A Novel “Petaloid” Mutant of *Torenia* (*Torenia fournieri* Lind. ex Fourn.) Bears Double Flowers through Insertion of the DNA Transposon *Tf1* into a C-class Floral Homeotic Gene

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A double-flowered torenia (*Torenia fournieri* Lind. ex Fourn.) mutant, “Petaloid”, was obtained from selfed progeny of the “Flecked” mutant, in which the transposition of the DNA transposon *Tf1* is active. A normal torenia flower has a synsepalous calyx consisting of 5 sepals, a synpetalous corolla consisting of 5 petals, 4 distinct stamens, and a syncarpous pistil consisting of 2 carpels. In contrast, a flower of the “Petaloid” mutant has 4 distinct petals converted from stamens, whereas the calyx, corolla, and pistil remain unchanged. The double-flower trait of the “Petaloid” mutant was unstable; some or all of the 4 petals converted from stamens frequently reverted to stamens. Furthermore, most S_1 plants obtained from self-pollination of the somatic revertant flower bore only normal single flowers. In petals converted from stamens, expression of the C-class floral homeotic gene *T. fournieri* *FARINELLI* (*TfFAR*) was almost completely inhibited. This inhibition was caused by insertion of *Tf1* into the 2nd intron of *TfFAR*, whereas reversion of converted petals to stamens was caused by excision of *Tf1* from *TfFAR*. The clear correspondence of the *TfFAR* genotype to the floral phenotype suggested that homozygous *TfFAR* alleles with the *Tf1* insertion caused the mutant phenotype. In contrast, *TfFAR* was moderately expressed in the pistil of the “Petaloid” mutant, leaving the pistil unchanged. We succeeded in inactivating *Tf1* transposition by cross-pollination between mutant and normal-type plants to genetically separate the transposon *Tf1* from the unidentified factor activating its transposition, which made the “Petaloid” mutation more stable.

Key Words: *En/Spm* superfamily, *euAG* lineage gene, mutable allele, *TfFAR*.

Introduction

Torenia (*Torenia fournieri* Lind. ex Fourn.) is primarily used as a bedding or potted plant in the summer in Japan. *Torenia* plants branch vigorously, and bear many flowers, forming a neat, compact arrangement (Morise, 2001; National Agriculture and Food Research Organization, 2006). *Torenia* grows well under a wide range of light intensities, from direct sunlight to weak light in the shade, and is also highly tolerant of high temperatures during mid-summer, which have been increasing by recent climate change. Breeding for flower colour variation has produced torenia cultivars with

white, pink, red, and yellow flowers, in addition to the original violet flower of the wild *T. fournieri*. Several breeding techniques have been employed to further extend the genetic variation of torenia. Interspecific crosses among *Torenia* species have increased the variety of flower colours (Miyazaki et al., 2007). However, fertile hybrids from interspecific crosses are limited, and variation in floral morphology is relatively poor among *Torenia* species. Although heavy ion-beam radiation and genetic transformation had increased the available variation in flower colour and morphology of *T. fournieri* (Aida et al., 2000; Sasaki et al., 2008), variation in floral morphology remains limited. Recently co-overexpression of B-class floral homeotic genes *TfDEF* (*TfDEFICIENS*) and *TfGLO* (*TfGLOBOSA*) induced conversion of sepals into petaloid organs, resulting in a kind of double flower (Sasaki et al., 2014). However, commercial use of transgenic torenia would be difficult at least in Japan because of the substantial

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cost for official registration and potential negative public perception (Oshima et al., 2010). Thus, there have been no double-flowered or large flowered torenia cultivars. If this limitation could be overcome, and a highly attractive appearance could be combined with the excellent growth properties mentioned above, the commercial value of torenia would increase significantly.

Although double flowers can result from several different morphological changes, the most frequently observed is the conversion of stamens and carpels into petals (Nishijima, 2012). This conversion is induced by a reduction in C-class gene function in floral homeotic genes. Floral organ identity is determined based on the mechanism described by the ABC model (Bowman et al., 1991; Coen and Meyerowitz, 1991). Combined A- and B-class gene expression establishes petal identity in whorl 2, and B- and C-class gene expression establishes stamen identity in whorl 3. Carpel identity is established by C-class gene expression alone in whorl 4. A- and C-class genes antagonistically control the expression of each other (Drews et al., 1991; Gustafson-Brown et al., 1994), and avoid overlap of A- and C-class gene expression. Thus, a reduction in C-class gene function permits A-class gene function in whorl 3, resulting in the conversion of stamens into petals. Further reduction in C-class gene function induces the indeterminate development of flowers in whorl 4 because reduction in C-class gene function promotes *WUS* expression in the floral meristem, preserving the meristematic competence of the floral bud, and inducing indeterminate development (Lenhard et al., 2001; Lohmann et al., 2001). These mechanisms were elucidated mainly in *Arabidopsis thaliana* and *Antirrhinum majus*. With regard to the reduction or loss of C-class gene function in *A. majus*, which belongs to Lamiales as torenia, a loss-of-function mutant of the C-class gene *PLENA* (*PLE*) causes the conversion of stamens into petals in *A. majus* (Bradley et al., 1993). Furthermore, double mutation of *ple* and another C-class gene, *farinelli* (*far*) decreases determinacy of flower development, inducing double flowers with many petaloid organs.

