Plant Biotechnology 25, 45–53 (2008)

Original Paper

Chimeric *AGAMOUS* repressor induces serrated petal phenotype in *Torenia fournieri* similar to that induced by cytokinin application

Takako Narumi[1], Ryutaro Aida[1], Tomoya Niki[1], Takaaki Nishijima[1], Nobutaka Mitsuda[2], Keiichiro Hiratsu[2,3], Masaru Ohme-Takagi[2], Norihiro Ohtsubo[1,*]

[1] National Institute of Floricultural Science, Tsukuba, Ibaraki 305-8519, Japan; [2] Research Institute of Genome-based Biofactory, National Institute of Advanced Industrial Science and Technology (AIST), Central 4, Tsukuba, Ibaraki 305-8562, Japan; [3] Department of Applied Chemistry, National Defense Academy of Japan, Yokosuka, Kanagawa 239-8686, Japan

* E-mail: nohtsubo@affrc.go.jp Tel & Fax: +81-29-838-6822

Received November 15, 2007; accepted December 17, 2007 (Edited by M. Sekine)

Abstract The homeotic protein AGAMOUS (AG) terminates the floral meristem and promotes the development of stamens and carpels in *Arabidopsis*. Disruption of its function or expression of the chimeric AG repressor (*AGSRDX*) results in redundant petals, known as a double flower phenotype. To investigate whether this morphological change in *Arabidopsis* is applicable to ornamental flowers to increase their horticultural value, we introduced *AGSRDX* into torenia (*Torenia fournieri* Lind.) plants. Transgenic torenia plants expressing *AGSRDX* showed no redundancy in petal number, although they exhibited serration in petal margins, anthocyanin accumulation and morphological change in the stigma surface, and formation of extra vascular bundles in petals and styles. Anatomical observation of petals and styles revealed that these phenotypes are highly similar to those of forchlorfenuron (CPPU)-treated torenia plants especially in the derangement of vascular bundles. Phenotypes similar to *AGSRDX* transgenic torenia plants were also observed when the chimeric repressors for torenia C-function genes *TfFAR* or *TfPLE1*, homologs of *Antirrhinum FARINELLI* and *PLENA* respectively, were expressed. These results suggest that the morphological changes in *AGSRDX* transgenic torenia plants are induced by the disruption of C-function. These novel phenotypes might be caused by the modification of cytokinin-dependent regulation in vascular bundle formation and ectopic expression of the chimeric repressors in all whorls by the cauliflower mosaic virus (CaMV) 35S promoter.

Key words: AGAMOUS, chimeric repressor, CRES-T, cytokinin, vascular bundle formation.

Flower shape is one of the most important characters for ornamental flowers. Floral morphology, such as double flowers and large-sized corollas, has been selected preferentially during the breeding of wild plants to produce floricultural crops.

Intensive studies on genetic and molecular analysis of floral homeotic genes have provided the ABC model that explains the development of floral structures depending on the action of three classes of floral homeotic genes, A, B, and C (Bowman et al. 1991; Coen and Meyerowiz 1991; Weigel and Meyerowiz 1994). These ABC floral homeotic genes function in overlapping domains to specify different floral organ identities. The ABC model of flower development was fine-tuned by adding two new functions, D and E, resulting in the ABCDE model (Pelaz et al. 2000, 2001). In the revised model, the D-function determines ovule development (Angenent et al. 1995; Pinyopich et al. 2003), while the E-function is required for development of all floral organs (Pelaz et al. 2000, 2001; Honma and Goto 2001; Piyopich et al. 2003; Ditta et al. 2004).

