sequenced.

#### Genomic Southern-blot analysis

A measure of 10 µg of genomic DNA isolated from young leaves were digested with *Nsi*I and then blotted onto Hybond N<sup>+</sup> membrane (Amersham, NJ, USA). A digoxigenin (DIG)-labelled partial *dPifTp1* DNA fragment was prepared with the PCR DIG Probe Synthesis kit according to the manufacturer's protocol (Roche, Penzberg, Germany) using the primers fbp2-f7 (5'-CTCTAAACCCCTCCCCA ACC-3') and fbp2-r11 (5'-TGAAATCCCCCAAAGAAA CAATATT-3'). The sample was hybridized at 55°C for 16 h, and the post-hybridization steps followed the manufacturer's recommendations (Roche).

#### Northern-blot analysis of total RNA

Whole flower buds or flowers of *P. inflata* were harvested at developmental stages defined as follows: stage I, <1.5 mm corolla length, not pigmented; stage II, 1.5–5.0 mm, pigmented corolla; stage III, 5–15 mm, flower bud just before opening; and stage IV, 15–20 mm, opened flower. Total RNA was extracted from these samples using Trizol reagent (Invitrogen). A DIG-labelled antisense riboprobe specific to the 3'-terminal sequence of the *FBP2* cDNA (635 bp) was prepared according to the manufacturer's protocol (Roche). Total RNA (10 µg) was denatured and separated on a 1% (w/v) agarose gel as described (Kodama et al. 1994). The hybridized probes were visualized as described (Hirai et al. 2007).

#### Association of the *dPifTp1* insertion with the *FBP2* gene and phenotype in the F<sub>2</sub> generation

In order to identify two *FBP2* alleles, PCR was conducted using the primers fbp2-f3 (5'-GCACCAGAGACTAAT ATATCCACACG-3') and fbp2-r3 (5'-TTGGCTGCTTAT TTCCTGTAATCAT-3'). The 1.7-kbp genomic region corresponding to the *FBP2* genomic DNA from nucleotides 1,519 to 3,256 (GenBank accession number AB375307) is expected to be amplified with these primers. When *dPifTp1* is inserted into the *FBP2* gene, a long fragment (3.8 kbp in length) is expected to be amplified. Each 50-µl reaction mixture consisted of ~50 ng total DNA, 1 × ExTaq buffer, 0.2 mM of each deoxynucleotide triphosphate, 1 mM of each primer, and 2.5 U of ExTaq (TaKaRa). The amplification was performed as follows: initial denaturation for 5 min at 95°C, followed by 30 cycles of 95°C for 45 s, 60°C for 1 min, and 72°C for 3 min, and a final extension at 72°C for 5 min. The PCR products were separated by agarose gel electrophoresis.

**Table 1** Numbers of stomata and trichomes on the adaxial and abaxial sides of corolla lobes and calyx lobes of the wild type (WT) and a green corolla segments mutant (GCS) of *Petunia inflata*

Location		Number of stomata/10,000 $\mu\text{m}^2$		Number of trichomes/ $\text{mm}^2$	
		Adaxial side	Abaxial side	Adaxial side	Abaxial side
Corolla lobes	Purple segments of WT	0.0 a*	0.0 a*	0.0 a*	7.1 a*
	Green segments of GCS	0.8 b	2.0 b	7.9 b	10.0 b
Calyx lobes	WT	2.5 c	4.6 c	11.7 c	10.3 b
	GCS	2.5 c	3.9 c	12.0 c	9.4 b

\* No significant difference at  $P = 0.05$  by Scheffe's test between the same letters within a column