Spontaneous mutations caused by transposable elements have been important mutagens in breeding floricultural plants (Inagaki et al., 1994; Matsubara et al., 2005; Nakajima et al., 2005; Nitasaka, 2003, 2007). However, transposable elements with high transposition activity often produce unstable traits (e.g. Goodrich et al., 1992; Inagaki et al., 1994). If transposition activity is moderate, and does not disrupt the practical uniformity of cultivars, transposable elements function as a useful mutagen for breeding (Matsubara et al., 2005; Nakajima et al., 2005; Nitasaka, 2003, 2007). We previously isolated a torenia mutant in which transposition of an *Enhancer/Suppressor-Mutator* (*En/Spm*)-like transposon, *Tifl* (*Transposon T.fournieri 1*), is activated (Nishijima et al., 2013). In this mutant, named

“Flecked”, *Tifl* is inserted in the 2nd intron of *TfMYB1*, which encodes a R2R3-MYB transcription factor promoting anthocyanin biosynthesis in petals. The insertion of *Tifl* reduces *TfMYB1* expression, which fades petal colour from violet to pale violet. Spontaneous excision of *Tifl* from *TfMYB1*, i.e. somatic reversion, causes small violet spots, semicircular violet sectors, or solid violet petal limbs. Self-pollination of complete somatic revertant flowers with solid violet petal limbs results in a high proportion of germinal revertant S_1 plants. In the S_1 plants, the allele in which *Tifl* is newly integrated may become homozygous, and the plants may express the new mutant phenotype.

We isolated, from the selfed progeny of “Flecked” mutant, a double-flowered mutant in which stamens were converted to petals. This is the first report of a double-flowered torenia mutation. This mutant, named “Petaloid”, is unstable, like the mutant “Flecked”. In this paper, we report the molecular mechanism underlying the double-flower trait of this mutant. Furthermore, we propose a method to stabilize the mutant trait in order to use the *Tifl*-induced mutants as breeding material.

Materials and Methods

Plant material and isolation of the “Petaloid” mutant

For isolation of the “Petaloid” mutant of torenia (*T. fournieri* Lind. ex Fourn.), flowers with apparent somatic reversion, i.e. flowers of the “Flecked” mutant with a solid violet lower lip (Nishijima et al., 2013) were self-pollinated by hand. We pollinated 2 flowers per plant and approximately 280 plants in total. Approximately 2000 S_1 plants were screened for novel mutations. The growth conditions of the plants were as follows. The seeds were planted in 288-cell plug trays (54 cm in length and 28 cm in width; each cell 1.8 cm in length and width and 3 cm in height) filled with a horticultural medium (Primemix; Sakata Seed Co. Ltd., Tokyo, Japan). To isolate novel mutants, seedlings were transplanted into 72-cell plug trays (54 cm in length and 28 cm wide; each cell 4 cm long, 4 cm wide and 6 cm high) filled with Kureha-Engei-Baido (Kureha Chemical Industry Co. Ltd., Tokyo, Japan) and Primemix [1:1 (v/v)]. Seedlings for genetic and molecular analysis and stabilization of the double-flowered trait, as described below, were planted in pots (9 cm in diameter and 12 cm deep; 3 seedlings per pot) filled with the same medium as that used in the 72-cell plug trays to obtain highly stable and uniform growth. The plants were grown in a glasshouse with natural sunlight. The temperature was kept between 18°C and 32°C. A slow-releasing coated fertilizer (Ecolong 100; Chisso Co., Tokyo, Japan) was used for top-dressing.

Microscopic observation

Young flower buds were fixed in a mixture of 70% (v/v) ethanol and 5% (v/v) acetic acid at 4°C overnight,

and then dehydrated through a graded ethanol series and transferred to *t*-butanol/ethanol (1:1 v/v). The samples were then transferred to 100% *t*-butanol, freeze-dried, and subjected to scanning electron microscopy (SEM) (VE-7800; Keyence, Osaka, Japan). Floral stages were identified according to Nishijima and Shima (2006). Surface structures of mature floral organs were observed by directly subjecting the raw samples to SEM.

Genetic analysis

A single normal type (NT) flower of “Flecked”, mutant-type (MT), and apparent somatic revertant (SR) flowers of “Petaloid” were cross-pollinated by hand in the combinations shown in Table 1. Six flowers from each of the seed and pollen parent plants were used for crossing.