AGAMOUS (AG) is a transcription factor which belongs to the MADS-box family (Yanofsky et al. 1990), and provides the C-function including determination of floral meristem growth and specification of reproductive organ identity (Gómez-Mena et al. 2005). The loss-of-function *ag* mutant exhibit indeterminate growth of floral meristems, producing double flowers (Yanofsky et al. 1990; Bowman et al. 1991). Furthermore, the *dp* mutant of Japanese morning glory (Nitasaka 2003) and the *ple*/*far* double mutant of *Antirrhinum* (Davies et al. 1999) also exhibit double flower phenotypes. Mitsuda et al. (2006) have shown that the expression of a chimeric AG repressor (*AGSRDX*), which consists of the coding

Abbreviations: AG, AGAMOUS; AGSRDX, chimeric AGAMOUS repressor; AG-ox, AGAMOUS overexpressor; CRES-T, chimeric repressor gene-silencing technology; CPPU, forchlorfenuron (*N*-[2-chloro-4-pyridyl]-*N′*-phenylurea); FAR, FARINELLI; PLE, PLENA.

This article can be found at http://www.jspcmb.jp/

region of *AG* and a modified EAR-like motif repression domain sequence called the *SRDX* (Hiratsu et al. 2003), also induced double flowers similar to the *ag* mutant in *Arabidopsis*. This repression domain-dependent gene silencing procedure is called the *C*himeric *RE*pressor Gene-*S*ilencing *T*echnology (CRES-T). It enables transcriptional activators to convert into transcriptional repressors. The resulting product is dominant and suppresses target gene expressions to confer loss-of-function phenotypes at high frequency even in the presence of functionally redundant transcriptional activators (Hiratsu et al. 2003; Matsui et al. 2004, 2005; Mitsuda et al. 2005, 2006, 2007; Koyama et al. 2007). If AGSRDX can repress the C-function and induce double flowers consistently in ornamental flowers, it would be an effective tool for enhancing their commercial value.

In this paper, we report the modification of flower traits in torenia using the *AGSRDX* gene of *Arabidopsis*. The *AGSRDX* transgenic torenia plants (abbreviated as "*AGSRDX* plants" hereafter) exhibited strongly serrated petal margins and deformed stigmas, but no redundancy in petal number. The unexpected phenotypes observed in the transgenic plants resembled those induced by treatment with a synthetic cytokinin analog, forchlorfenuron (*N*-[2-chloro-4-pyridyl]-*N′*-phenylurea, CPPU), as reported recently by Nishijima and Shima (2006), especially in the formation of perturbed vascular bundles in petals and styles. The relationship between the observed morphological changes and the function of the AGSRDX in controlling such cytokinin-dependent morphological events is discussed.

Materials and methods

Plant material

Torenia fournieri Lind. cultivar 'Crown Violet' was used as a wild-type, non-transgenic control plant and the starting material for producing transgenic plants. Plants were maintained under sterile conditions in a plantbox supplemented with 1/2 Murashige and Skoog (MS) medium containing 0.2% gellan gum at 25°C under fluorescent light (16L/8D, 160 μmol m^{-2} s^{-1}), and propagated vegetatively by herbaceous cuttings as described previously (Aida and Shibata 2001).

Generation of transgenic plants

The *AGSRDX* gene was reported previously (Mitsuda et al. 2006). To construct the *AG-overexpressor* (*AG-ox*), the *AGSRDX* sequence was replaced with the *AG* coding sequence amplified from *AGSRDX*. Each construct contains a cauliflower mosaic virus (CaMV) 35S promoter followed by Ω translation enhancer sequence, nopaline synthase (NOS) terminator, the *att*L1 and *att*L2 recombination sites (Invitrogen Corp., Carlsbad, CA, USA) outside the regions of the CaMV35S promoter, and the NOS terminator in the pUC119 vector. These transgene cassettes were transferred into the destination vector pBCKK (Mitsuda et al. 2006), which was derived from the plant transformation vector pBIG-Km (Becker 1990) using the Gateway LR clonase reaction (Invitrogen Corp.). Each transgene vector was then introduced into torenia by *Agrobacterium*-mediated transformation as described previously (Aida and Shibata 2001). Transgenic plants regenerated *via* adventitious shoots were grown in a contained greenhouse at the National Institute of Floricultural Science (Tsukuba, Ibaraki, Japan). Fifty-one *AGSRDX* and 21 *AG-ox* transgenic torenia plants were used for the observations (Table 1).