### cDNA microarray analysis

A *Petunia* microarray with 2,976 probes was prepared as described (Shimamura et al. 2007). The probes were the amplified products of cDNAs selected from cDNA libraries constructed using total floral RNAs isolated from an interspecific hybrid plant between *P. axillaris* (Lam.) Britton, Sterns & Poggenb. and *P. integrifolia* (Hook.) Schinz & Thell. (Shimamura et al. 2007). The region corresponding to the multiple cloning site of pCMVFL3 with a short flanking sequence was amplified using a Cy3-conjugated primer (5'-Cy3-ATTAGGCCTATTTAGGTGACACTATAGAAC-3') and an M13-M3-24 primer in the presence of Cy3-dCTP (GE Healthcare, Buckinghamshire, UK). Trizol reagent (Invitrogen) was used to isolate total RNAs from GCS of GCS mutants, purple corolla limb of GCS mutants, purple corolla segments plus purple corolla limbs of the WT plants, and calyx segments of the WT. Cy5-labeled targets corresponding to each floral sample were prepared by reverse transcription of total RNA with a CyScribe first-strand cDNA labelling kit (GE Healthcare). The hybridization reaction was carried out with the Cy5-labeled targets and a Cy3-labeled fragment corresponding to the multiple cloning site sequence of pCMVFL3. Hybridization and scanning were performed as described (Shimamura et al. 2005; Nagira et al. 2006; Shimamura et al. 2007). All hybridizations were performed twice using RNAs prepared from independently sampled flowers. Duplicate probes were spotted onto each microarray glass. Thus, the expression level of each gene was estimated from a total of four fluorescence intensities of hybridization signals. The signal to noise (SN) ratio of each spot was calculated. Spots with a low SN ratio (<2 in Cy3 and <1 in Cy5 fluorescence intensities) were not included in further analysis. The amount of each probe on the microarray glass was normalized by measurement of the Cy3 fluorescence intensity. The mRNA level was estimated by the Cy5 fluorescence intensity of the probe-target duplex. *Petunia* ESTs with a more than 1.5-fold change in expression were selected as up- or downregulated genes (Shimamura et al. 2007).

### Results

#### Morphology of the GCS flower

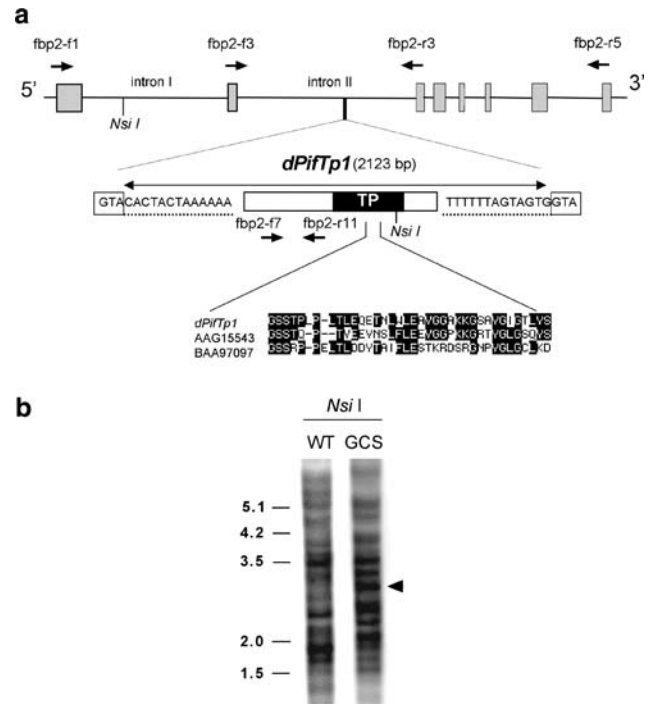
The adaxial surface of the corolla limb and segments of a wild-type (WT) *Petunia inflata* flower was completely covered with conical epidermal cells that accumulated anthocyanin pigments (Fig. 1d). The abaxial surface was covered with flat epidermal cells that did not accumulate anthocyanin (data not shown). Stomata were absent on both sides. Trichomes were only found on the abaxial side (Table 1). These features were similar to those of the purple corolla limb of a GCS flower. In the green area of the corolla segments of a GCS flower, however, the epidermal cells on the adaxial surface were flat (Fig. 1e, f), and stomata and trichomes were also present (Fig. 1f, Table 1). Anthocyanin pigments did not accumulate in the flat epidermal cells on the adaxial side. These features of the green segments of GCS flowers resembled those of calyx segments, but the number of stomata on the adaxial and abaxial surfaces of the green segments of GCS flowers was significantly lower than those of the calyx segments of WT and GCS plants. There were also fewer trichomes on the adaxial side of the green segments of GCS flowers than on calyx segments of WT and GCS plants (Table 1). The morphologies of the other floral organs, such as stamens and carpels, were well conserved in GCS flowers. The GCS plant was functional as a seed and pollen parent. The flowering habit of the GCS plants was also similar to that of WT plants.