Expression analysis of floral homeotic genes

To investigate expression of floral homeotic genes in NT, MT, and SR flowers, the corolla, stamen, and pistil were collected from flower buds during the early and late portions of the petal development stage (stage 6; Nishijima and Shima, 2006). Corolla length was 4–6 and 14–16 mm early and late in stage 6, respectively. The samples were frozen in liquid nitrogen, and stored at -80°C until use. Total RNA was obtained using an RNeasy Plant Mini Kit (QIAGEN K. K., Tokyo, Japan). cDNA was synthesized using a PrimeScript 1st strand cDNA Synthesis Kit (TakaraBio, Shiga, Japan). For total RNA extraction for cDNA synthesis, we treated the samples with DNase to avoid potential contamination with genomic DNA. Gene-specific primers for *T. fournieri* floral homeotic genes and the actin gene [*T. fournieri* *ACTIN3* (*TfACT3*); AB330989], which was used as an internal control, were designed for the 3'-terminal regions of the open reading frame and the 3'-untranslated regions of each gene. Primer sequences and the lengths of PCR products used for the quantitative real-time PCR reactions were as follows: forward 5'-GCTTTGCTGCATGATGATATA-3' and reverse 5'-GCGTTGTTTTGTTGCATCT-3' for *TfSQUA* (*TfSQUAMOSA*, 151 bp; AB359949), forward 5'-GGTACTACTAATTTTCGTAGGG-3' and reverse 5'-

TAATATGGATCGAAATCATC-3' for *TfDEF* (103 bp; AB359951), forward 5'-CGAATCTTCAGGAACGTTTC-3' and reverse 5'-AAGGTTTTGGCTTAACGAGAG-3' for *TfGLO* (111 bp; AB359952), forward 5'-CCTTTGGCTGTTAGGATG-3' and reverse 5'-GACACAGCCCGAGTCGATGAG-3' for *TfPLE1* (172 bp; 359954), forward 5'-ATGGGATCCTCTGCTGATTAT-3' and reverse 5'-TTCAAATTGAACAACACATGG-3' for *TfFAR* (129 bp; AB359953) and forward 5'-TGCAGTAAAGTGTATTGTGGAAG-3' and reverse 5'-GGAATCTCTGGGTAGGATC-3' for *TfACT3* (145 bp). We previously isolated two other class-C floral homeotic genes, *TfPLE2A* (AB359955) and *TfPLE2B* (AB359956); however, we did not analyze these genes because their deduced amino acid sequences have marked truncation. Expression of the genes was quantified by a quantitative real-time PCR (qPCR) (Thermal Cycler Dice TP-800; TakaraBio) and SYBR Premix Ex Taq (TakaraBio). The reactions were cycled with a preincubation step of 10 s at 95°C , followed by 45 cycles of 5 s at 95°C and 30 s at 60°C . The raw data were analysed with Thermal Cycler Dice Real Time System Software (TakaraBio). Plasmids (pGEM-T Easy vector; Promega, Madison, WI, USA) harbouring cDNAs of the target genes were used to obtain standard curves. The ratio of the expression of each gene to that of *TfACT3* was calculated. The expression analysis was independently conducted in triplicate.

Molecular cloning of the gene responsible for the “Petaloid” mutation

Stamens from NT flowers, stamens converted to petals from MT flowers and stamens from SR flowers were collected late in stage 6. The samples were frozen in liquid nitrogen and stored at -80°C until use. Genomic DNA was extracted using a MagExtractor Plant Genome Kit (TOYOBO, Osaka, Japan). The genomic sequence, including the full-length open reading frames of class-C floral homeotic genes, was amplified by nested PCR using the following primer sets; *TfPLE1*, 1st PCR: forward 5'-CTGCAACTCTCTGTCCACAA-3' and reverse 5'-GAACAAAAGCCATGCAATGA-3', 2nd PCR: forward 5'-AAATCTCTTTCTTTCTCGCTCTC-3' and reverse 5'-CACTTAATAACGTGTGCG

Table 1. Trait segregation of F_1 and S_1 plants derived from crosses among normal type flowers (NT), mutant type flowers (MT), and flowers apparently with complete somatic reversion (SR).

Cross	Plant solely with the NT flower (No. of plants (%))	Plant with MT flower ^z (No. of plants (%))
MT × NT	48 (100)	0 (0)
NT × SR	51 (100)	0 (0)
SR × NT	47 (100)	0 (0)
MT × SR	31 (63)	18 (37)
SR × SR	43 (84)	8 (16)

^z Plants with MT flowers include ones bearing both MT and SR flowers within a plant.

ACAC-3'; *TfFAR*, 1st PCR: forward 5'-CTGCAACTCTCCTGTCCACAA-3' and reverse 5'-GTAAATAATTGTCCCTTGACTTC-3', 2nd PCR: forward 5'-TCAGAAAAGCAGAACTTGAGC-3' and reverse 5'-AATATAGCAGCAATCGAAGGTT-3'. The PCR fragments were cloned into pGEM T-easy vectors (Promega), and analysed using a DNA sequencer (ABI 3100 Genetic Analyser; Life Technologies Co., Foster City, CA, USA).