RNA preparation and real-time PCR

Total RNA was isolated from leaves using the SV Total RNA Isolation System (Promega), and from sepals, petals, stamens, and carpels using RNeasy Plant Mini Kit (QIAGEN). Each floral organ was prepared from 7 pieces of flower buds at the stage when the petals reached 7 mm in size. First-strand cDNA was synthesized using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). Expressions of transgene and endogenous ABC-function genes, *TfSQUA* (A-function), *TfDEF* and *TfGLO* (B-function), and *TfFAR* and *TfPLE1* (C-function) were analyzed by real-time RT-PCR with the SYBR *Premix Ex Taq* (TaKaRa). Reactions were carried out using LightCycler ST300 system (Roche Diagnostics). A plasmid containing each transgene and the endogenous ABC-function genes sequence was used for the standard curve assay, and the levels of gene transcripts are provided as copy number per 50 ng of total RNA. Gene specific primers for real-time RT-PCR are as follows: *AGSRDX* and *AG-ox* forward, 5′-ACGGAATTATTTCCAAGTCGCGGC-3′; *AGSRDX* reverse, 5′-AGCGAAACCCAAACGGAGTTCTAG-3′; *AG-ox* reverse, 5′-AGACCGGCAACAGGATTCAATC-3′; *TfSQUA* forward, 5′-GCTTTGCTGCATGATGATATA-3′; *TfSQUA* reverse, 5′-GCGTTGTTTTGTTGCATCT-3′; *TfDEF* forward, 5′-GGTACTACTAATTTCGTAGGG-3′; *TfDEF* reverse, 5′-TAATATGGATCGAAATCATC-3′; *TfGLO* forward, 5′-GCAACCGAATCTTCAGGAACGTTT-3′; *TfGLO* reverse, 5′-GGTTTTGGCTTAACGAGAGACAGG-3′; *TfFAR* forward, 5′-ACTAACAACTATCCAGCGAGGCAC-3′; *TfFAR* reverse, 5′-ATGCATGCCCAATCTTCTGTAAGC-3′; *TfPLE1* forward, 5′-CCTTTGGCTGTTAGGATG-3′; and *TfPLE1* reverse, 5′-GACACAGCCCGAGTCGATGAG-3′. The expression level of these genes relative to the torenia *Actin3* (*TfACT3*) was calculated for each sample by triplicate experiments. Primers for *TfACT3* were as follows: forward, 5′-TGCAGTAAAGTGTATTGTGGAAG-3′ and reverse, 5′-GGAACTATCTGGGTAGGATC-3′.

Table 1. The comparison of the phenotype in *AGSRDX* and *AG-ox* transgenic torenia

Transgene	Number of transgenic plants	Serrated petal				Pistil-like sepal
		Normal	Mild	Moderate	Severe	
AGSRDX	51	19	8	22	2	0
AG-ox	21	7	0	0	0	14

The GenBank accession numbers for the cDNAs mentioned in this article are as follows: *TfSQUA*, AB359949; *TfDEF*, AB359951; *TfGLO*, AB359952; *TfFAR*, AB359953; *TfPLE1*, AB359954; *TfACT3*, AB330989.

Southern blot analysis

Genomic DNA was isolated from torenia leaves by the CTAB method (Doyle and Dickson 1987). Ten micrograms of torenia genomic DNA was digested with *Eco*R I or *Pst* I, separated on a 0.8% agarose gel, and transferred to a nylon membrane (Hybond N^+, Amersham Pharmacia Biotech). The full length *AGSRDX* cDNA was used as the probe. The DNA probe was labeled using the DIG DNA labeling kit (Roche). Hybridization signals were detected by chemiluminescence with CSPD-Star (Roche) as the substrate, and recorded on X-ray film (RX-U, Fuji Photo Film, Tokyo).