The epidermal morphology of several commercial cultivars of the Picotee phenotype, including 'Baccara Blue Picotee', 'Baccara Red Picotee' (Fig. 1a), and 'Baccara Rose Picotee', was also examined. When comparing the marginal white site and the inside coloured site, no difference was found in terms of the shape of the epidermal cells or the density of trichomes, and stomata were absent from both sides. The exception was the degree of anthocyanin accumulation in the conical epidermal cells on the adaxial side. The border between the green and purple sites on GCS flowers was clear, but that between the white and coloured sites in Picotee flowers was obscure.

Identification of a locus causing the GCS phenotype

Since GCS plants resemble an *FBP2* gene mutant in the floral phenotype (Angenent et al. 1994; Ferrario et al. 2003; Vandebussche et al. 2003), the *FBP2* genomic sequence corresponding to the *Petunia × hybrida* *FBP2* cDNA (GenBank accession number M91666; Angenent et al. 1992) from base pairs 35 to 755 was amplified from WT *P. inflata*, and the resulting 5,053-bp sequence was determined (GenBank accession number AB375307). The *FBP2* gene of WT *P. inflata* consisted of eight exons separated by seven introns. The WT *P. inflata* *FBP2* transcript had five silent substitution mutations in the corresponding open reading frame as compared with that of *Petunia × hybrida* *FBP2* (M91666). When the corresponding *FBP2* genomic sequence was amplified from a GCS plant, a longer fragment (about 7 kb in length) was obtained. The introns IV and V of the GCS *FBP2* genomic DNA were identical to those of WT *FBP2*, except for one base substitution in intron V. Although the lengths of introns I (1,315 bp), III (90 bp), VI (394 bp), and VII (268 bp) of a GCS plant were found to be considerably different from those of a WT plant (intron I, 1,289 bp; III, 100 bp; VI, 387 bp; VII, 517 bp), we could not find any significant difference in structure. The most noticeable difference was found in intron II: a 2,123-bp insertion in the GCS plant (Fig. 2). A short open reading frame (about 550 bp in length) was found in this insertion sequence, and its predicted amino acid sequence showed limited similarity to those of the *En/Spm*-like transposases of *P. × hybrida* (AAG15543) and *Arabidopsis thaliana* (BAA97097) when the BLAST program was used to confirm homology and sequence identity (Altschul et al. 1997). This insertion sequence had 13-bp terminal inverted repeats starting with the nucleotides CACTA and a 3-bp target-site duplication on the insertion (Fig. 2). Except for the *En/Spm*-like transposase domain and characteristics of the terminal inverted repeats, this insertion element shared little sequence similarity over its entire length with any other transposable element. These structural characteristics indicated that this insertion sequence is an inhibitor or defective suppressor mutator (*Idspm*)-like transposable element. This element was designated *dPifTp1*.

The transposition of *dPifTp1* was investigated using Southern-blot analysis (Fig. 2). Genomic DNAs from WT and GCS plants were digested with *NsiI*, and then fragments were visualized by hybridization with a *dPifTp1* probe corresponds to an insertion of *dPifTp1* (Fig. 2a). Since only a limited number of hybridizing bands (7–8 strongly hybridizing bands and several weakly hybridizing bands) was detected in both plants (Fig. 2b), the copy number of the *dPifTp1* was considered probably low. A strongly hybridizing fragment of 2.8 kbp was unique to the GCS plant (Fig. 2b). The size of this fragment was in good