Analysis of TfFAR genotype correspondence to the mutant phenotype

S₁ plants descended from somatic revertant flowers were used. We analysed 12 S₁ mutant plants bearing both MT and apparent SR flowers on a single plant and 12 apparent germinal revertant (GR) plants bearing only revertant flowers. Petals converted from stamens were collected from MT flowers of mutant plants, whereas stamens were collected from apparent GR plants. Genomic DNA was extracted as described above. qPCR analysis was performed as described above to confirm the *TfFAR* genotype. In the following text, the allele of *TfFAR1* bearing the *TfI* insertion is indicated as *f^{TfI}*, whereas the allele of *TfFAR* without *TfI* insertion is indicated as *F*. We used the following 2 primer sets. Primer set A, forward 5'-ATTTTCTGTCGCCGATCC-3' and reverse 5'-CTGATACATCTGAACTCAGATCTGAAG-3', amplifies the 127-bp sequence between the right subterminal region of *TfI* and the 2nd intron of *TfFAR* in *f^{TfI}*, but does not amplify any sequence of *F*. Primer set B, forward 5'-GTAAAA CATTAGTAAAGGGCAAAGTGTC-3' and reverse 5'-CTGATACATCTGAACTCAGATCTGAAG-3' (identical to the reverse primer of set A), amplifies a 149-bp sequence in *F*, but does not amplify any sequence in *f^{TfI}* because the sequence to be amplified is too long (3042 bp). Quantitative real-time PCR analysis was performed as described above using genomic DNA as a template. Plasmids (pGEM-T Easy vector) harbouring genomic sequences of *f^{TfI}* and *F* were used to obtain standard curves. *TfACT3* was employed as an internal standard using the primers described above. The genomic sequence amplified by this primer set was the same length as the cDNA sequence.

Inactivation of a TfI excision from TfFAR

Pollen from "Common Violet" in which *TfI* transposition is inactive was placed on the stigmas of MT flowers of "Petaloid" by hand. The resultant 4 F₁ plants were self-pollinated to produce F₂ plants. Stability of the double-flower trait was evaluated in F₂ plants. The *TfFAR* genotype of F₂ plants was analysed by qPCR as described above. Petals converted from stamens from 3 mutant flowers per plant were analysed.

Results and Discussion

Isolation of the "Petaloid" mutant

We obtained a double-flowered mutant from among approximately 2000 S₁ plants of the "Flecked" mutant. Since this double-flowered mutant occasionally bore revertant flowers with normal stamens, we placed pollen from revertant flowers on the stigmas of mutant flowers to obtain M₂ seeds. A similar method was repeated in the following generations to maintain the mutant. Double-flowered M₄ plants were selected and used in the experiments.

Morphological characterization of the "Petaloid" mutant

A normal-type (Fig. 1, NT) torenia flower has 4 stamens, and the basal part of the filaments are fused to the corolla tube. The upper two stamens are located on the margin of the upper and lower lips, whereas the lower two are located on the lower lip (indicated by a black arrowhead with white margin in Figure 1). In contrast, the stamens of the double-flowered mutant (Fig. 1, MT, i.e. mutant type) were converted to petals (indicated by a white arrowhead with black margin in Figure 1). In NT flowers, the epidermal cells of the anther and filament were flat (Fig. 2E, F). In contrast, both the adaxial and abaxial epidermal cells were cone-like in petals converted from stamens (Fig. 2C, D), identical to other petal cells (Fig. 2A, B). These results suggest that stamens of the MT flower are completely converted to petals. The double-flower trait of the mutant was unstable; all 53 plants tested bore SR flowers until 42 days after the first flower opened. Once an SR flower developed, further apical flowers, seemingly generated from the same cell lineage, were mostly SR. Flowers with both stamens and petals converted from stamens (Fig. 1, IR, i.e. incomplete somatic revertants) occurred frequently, perhaps caused by localized reversion of the double-flowered trait. Because this mutant trait resembles that of the "Petaloid" of Japanese morning glory (*Ipomoea nil*), which is caused by mutation of a C-class floral homeotic gene (Nitasaka, 2007), we named the torenia mutant and its double-flowered trait "Petaloid", although the mutant trait of the "Petaloid" of Japanese morning glory is stable.

In developing flower buds, no morphological differences were observed between NT and MT plants before differentiation of the corolla, stamen, and pistil primordia (Fig. 3, in stage 2, the sepal initiation stage); however, in stage 4, the sex organ and corolla initiation stage, stamen primordia grew thicker than petal primordia in NT flower buds, whereas there were no clear morphological differences between petals converted from stamens and petals in MT flower buds. In stage 5, the early corolla development stage, globular anthers and plate-like petals were clearly distinguishable in NT flower buds; however, both petals converted from sta-

Fig. 1. Flower of the “Petaloid” torenia mutant. Upper column shows a front view of the flowers, and the lower column shows a side view of longitudinally bisected flowers. NT, normal type; MT, mutant type; IR, flower with incomplete somatic reversion; and SR, flower with complete somatic reversion. Black arrowhead with white margin indicates stamen, while white arrowhead with black margin indicates the petal converted from stamen. Black bar indicates 10 mm.