Application of cytokinin

CPPU (Sigma-Aldrich, Japan) was dissolved in 20% (v/v) aqueous acetone as described previously (Nishijima and Shima 2006). Eight microliters of 3.0 μM solution was applied to the apex of each inflorescence (n=5 inflorescences per treatment) of wild-type and *AGSRDX* plants. Aqueous acetone of 20% (v/v) without CPPU was used as a control.

Anatomical observations

For microscopic observations, petals and pistils were fixed with a mixture of ethanol, formalin, and propionic acid (8 : 2 : 1 v/v/v, FPA_{50}) and stored at 4°C. For observations of vascular bundles of petal and pistil, fixed samples were first immersed twice in 50% (v/v) ethanol for 30 min, twice in 30% (v/v) ethanol for 30 min, then incubated for 1 h in a chloral hydrate solution (chloral hydrate, 8 g; glycerol, 1 ml; distilled water, 2 ml) to make the tissues transparent. The samples were then examined under dark-field microscopy.

For observations of the stigma surface, fresh tissues were prepared and used for scanning electron microscopy (SEM) without fixing (VE-7000, Keyence Co., Osaka, Japan).

Results

The chimeric AG repressor induces serration in petals of torenia

In *Arabidopsis*, expression of *AGSRDX* resulted in the induction of redundant sepals and petals by the conversion of stamens and carpels into these organs (Mitsuda et al. 2006). To examine the function of AGSRDX derived from *Arabidopsis* in ornamental plants, we expressed *AGSRDX* in torenia. Unlike *Arabidopsis*, none of the 51 *AGSRDX* plants expressed a double flower phenotype, and 32 of them showed a distinctive serration of the petal margin and a reduction of corolla size (Figure 1B, C). Other external features such as plant height, size, leaf shape, and inflorescence development were the same as the wild type (Figure 1A). Furthermore, some of the *AGSRDX* plants exhibited greening of the petal tips, which might be induced by the transition of petals to sepal-like structures (Figure 1F, arrowheads).

On the other hand, 14 of 21 *AG-ox* transgenic torenia plants (abbreviate as "*AG-ox* plants" hereafter) were produced as repression domainless control plants, and exhibited undersized corolla and a narrow corolla tube (Figure 1B, C). The sepal tips of *AG-ox* transgenic torenia exhibited pistil-like structures (Figure 1D, arrowhead). This phenotypic change is similar to that induced by the ectopic expression of *AG* in *Arabidopsis* (Mizukami and Ma 1992). These results suggest that the novel phenotypes observed in the *AGSRDX* plants were induced by the chimeric AG repressor, although it did not induce the double flower in torenia.

Relationship between the transgene expression and phenotypic strength

The *AGSRDX* plants with altered phenotypes were classified into three groups based on the strength of petal serration. Out of the 51 transgenic plants, 8 had mild, 22 had moderate, and 2 had severe serrated petals (Table 1). To examine whether there is a correlation between the severity of phenotype and the expression level of the transgene, we analyzed the mRNA amount of *AGSRDX* using real-time PCR. Total RNA was extracted from the leaves of some typical lines in these three petal phenotype groups. As shown in Figure 2, expression of the *AGSRDX* gene was detected in all the transgenic plants used in this assay. However, the *AGSRDX* mRNA amount in each line did not reflect the strength of serrated petal phenotype (Figure 2), whereas the phenotype of *AG-ox* transgenic torenia depended largely on *AG* transgene expression (data not shown).