**Fig. 2** Structural organization of the *fbp2-3* allele containing *dPifTp1* and genomic Southern-blot analysis of *dPifTp1*. **a** The solid box in intron II shows the position of the *dPifTp1* insert. The target site duplications are boxed, and terminal inverted repeats are indicated by dotted underlines. A sequence alignment of part of the predicted *En/Spm* transposase-like protein (shown by TP) of *dPifTp1*, AAG15543 from *Petunia × hybrida*, and BAA97097 from *Arabidopsis thaliana* is shown. Conserved amino acids are shaded black. The positions of *NsiI* sites are indicated. **b** Southern-blot of *NsiI*-digested genomic DNA. Lane WT, wild type; lane GCS, green corolla segment type. The arrowhead indicates the 2.8-kbp *NsiI* fragment that is possibly generated from the *fbp2* gene containing *dPifTp1*. The numbers to the left indicate the positions of DNA size markers (in kb)

agreement with the size expected from the *FBP2* sequence isolated from the GCS plant, suggesting that the 2.8-kbp fragment corresponds to the transposition of *dPifTp1*. In addition, a few other fragments were also unique to the GCS plant, suggesting that the transposition of several *dPifTp1*-related transposable elements occurred in the GCS plant.

Inheritance of the GCS phenotype and cosegregation of *dPifTp1*

A GCS plant of *Petunia inflata* was bud self-pollinated to obtain progeny. All of the progeny (112 individuals) had the GCS phenotype. A GCS plant of *P. inflata* was crossed reciprocally with WT *P. inflata* plants and the commercial cultivar ‘Baccara Light Salmon’, which has a solid salmon corolla. All of the F<sub>1</sub> progeny displayed the WT phenotype. F<sub>1</sub> progeny plants were randomly crossed with one another to obtain the F<sub>2</sub> generation. The WT phenotype (2,358 individuals) and the GCS phenotype (801 individuals) segre-

gated in the  $F_2$  generation. Testing of the actual segregation ratio against the ratio expected with the hypothesis (3:1) revealed that the GCS phenotype was caused by a single recessive gene, and the null hypothesis (the actual ratio equals the expected ratio) could not be discarded statistically ( $\chi^2 = 0.2042$ ).

We then investigated the segregation of *dPifTp1* in  $F_1$  (50 individuals) and  $F_2$  (100 individuals) progeny between the GCS *P. inflata* plant and the WT *P. inflata* plant. Partial fragments of the *FBP2* gene including intron II were amplified by PCR using the primers *fbp-f3* and *fbp-r3* (Fig. 2a). An *FBP2* fragment of 1.7 kbp was detected in all plants expressing the WT phenotype, but this fragment was replaced with a longer fragment of 3.8 kbp in plants expressing the GCS phenotype (Fig. S1).

### Expression of the *FBP2* gene

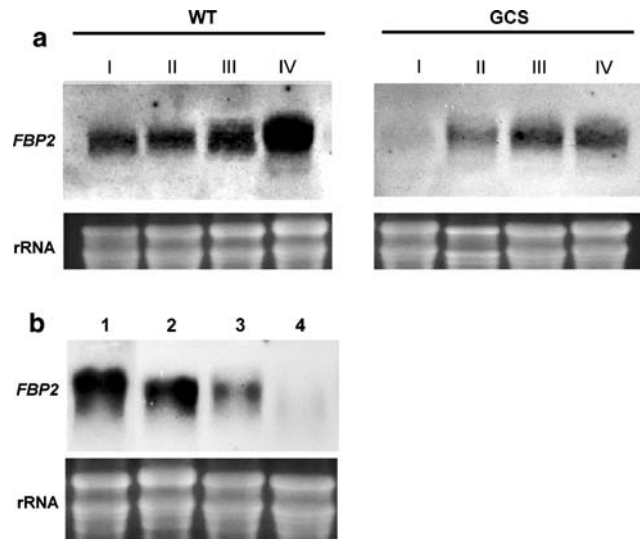
The expression of the *FBP2* gene in buds and flowers at stages I–IV was examined by Northern-blot analysis. The *FBP2* transcripts in the GCS flower were similar in length to those in the WT flower. In the WT plant, the *FBP2* mRNA was expressed consistently in whole buds and flowers at all stages tested, and increased markedly at stage IV. In GCS plants, however, the level of *FBP2* transcripts was very limited in buds and flowers at stage I. Although the *FBP2* transcript level increased gradually at stages II and III, the level remained constant in stage-IV GCS flowers (Fig. 3a).