Fig. 2. Scanning electron microscopy of floral organs of the “Petaloid” mutant. Adaxial (A) and abaxial (B) petal faces of the normal-type flower; adaxial (C) and abaxial (D) faces of a petal converted from the stamen in the mutant-type flower; anther (E) and filament (F) of the normal-type flower.

mens and petals were plate-like in MT flower buds, although petals converted from stamens were narrower than petals. In stage 6, the middle corolla development stage, the pollen sac was clearly observable in stamens of NT flower buds, whereas petals developed a wide plate-like shape. In contrast, both petals converted from stamens and petals of MT flower buds developed the plate-like shape characteristic of petals. No differences in pistil development were observed between NT and MT flowers.

Genetic analysis of the double-flowered trait

All F_1 plants derived from the cross between the MT and NT flowers bore only NT flowers (Table 1). When an NT flower was crossed with an SR flower, all F_1 plants also bore only NT flowers. These results suggest that the double-flowered trait is recessive. In contrast, the cross between MT and SR flowers and self-pollination of an SR flower resulted in F_1 and S_1 plants bearing MT flowers (i.e. MT plants). In these combinations, the resulting percentage of MT plants was higher in the cross between MT and SR flowers than in the

Fig. 3. Floral development of the “Petaloid” mutant. NT, normal type; and MT, mutant type. Black arrowhead with white margin indicates the stamen, while white arrowhead with black margin indicates a petal converted from the stamen. P, petal; St, stigma; and Sp, sepal. White bar indicates 50 μ m.

self-pollinated SR flowers. If the double-flowered mutation is caused by a single recessive allele *d*, 50% of the F_1 plants derived from a cross between the MT (*dd*) and SR (*Dd*) flowers would be MT (*dd*) plants; however, the actual ratio of the MT F_1 plants was 37%. Similarly, 25% of the S_1 plants derived from self-pollination of the SR (*Dd*) flowers would be MT (*dd*) plants; however, the actual ratio of MT S_1 plants was 16%. The lower observed percentages of MT plants compared with the theoretical estimations indicates substantial reversion of the mutant allele.

Expression of floral homeotic genes in floral organs of the “Petaloid” mutant

In NT flower buds in early stage 6, petals had marked expression of the B-class floral homeotic genes *TfDEF* and *TfGLO*, as predicted by the ABC model; however, expression of the A-class floral homeotic gene *TfSQUA* was slight. Stamens showed marked expression of *TfDEF* and *TfGLO* and of the C-class floral homeotic genes *TfPLE1* and *TfFAR* (Fig. 4), whereas *TfPLE1* and *TfFAR* were the main floral homeotic genes expressed in pistils. These results are as predicted by the ABC model, except expression of *TfSQUA* was slight in petals. The expression profile of these genes in stamens and pistils were essentially the same in late stage 6 (Fig. 5). At this stage, the tube and limb of petals were separately analysed. Marked expression of *TfDEF* and *TfGLO* was observed both in the tube and limb, but only slight or no *TfSQUA* expression was detected as in petals in the early stage 6 (Fig. 4). Although *TfSQUA* is markedly expressed in petal primordia (Niki et al., 2012), our results show that *TfSQUA* is barely ex-

pressed in more developed petals.

The basal portion of the stamen is fused to the corolla tube in late stage 6, meaning each tube sample contains a small amount of filament; however, the expression of *TfPLE1* and *TfFAR*, which are expressed in stamens, was very low in the corolla tube. This is possibly because expression of C-class genes is relatively low in filaments, as shown in *A. majus* (Yamaguchi et al., 2010).

In petals converted from stamens in MT flower buds, expression of *TfPLE1* and *TfFAR* almost completely disappeared in both early and late stage 6, whereas *TfDEF* and *TfGLO* were markedly expressed. Furthermore, *TfSQUA* was significantly expressed in early stage 6. Therefore, the expression profile of floral homeotic genes follows the “petal” pattern in early stage 6; it is clear that this gene expression profile converted stamens into petals.

In the pistils of MT flower buds, mainly *TfPLE1* and *TfFAR* were expressed, both in early and late stage 6; however, *TfFAR* expression was lower than in NT flower buds (Figs. 4 and 5). In contrast, the expression of *TfPLE1* was not lower than in NT flower buds. The expression profile of petals of MT flower buds was almost identical to that of NT flower buds, with the exception that in early stage 6, *TfSQUA* was significantly expressed (Fig. 4). The “Petaloid” mutation further decreased the slight *TfFAR* expression in the petals, although expression was too slight to clearly show the decrease in Figure 4. This decrease may have released repression of *TfSQUA* expression (Fig. 4) because expression of A- and C-class genes is reciprocally antagonistic (Drews et al., 1991; Gustafson-Brown et al., 1994).

Fig. 4. Expression of floral homeotic genes in young flower buds of the “Petaloid” mutant. Flower buds in the early petal development stage (early stage 6, 4–6 mm corolla length) were used for the experiment. NT, normal type; MT, mutant type; and SR, flower with complete somatic reversion. Vertical bars indicate SE ($n = 3$).