We also confirmed the integration and copy number of the transgene by Southern blot analysis. Because no restriction site of either *Eco*R I or *Pst* I exists within the transgene sequence, we used these two restriction enzymes for genomic DNA digestion. Six independent *AGSRDX* plants with various strength of serration phenotype were subjected to the experiment. As shown in Figure 3, two to three copies of the *AGSRDX* gene were found to be integrated in the genomic DNA of the *AGSRDX* plants. These results suggest that neither the amount of *AGSRDX* mRNA nor the copy number of the transgene correlated with strength of the serration phenotype, at least within the range observed in these experiments.

AGSRDX transgene affects the expression of torenia endogenous B-function genes

It is possible that the distinctive phenotypic changes observed in the *AGSRDX* plants are caused by some indirect effects of the chimeric repressor on the regulation of endogenous floral homeotic genes. To test this hypothesis, we investigated the expression of the *AGSRDX* transgene and endogenous floral homeotic

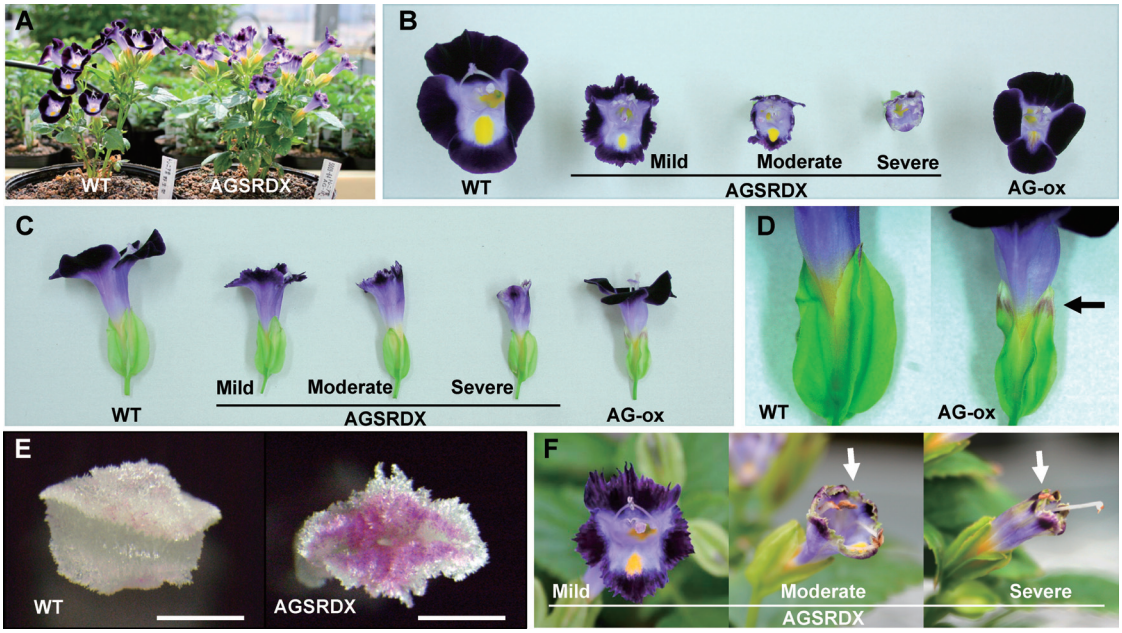

Figure 1. Comparison of phenotypes in wild-type, *AGSRDX*, and *AG-ox* transgenic torenia plants. (A) Wild-type *Torenia fournieri* 'Crown Violet' (left) and the *AGSRDX* plant (right). External phenotypes except for the floral organs appeared unchanged in the transgenic plants. (B) Comparison of corolla size, divergence, and petal shape. Typical mature flowers in wild-type torenia (left), the *AGSRDX* plants with mild, moderate, and severe phenotypes (central three images from left to right), and the *AG-ox* plants (right) are shown. (C) Comparison of flower size by lateral view of the same flowers as in B. (D) Sepals of wild-type (left) and of *AG-ox* transgenic plant with a pistil-like structure (right). (E) Stigma surfaces of wild-type (left) and *AGSRDX* plant (right). (F) Close-up photographs of the *AGSRDX* plants with mild (left), moderate (center) and severe (right) phenotype. Greening in petal tips are observed in the latter two. Bar=1 mm