The spatial expression pattern of the *FBP2* gene was investigated using flowers at stage III. In the purple site of the GCS corolla limb, *FBP2* mRNA accumulated at high levels. It also accumulated in the green site of the GCS corolla segments, but at a much lower level than in the corresponding site in WT corolla segments. In the calyx segments of WT plants, the *FBP2* transcript level was very low (Fig. 3b).

Reverse transcription PCR products of the *FBP2* mRNAs in the GCS flower were cloned and sequenced. Although the sequence corresponding to the open reading frame in the *FBP2* transcripts produced in the GCS flower showed two base substitutions as compared to the WT *FBP2* transcript sequence, the predicted amino acid sequence of the *FBP2* protein did not differ between the two flower types, indicating that the mutations were silent.

### Expression levels of 2,976 genes in GCS flowers

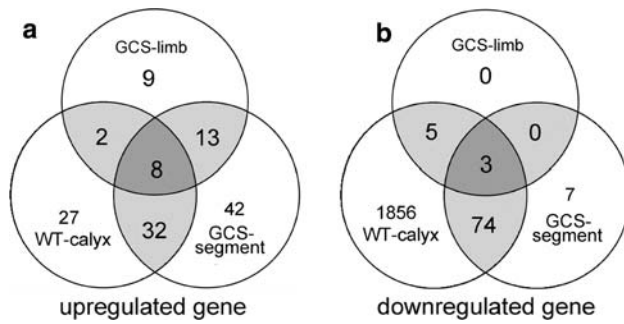
The expression of 2,976 genes was compared between GCS and WT flowers using a microarray constructed from *Petunia* flowers (Shimamura et al. 2007). The global gene expression in the corolla limb plus corolla segments of WT flowers (referred to hereafter as WT corolla) was compared



**Fig. 3** Northern-blot analysis of the *FBP2* gene. **a** Total RNA was isolated from floral tissues of WT and GCS plants at four different floral developmental stages. Stages I–IV indicate the floral developmental stages (see “Materials and methods”). **b** Total RNA was isolated from the corolla limb including the corolla segments of WT (lane 1), the purple corolla limb of GCS (lane 2), green corolla segments of GCS (lane 3), and calyx segments of WT plants (lane 4). The equivalence of RNA loading among the lanes is demonstrated by ethidium bromide staining of rRNA bands

with that of the purple corolla limb of GCS flowers (GCS limb), the GCS of GCS flowers (GCS segment), and the calyx segments of WT flowers (WT calyx). We chose a minimum threshold of a 1.5-fold change in the intensity ratio. Scatter plots showing signal intensities for each gene on the microarray are shown in Fig. S2.

Almost all of the tested genes were expressed at similar levels in the WT corolla and GCS limb (Fig. S2a). Only 32 and eight genes were up- and down-regulated, respectively, in the GCS limb. In contrast, 95 and 84 genes were up- and down-regulated, respectively, in the GCS segment as compared to the WT corolla (Fig. 4). *Petunia* genes encoding photosynthesis-related proteins, such as genes encoding the small subunit of ribulose 1,5-bisphosphate carboxylase, were expressed at higher levels in both the segment of GCS and the calyx of WT phenotypes as compared with expression in the corolla of WT phenotype (Table 2). One interesting feature of the ESTs on the microarray was revealed by the comparison of gene expression in the WT-corolla and WT-calyx. About 66% of the total ESTs on the microarray (1,963 of 2,976) were expressed less in WT-calyx than in WT-corolla, while only 72 of 2,976 genes were up-regulated in the WT-calyx. Thus, most ESTs on the microarray represented the genes that were preferentially expressed in corolla tissue but not in calyx-tissue. Therefore, the expression of genes corresponding to these 1,963 ESTs are expected to be weakened in the GCS segments if the corolla segments are converted into the calyx-like



**Fig. 4** Venn diagrams of the numbers of overlapping and non-overlapping genes that were up-regulated **a** or downregulated **b** by at least 1.5-fold or more in GCS over the expression level in WT corolla. Abbreviations of floral parts are three different floral tissues: GCS limb (purple corolla limb of GCS mutant), GCS segment (green corolla segments of GCS mutant) and WT calyx (calyx segments of WT)

segments in the green site of the GCS flowers. Of 1,963 genes mentioned above, however, only 77 genes were expressed at lower levels in the GCS segments than in the WT corolla. Therefore, the weakened expression of *FBP2* gene in the GCS plants caused the calyx-like morphogenesis in the GCS, but did not shift the global gene expression in corolla segments towards that of calyx segments.