The expression profile of floral homeotic genes in SR flower buds was essentially identical to that of NT flower buds in both early and late stage 6 (Figs. 4 and 5); however, *TfSQUA* expression was higher in petals than in NT flower buds in early stage 6, likely due to lower levels of *TfFAR* expression in SR petals than in NT petals, although expression was too slight to clearly show the difference in Figure 4. It should be noted that *TfPLE1* expression was higher in stamens and pistils in SR flower buds than in NT flower buds.

Because stamens of “Petaloid” were converted to petals, it was expected that a C-class floral homeotic gene had mutated, as observed in the aforementioned “Petaloid” morning glory (Nitasaka, 2007). As expected, *TfPLE1* and *TfFAR* expression in MT petals converted from stamens were almost completely absent. In contrast, substantial expression of these genes remained in pistils, which may have inhibited the conver-

Fig. 5. Expression of floral homeotic genes in young flower buds of the “Petaloid” mutant. Flower buds in the late petal development stage (late stage 6, 14–16 mm corolla length) were used for the experiment. NT, normal type; MT, mutant type; and SR, flower with complete somatic reversion. Vertical bars indicate SE ($n = 3$).

sion of the pistil to new flowers, as observed in mild phenotypes of *agamous* (*ag*) of *Arabidopsis thaliana* (Yanofsky et al., 1990) and *plena* (*ple*) of *A. majus* (Carpenter and Coen, 1990).

Identification of the mutation responsible for the “Petaloid” phenotype

When the genomic sequence, including the full-length open reading frames of the C-class floral homeotic genes, was amplified by PCR, 4.0- and 4.8-kbp DNA fragments of *TfPLE1* and *TfFAR*, respectively, were detected in all of the NT, MT, and SR flowers, whereas an additional 7.7-kbp fragment of *TfFAR* was detected in the MT flower (Fig. 6). These results suggest the insertion of the 2.9-kbp element *Tf1* in *TfFAR* in MT flowers. Thus, we cloned the 4.8- and 7.7-kbp fragments of *TfFAR*. Sequence analysis of the 4.8-kbp fragment showed that *TfFAR* consists of 9 exons and 8

introns (Fig. 7A, LC075590). Further, sequence analysis of the 7.7-kbp fragment showed insertion of *Tf1* (2893 bp) in the long 2nd intron (2401 bp) of *TfFAR* with the left terminal placed in the 5' direction (Fig. 7A). The long 2nd intron is also known from the *AG* of *A. thaliana* and *PLE* of *A. majus*, which belong to C-class floral homeotic genes. There is evidence that these introns contain regulatory elements, which contribute to the control of spatial and temporal expression (Bradley et al., 1993; Busch et al., 1999; Deyholos and Sieburth, 2000; Hong et al., 2003; Sieburth and Meyerowitz, 1997). Insertion of a DNA transposon, *Tam3*, into the 2nd intron of *PLE* inhibits expression of, or induces ectopic expression of, *PLE* depending on the site and direction of *Tam3* insertion (Bradley et al., 1993). Inhibition of *PLE* expression by *Tam3* induces the conversion of stamens and carpels into petals and new flowers, respectively. Therefore, *Tf1* insertion into the 2nd intron of *TfFAR* in *torenia* inhibited *TfFAR* expression, which induced the conversion of stamens into petals.

It should be noted that *Tf1* insertion inhibited *TfFAR* expression to a greater extent in stamens than in pistils in early stage 6 (Fig. 4). Thus, *Tf1* insertion may have

affected organ-specific regulation of *TfFAR* expression. A regulatory element CARG box, which inhibits ectopic expression of C-class floral homeotic genes (Hong et al., 2003), was located 24-bp 5'-upstream of the *Tf1* insertion site of *TfFAR*. However, this structure does not appear to inhibit *TfFAR* expression through reduction of the CARG box function, which would induce ectopic expression of *TfFAR* (Hong et al., 2003). We could not find, near the *Tf1* insertion site, any other known regulatory sequences involved in spatial and temporal regulation of C-class floral homeotic genes including the *LEAFY* and *WUSCHEL* binding sites (Busch et al., 1999; Lohmann et al., 2001), AAGAAT box and CCAAT box (Hong et al., 2003).

The 3-bp target duplication sequences flanking *Tf1* (Fig. 7B, TTT) were not present in NT flowers, which were formed on the integration of *Tf1*, as observed in other *En/Spm* transposons (Gierl and Saedler, 1992). Since the target duplication sequence is TGA when *Tf1* is integrated in *TfMYB1* (Nishijima et al., 2013), the target sequence for *Tf1* integration is not only TGA, but also TTT. In SR flowers, the total target duplication sequence, TTTTTT, was converted to the new 3- and 4-bp sequences TTT and TTTT (Fig. 7B), which were generated as footprints of *Tf1* excision.