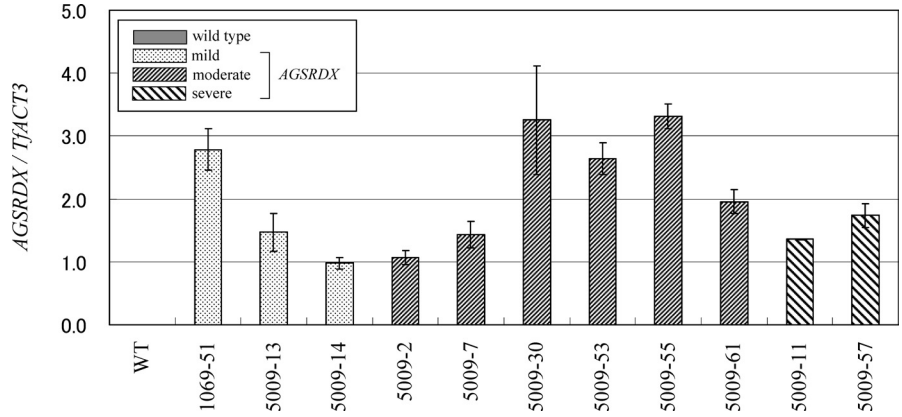

Figure 2. Expression of the *AGSRDX* transgene in wild-type and *AGSRDX* plants. Total RNA was prepared for the wild-type and 11 independent *AGSRDX* plants including 3 lines of mild, 7 lines of moderate, and 2 lines of severe phenotypes. RNA preparation and quantitative real-time RT-PCR reactions are described in Materials and methods. Expression of the *AGSRDX* transgene in each lines is indicated as a mean value ±SE.

genes in the four floral organs; sepal, petal, stamen, and pistil, respectively.

As shown in Figure 4A, there was no significant difference in the relative proportion of *AGSRDX* gene expressions between the four organs, and the expression in stamens and pistils was lower than those in sepals and petals. This whorl-specific expression reflects the nature of the CaMV35S promoter as reported by de Mesa et al. (2004). In that study, the *GUS* gene under the control of CaMV35S promoter showed low expression in the stamen and style in comparison to the other organs.

As shown in Figure 4B, *TfSQUA*, a homolog of *SQUAMOSA* in *Antirrhinum*, is expressed preferentially in sepals and petals (i.e., whorl 1 and 2), reflecting its native A-function. This is also the case for the class-B genes *TfDEF* and *TfGLO* (homolog of *Antirrhinum DEFICIENCE* and *GLOBOSA*, respectively) in whorl 2 and 3 (Figure 4C, D), as well as for the class C genes *TfPLE1* and *TfFAR* (homolog of *Antirrhinum PLENA* and *FARRINERRI*, respectively) in whorl 3 and 4 (Figure 4E, F) (Huijser et al. 1992; Tröbner et al. 1992; Egea-Cortines et al. 1999). Expression of the endogenous floral

homeotic genes in *AGSRDX* plants showed the same organ specificity as in the wild type, though their mRNA amounts were slightly reduced (Figure 4B–F). On the other hand, *TfDEF* transcripts in petals gradually decreased in parallel with the phenotypic strength of sepal-like structures (Figure 4C), in contrast to the equivalency in the *TfSQUA* expression (Figure 4B). It is probable that this sepal-like structure in the petal tips of *AGSRDX* plants is caused by the partial suppression of B-function through the reduction of *TfDEF* transcripts. In other words, reduction of B-function in whorl 2 allowed exhibition of an A-function-dependent phenotype in the petal margin. These results suggest that the phenotypic changes in the petals of *AGSRDX* plants were caused by the suppression of B-function by expressing *AGSRDX* in whorl 2 ectopically under the control of the CaMV35S promoter.