**Discussion**

The green segments of GCS flowers appeared similar to the calyx segments in terms of epidermal morphology, such as presence of stomata and trichomes (Fig. 1), but a close inspection revealed the difference in their frequencies (Table 1). This probably confers sepaloïd features occurring at the corolla segments. Although similar pattern of corolla part without anthocyanin is found in the Picotee phenotype (green part in the GCS and white part in the Picotee), two phenotypes are different in morphology. GCS phenotype is caused by a single recessive mutation, and co-segregates with an insertion of transposable element, *dPifTp1*, into the *FBP2* gene. The GCS plant found in the present study is wild origin; it was discovered in cultivated plants raised from the seeds collected from the natural field of South America. This confirms the transposon activity in natural conditions, not in-laboratory conditions. The open reading frame sequence and several introns of the mutated *FBP2* gene in the GCS plant differed from those of the WT counterpart. They hardly shared similar patterns of *dPifTp1* insertion (Fig. 2b). The genetic background seems different between the GCS and the WT plants. These evidences imply that these alleles co-existed in nature for sometime, and therefore natural allele variations exist among *FBP2* gene of wild plants. We designated this mutant allele as *fbp2-3*.

Although the *fbp2-3* allele had a *dPifTp1* insertion, the insertion sequence was spliced out and the coding region sequence of the mature mRNAs was the same as that of *FBP2* mRNA. The level of *FBP2* transcripts was very limited in buds and flowers at stage I, and remained constant in stage-IV GCS flowers (Fig. 3a). Although *FBP2* mRNA accumulated in the green site of the GCS corolla segments, it was at a much lower level than in the corresponding site in WT corolla (Fig. 3b). In the calyx of WT plants, the *FBP2* transcript level was very low. Therefore, weakened accumulation of the *FBP2* mRNA in the green site of GCS flower disturbs the determination of cell fate in the corolla segments. The weakened expression of a plant gene due to the insertion of *l1dspm*-type transposable elements was reported in the maize *A1* gene (Fedoroff 1989). This *l1dspm*-type element was removed after splicing of the *A1* transcripts, and the level of the *A1* mRNA was reduced, as in the *fbp2-3* gene in GCS plants. The cause of the reduced *FBP2* mRNA level in GCS plant is not clear. We considered that inefficient processing of its nascent transcripts may account for this, although the sequence differences found in the other introns cannot be excluded as additional causes.

The *FBP2* gene is one of the *SEPALLATA*-like MADS box genes and encodes the E function, which, in *Arabidopsis*, has been shown to be required for B and C floral organ identity (Pelaz et al. 2000). An *fbp2* mutant has been reported to have a calyx-like corolla (Angenent et al. 1994; Ferrario et al. 2003; Vandenbussche et al. 2003). The morphology of the GCS flowers suggests that the adaxial epidermis at the green site on corolla segments of the GCS mutant may lose petal identity and may express several properties of the epidermis of calyx segments. From these observations, we expected that the global gene expression in the green segments of the GCS flowers was shifted toward that of the calyx segments. To address this possibility, we investigated the expression of the 2,976 genes in the GL and WT flowers by using a microarray constructed from *Petunia* flowers (Shimamura et al. 2007). As mentioned in “Results” section, most of the ESTs on the microarray represented that genes were preferentially expressed in corolla tissue and their expression were weakened in calyx tissue. If the corolla segments are converted into calyx-like segments in the green areas of the GCS flowers, the expression of genes corresponding to the ESTs that were preferentially found in the corolla is expected to be lower in the GCS segment. However, only a very limited fraction of ESTs were expressed at lower levels in the GCS segment than in the WT corolla. These results indicate that most of genes were expressed at a similar level in the GCS segments to those observed in the WT corolla, even though the weakened expression of the *FBP2* gene in GCS plants

**Table 2** Expressed profile of up-regulated genes in both green corolla segments of GCS flowers and calyx lobes as compared with that in the WT corolla