Correspondence of the *TfFAR* genotype with flower traits

To confirm whether the *TfFAR* genotype actually corresponds with the observed floral traits, S_1 segregants for the “Petaloid” trait were assessed to see whether they contained *TfFAR* with or without *Tf1* insertion, i.e. alleles f^{Tf1} or *F*, respectively, using qPCR. There was clear correspondence between the *TfFAR* genotype and the floral traits (Fig. 8). Most MT plants exhibited a high quantity of f^{Tf1} , although a small quantity of *F* was detected. This result indicates that the genotype of MT plants is essentially f^{Tf1}/f^{Tf1} , although a small amount of

Fig. 6. PCR products from genomic DNA, including full-length class-C floral homeotic genes. NT, normal type; MT, mutant type; and SR, flower with complete somatic reversion. M, molecular weight marker.

Fig. 7. Insertion of *Tf1* in *TfFAR*. A: Structure of *TfFAR* carrying *Tf1*. Open square, 5' UTR; grey square, coding region; horizontal line, intron. B: DNA sequence around *Tf1* insertion site of *TfFAR* and footprint sequences formed by excision of *Tf1* (TTTT and TTTTT). Sequences of TTT underlined in the *TfMYB1* sequence are 3-bp target duplications.

Fig. 8. *TfAR* genotypes analysed by qPCR in S_1 plants derived from somatic revertant flowers. The open column indicates a mutant-type allele with *TfI* insertion (f^{TfI}), while the grey column indicates a normal-type allele without *TfI* insertion (F). For clarity, data were arranged in descending order in quantities of f^{TfI} of mutant type plants and ascending order in quantities of F of revertant plants.

somatic revertant tissue is included. In contrast, all GR plants exhibited a high quantity of F . f^{TfI} was detected in 8 out of 12 GR plants, suggesting they have the F/f^{TfI} genotype, whereas the other 4 GR plants had no detectable f^{TfI} , suggesting they have the F/F genotype. Overall, we concluded that the “Petaloid” mutation is recessive; homozygous f^{TfI}/f^{TfI} genotype causes the “Petaloid” phenotype, whereas the F/f^{TfI} and F/F genotypes cause the normal phenotype.

Inactivation of *TfI* transposition

Since *TfI* is a non-autonomous element, the genetic separation of *TfI* from the unidentified factor activating *TfI* transposition may stabilize *TfI*-caused mutations, as shown in rice cotransfected with transposon *Ds* and the transposase gene *Ac* of maize (Shimamoto et al., 1993). Therefore, we crossed the “Petaloid” mutant and ‘Common Violet’ in which the *TfI* transposition is inactive. All 48 F_1 plants bore single flowers (Table 2). In F_2 plants descended from self-pollination of 3 F_1 plants, 6 of 60 plants (10%) bore double flowers and 54 of 60 plants (90%) bore only single flowers. Within the first 40 flowers opened per plant, the 4 unstable plants bore 18–30 (36%–75%) revertant flowers, while 2 moderately stable plants bore 2 and 4 (5% and 10%, respectively) revertant flowers. qPCR analysis of genomic DNA of those double-flowered plants showed that 2 moderately stable double-flowered plants had the complete f^{TfI}/f^{TfI} genotype with no reversion to F (Fig. 9), while the 4 unstable plants showed substantial F allele levels in mutant flowers analysed. Therefore, phenotypic reversion observed in the 2 moderately stable plants is not likely due to genetic reversion but to inconsistency of the double-flowered phenotype expressed by the far^{TfI}/far^{TfI} genotype.

These results suggest that the mutability of the double-flowered trait was reduced through the separation of the *TfI* insertion and the unidentified activator of *TfI* transposition. The “Petaloid” mutation is recessive

Fig. 9. Quantification of *TfAR* genotypes in mutable and moderately stable double-flowered F_2 plants derived from a cross between “Petaloid” mutant and normal type plant. The open column indicates a mutant-type allele with *TfI* insertion (f^{TfI}), while the grey column indicates a normal-type allele without *TfI* insertion (F). Numbers in horizontal axis indicate plant number. Vertical bars indicate SE ($n = 3$).

as discussed above, while trait of active *TfI* transposition inherits dominantly. Thus, 19% mutable and 19% stable double-flowered plants should have theoretically occurred in the F_2 generation; however, only 7% mutable and 3% stable double-flowered plants occurred in our experiment. This result suggests that *TfI* was excised from *TfAR* during growth and reproduction.