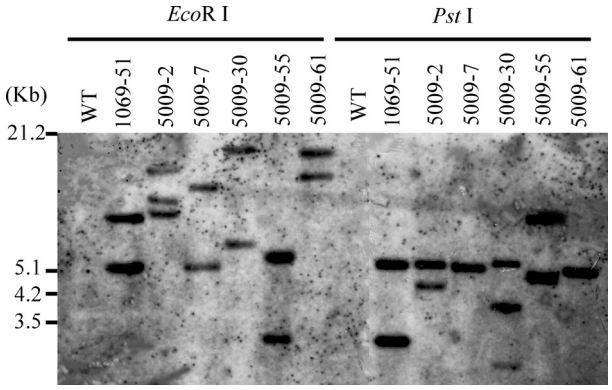

Figure 3. Copy numbers of integrated transgenes in *AGSRDX* plants. Three independent lines each were selected from the *AGSRDX* plants with mild (1069-51, 5009-2, 5009-7) and moderate (5009-30, 5009-55, 5009-61) phenotype. Genomic DNA preparation, enzymatic digestion, agarose gel electrophoresis, probe hybridization, and detection are described in Materials and methods.

Similarity in the phenotypes between AGSRDX plants and cytokinin-treated wild-type plants

The observations revealed that the serrated petal phenotype in *AGSRDX* plants is highly similar to that in the CPPU-treated torenia reported by Nishijima and Shima (2006). They have shown that the application of the synthetic cytokinin CPPU to torenia inflorescences induces strong serration on petal margins. To further investigate the morphological similarity between the *AGSRDX* plants and the CPPU-treated plants, we performed anatomical observations (Figure 5).

CPPU-treated wild-type torenia showed strong serration of petals (Figure 5B) as observed in the *AGSRDX* plants; however, the corolla size was not affected (Figure 5C). Application of CPPU to *AGSRDX* plants of mild phenotype intensified the morphological change and resulted in the severe serrated petal phenotype (Figure 5D). Vascular bundles in the wild-type petal spread uniformly over the tissue resulting in a smooth petal margin (Figure 5E). In contrast, petals of the *AGSRDX* plants and CPPU-treated wild-type plants showed thick and sparsely distributed vascular bundles (Figure 5F, G). In these petals, indentations were generated between the tips of vascular bundles, thereby forming serrated margins. Petals of *AGSRDX* plants treated with CPPU exhibited an extremely irregular distribution of vascular bundles, especially around the petal margin (Figure 5H). Imperfect unfolding of the corolla in the transgenic lines with the severe phenotype (Figure 1F, 5D) might be caused by these extra vascular bundles along the petal edge (Figure 5H).