EST	BLAST × homology search								Species	Expected value	Functional classifications
	Level of expression (%)		GCS-segmen		WT-calyx		Annotation				
	WT-corolla	GCS-limb	GCS-segmen	WT-calyx	Gene ID	Annotation					
DC239809	51	69	99	100	CAA28450	ST-LS1 protein		<i>Solanum tuberosum</i>	9.00E-53	Energy	
DC239969	31	45	76	100	CAA26211	Unnamed protein product		<i>Petunia</i> sp.	4.00E-88	Energy	
DC240147	52	58	95	100	CAA27445	Ribulose 1,5-bisphosphate carboxylase		<i>Petunia × hybrida</i>	1.00E-69	Energy	
DC240329	34	50	92	100	CAA27445	Ribulose 1,5-bisphosphate carboxylase		<i>Petunia × hybrida</i>	4.00E-59	Energy	
DC240416	34	48	99	100	CAA27444	Ribulose 1,5-bisphosphate carboxylase		<i>Petunia × hybrida</i>	3.00E-71	Energy	
DC240621	31	42	100	87	CAA27445	Ribulose 1,5-bisphosphate carboxylase		<i>Petunia × hybrida</i>	1.00E-65	Energy	
DC240870	32	57	91	100	CAA27444	Ribulose 1,5-bisphosphate carboxylase		<i>Petunia × hybrida</i>	2.00E-35	Energy	
DC240970	50	56	100	78	ABE83768	Photosystem I reaction center subunit N		<i>Medicago truncatula</i>	3.00E-25	Energy	
DC241027	36	57	100	98	CAA27445	Ribulose 1,5-bisphosphate carboxylase		<i>Petunia × hybrida</i>	1.00E-80	Energy	
DC241034	41	61	98	100	ABE78743	Ribulose bisphosphate carboxylase, small chain		<i>Medicago truncatula</i>	8.00E-56	Energy	
DC241409	41	60	93	100	CAA28639	Chlorophyll <i>a/b</i> binding protein		<i>Petunia × hybrida</i>	9.00E-69	Energy	
DC242029	43	56	100	85	AAO85557	Photosystem I subunit XI		<i>Nicotiana attenuata</i>	3.00E-50	Energy	
DC242477	64	78	100	99	BAA77603	Plastidic aldolase		<i>Nicotiana paniculata</i>	9.00E-57	Metabolism	
DC242741	28	52	92	100	CAA41187	Chlorophyll <i>a/b</i> binding protein		<i>Nicotiana tabacum</i>	1.00E-76	Energy	
DC242755	56	77	100	87	CAA43590	Type I (26 kD) CP29 polypeptide		<i>Lycopersicon esculentum</i>	7.00E-58	Energy	



caused calyx segment-like morphogenesis in the corolla segments.

Vandenbussche et al. (2003) isolated two independent transposon-insertion mutants in the *FBP2* gene. Both mutants contained the *dTph1* insertion in the K domain region of the *FBP2* gene, suggesting that they are null mutants. Flowers of these two mutants had corolla limb with an overall diffuse green hue, and the strongest effects were evident in the area surrounding the main veins on the corolla limb and at the edges of the corolla limb, probably including the segment. In addition, secondary inflorescences developed in the third whorl. Unlike these *fbp2* mutants, the GCS plants used in the present study had very limited phenotypic aberration. In fact, the GCS flowers showed no clear morphological changes except on the corolla segments. The global gene expression in the GCS segment was similar to that of the WT corolla, not to that in the WT calyx (Figs. 4 and S2), suggesting that a limited number of genes involved in the establishment of calyx segment-like morphogenesis was up-regulated by the partial loss of the *FBP2* protein. The E function mediated by the *FBP2* gene was fulfilled by the residual *FBP2* protein in most parts of the GCS floral organ, which allowed the establishment of the B and C floral-organ identity functions. Only in the corolla segments an additional factor might interact with the *FBP2* protein in an *FBP2* protein dose-dependent manner. Possibly, the *FBP2* protein level gradually decreases towards the corolla segment in the WT plants as it does in the GCS plants, but arrives at a threshold of functional insufficiency in the GCS plants not in the WT plants.