Function of *torenia* C-class floral homeotic genes

In angiosperms, a duplication event in the *AG* family resulted in the *euAG* and *PLE* lineages within the core eudicots (Kramer et al., 2004). Genes in the *euAG* and *PLE* lineages show species-dependent functional differences. Genes belonging to the *euAG* lineage, such as *AG* of *A. thaliana* (Favaro et al., 2003; Yanofsky et al., 1990), *TOMATO AGAMOUS1* of the tomato (Pnueli

et al., 1994), *PMADS3* of the petunia (Kapoor et al., 2002, 2005; Kater et al., 1998; Tsuchimoto et al., 1993), and *DUPLICATED* of the Japanese morning glory (Nitasaka, 2003) are necessary for stamen and carpel development. In contrast, genes belonging to the *PLE* lineage, such as *SHATTERPROOF1/2* of *A. thaliana*, are necessary for dehiscence zone formation in the silique (Liljegren et al., 2000; Pinyopich et al., 2003). *TOMATO AGAMOUS-like1* of the tomato is necessary for fruit development and maturation (Itkin et al., 2009; Vrebalov et al., 2009). The functions of *FBP6* of petunia (Kater et al., 1998) and *PEONY* of Japanese morning glory (Nitasaka, 2003) are unknown.

However, *AG*-family genes in *A. majus*, which like *torenia* belongs to the Lamiales, have the opposite functional assignment from those in the aforementioned 4 plant species; the *PLE*-lineage gene *PLE* is necessary for the development of stamens and carpels (Bradley et al., 1993; Carpenter and Coen, 1990), whereas the *euAG* lineage gene *FAR* is necessary for pollen development (Davies et al., 1999). *FAR* also has weak and partial function for the promotion of stamen and carpel development because a double mutation of *PLE* and *FAR* enhances homeotic conversion of carpels into petals and indeterminate development of flowers more extensively than mutation of *PLE* alone (Bradley et al., 1993). In summary, either *euAG*- or *PLE*-lineage genes function primarily as C-class floral homeotic genes, and genes of the other lineage either perform a supplementary C-class floral homeotic function, or have a different function.

In the present study, stamens of *torenia* were converted to petals through the inhibition of *TjFAR* expression caused by *Tjfl* insertion. This result suggests that *TjFAR* functions mainly for determination of stamen identity. This is contrary to the understanding that the *PLE* lineage functions mainly in the determination of stamen and carpel identity in *A. majus*, as mentioned above. Therefore, it is evident that this divergence in functional assignment of *AG*- and *PLE*-lineage genes occurred after the phylogenetic differentiation of Lamiales.

Although the function of *TjPLE1* has not yet been identified, inconsistency of double-flowered traits shown in the plant with stabilized *far^{Tjfl}/far^{Tjfl}* genotype may indicate that reduction of *TjFAR* function is not sufficient for complete conversion of stamens to petals; i.e., *TjPLE* functions partially for determination of stamen identity with *TjFAR*. In addition, it should be noted that *TjPLE1* expression decreased in petals converted from stamens (Figs. 4 and 5). In *A. majus*, homeotic conversion of floral organs into staminoid organs through ectopic expression of *PLE* induces expression of *FAR*, which is necessary for pollen development (Davies et al., 1999). In *Arabidopsis*, *AG* induces *SHP1/2* which are necessary for dehiscence zone formation in the silique as mentioned above (Gómez-Mena et al., 2004).

These findings indicate that either *euAG* or *PLE* lineage genes functioning as C-class floral homeotic genes induce the other lineage genes necessary for stamen and carpel development. Therefore, if *TjPLE1* performs a function necessary for stamen development, it follows that *TjPLE1* expression was reduced in petals converted from stamens (Fig. 4). Those 2 hypothesis for possible *TjPLE* function remain to be elucidated.

Future perspectives

Construction of transgenic plants overexpressing the chimeric *AG* repressor was previously attempted to induce double flowers in *torenia*, but resulted in only partial reduction of C-class function and incomplete conversion of stamens into petals (Narumi et al., 2008). The “Petaloid” mutant reported in the present paper is the first published *torenia* germplasm bearing double flowers with complete conversion of stamens into petals. Further, genetic separation between the *Tjfl* insertion responsible for the desired phenotype, and the unidentified factor activating *Tjfl* transposition was found to be effective to increase stability of the mutant trait.

Although the double-flowered mutation is currently caused solely by conversion of stamens into petals, it may be possible to further increase the ornamental value of double-flowered *torenia* by inducing the conversion of carpels into new indeterminate flowers. This result could be attained in the following 2 ways. One is to induce mutations that more strongly inhibit *TjFAR* function. In the Japanese morning glory, the reduction of C-class function by insertion of the transposon *Tpn* in the C-class gene *DUPLICATED* (*DP*) induces conversion of stamens into petals (Nitasaka, 2003, 2007). Furthermore, deletion of a large portion of *DP*, presumably as a result of incomplete excision of *Tpn* and subsequent illegitimate recombination of *DP*, induces formation of an indeterminate double flower. In the present study, carpels of *torenia* remained unaltered even when *TjFAR* expression was inhibited by *Tjfl* insertion, probably because *TjFAR* expression remained in the pistil, as discussed above. Thus, the null mutation of *TjFAR* may substitute indeterminately developed new flowers for the pistil. The other way is to obtain double mutants of *TjFAR* and *TjPLE1* to inhibit C-class floral homeotic function more intensively. This will be effective if *TjPLE1* has partial C-class function, similar to *FAR* of *A. majus*, as mentioned above (Davies et al., 1999).

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