The spatial pattern of the green site in corolla segments of the GCS plant resembles the pattern of the whitish area observed in the *P. × hybrida* cultivar Picotee, suggesting the presence of a factor conferring spatial information in the corolla segments. It would be interesting to identify this factor and analyse its relation to *FBP2*, both in the GCS and Picotee phenotypes. This unique properties caused by *fbp2-3* would be attractive for breeders because of a limited aberration in flower organs. The flower shape, size and fertility were nearly as in the WT, which allow us to use the GCS phenotype as a new genetic source of a bi-colored flower.

## References

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new gen-

- eration of protein database search programs. *Nucleic Acids Res* 25:3389–3402
- Ando T, Hashimoto G (1993) Two new species of *Petunia* (Solana-ceae) from southern Brazil. *Bot J Linn Soc* 111:265–280
- Angenent GC, Busscher M, Franken J, Mol JNM, Van Tunen AJ (1992) Differential expression of two MADS box genes in wild-type and mutant petunia flowers. *Plant Cell* 4:983–993
- Angenent GC, Franken J, Busscher M, Weiss D, Van Tunen AJ (1994) Co-suppression of the petunia homeotic gene *fbp2* affects the identity of the generative meristem. *Plant J* 5:33–44
- Bailey LH (1896) Evolution of the *Petunia*. In: The survival of the un-like, Macmillan, London, pp 465–472
- Esau K (1977) Anatomy of seed plants, 2nd edn. Wiley, New York
- Fedoroff NV (1989) Maize transposable elements. In: Howe M, Berg P (eds) Mobile DNA. American Society for Microbiology, Wash-ington, pp 375–411
- Ferrario S, Immink RG, Shchennikova A, Busscher-Lange J, Angenent GC (2003) The MADS box gene *FBP2* is required for SEPALLA-TA function in petunia. *Plant Cell* 15:914–925
- Hirai S, Oka S, Adachi E, Kodama H (2007) The effects of spacer sequences on silencing efficiency of plant RNAi vectors. *Plant Cell Rep* 26:651–659
- Jernstedt UA, Cutter EG, Gifford EM, Lu P (1992) Angle meristem ori-gin and development in *Selaginella martensii*. *Ann Bot* 69:351–363
- Kodama H, Hamada T, Horiguchi G, Nishimura M, Iba K (1994) Ge-netic enhancement of cold tolerance by expression of a gene for chloroplast omega-3 fatty acid desaturase in transgenic tobacco. *Plant Physiol* 105:601–605
- Lassner MW, Peterson P, Yoder JI (1989) Simultaneous amplification of multiple DNA fragments by polymerase chain reaction in the analysis of transgenic plants and their progeny. *Plant Mol Biol Rep* 7:116–128
- Nagira Y, Shimamura K, Hirai S, Shimanuki M, Kodama H, Ozeki Y (2006) Identification and characterization of genes induced for anthocyanin synthesis and chlorophyll degradation in regenerated torenia shoots using suppression subtractive hybridization, cDNA microarrays, and RNAi techniques. *J Plant Res* 119:217–230
- Paxton J (1836) *Petunia nyctaginiflora violacea*. *Paxton's Mag Bot* 2:173
- Pelaz S, Ditta GS, Baumann E, Wisman E, Yanofsky MF (2000) B and C floral organ identity functions require *SEPALLATA* MADS box genes. *Nature* 405:200–203
- Shimamura K, Ishimizu T, Nishimura K, Matsubara K, Kodama H, Watanabe H, Hase S, Ando T (2007) Analysis of expressed se-quence tags from *Petunia* flowers. *Plant Sci* 173:495–500
- Shimanuki M, Shimamura K, Hirai S, Nishiuchi T, Suzuki K, Kodama H (2005) Polyethylene glycol-mediated enhancement of the hybridization rate on cDNA microarrays. *Anal Biochem* 344:284–286
- Vandenbussche M, Zethof J, Souer E, Koes R, Tornielli GB, Pezzotti M, Ferrario S, Angenent GC, Gerats T (2003) Toward the analysis of the petunia MADS box gene family by reverse and forward transposon insertion mutagenesis approaches: B, C, and D floral organ identity functions require *SEPALLATA*-like MADS box genes in petunia. *Plant Cell* 15:2680–2693