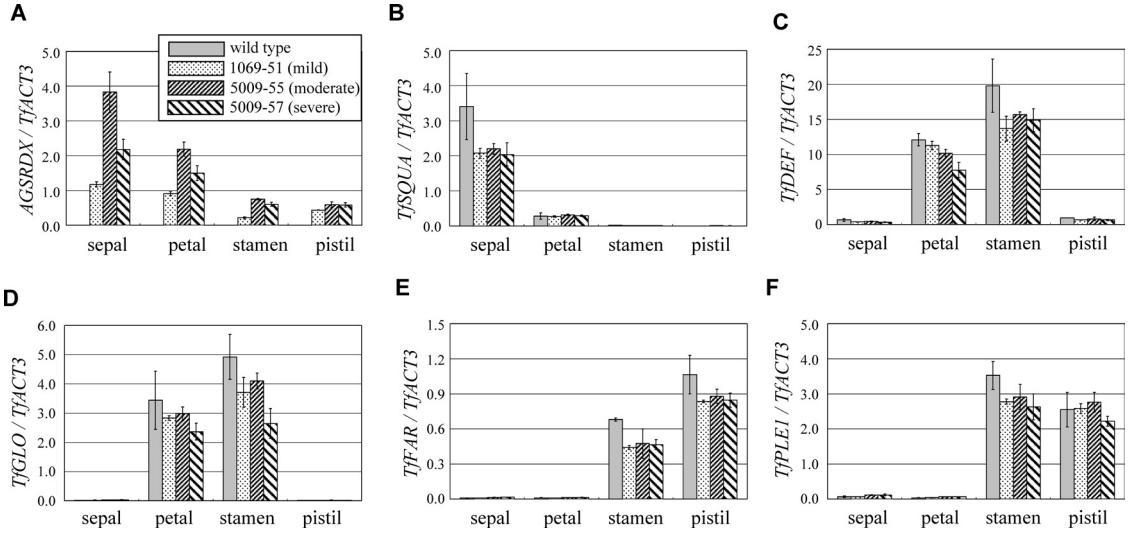

Figure 4. Expression of endogenous ABC-function genes in each floral organ of wild-type and *AGSRDX* plants. Seven flower buds of the *AGSRDX* plants with mild (1069-51), moderate (5009-55), and severe (5009-57) phenotype were independently dissected by each whorl, namely sepal, petal, stamen, and pistil, and then used for the preparation of total RNA. Quantitative real-time RT-PCR analyses were performed as described in Materials and methods. Expression of the *AGSRDX* transgene (A), endogenous A-function gene *TfSQUA* (B), B-function genes *TfDEF* (C) and *TfGLO* (D), and C-function genes *TfFAR* (E) and *TfPLE1* (F) are indicated as mean values ±SE.

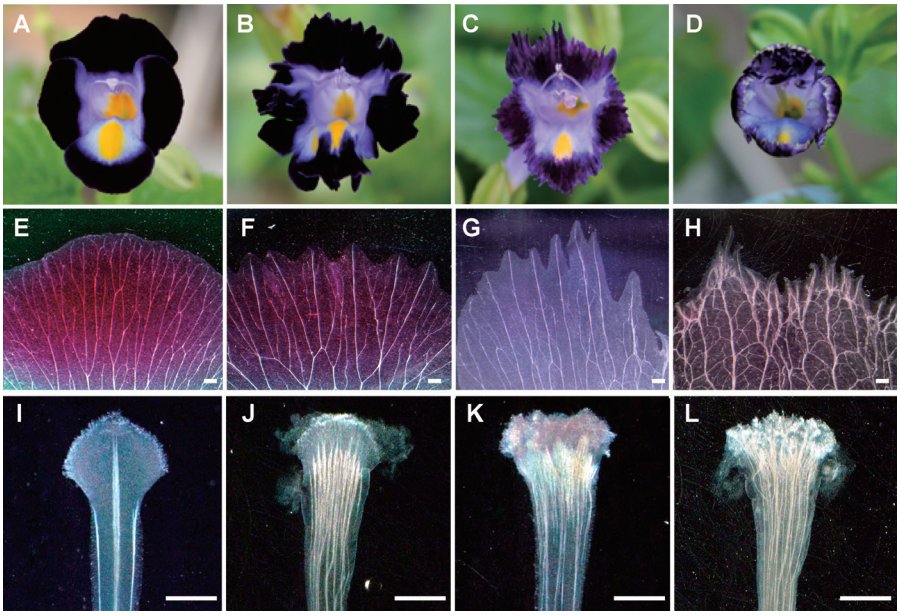

Figure 5. Morphological and anatomical comparison of serrated petals and styles in CPPU-applied plants and *AGSRDX* plants. Flower aspect, pattern of vascular bundles in petal and style of wild-type plants (A, E, I), CPPU applied torenia (B, F, J), *AGSRDX* plant (C, G, K), and CPPU-applied *AGSRDX* plant (D, H, L) are shown. Procedures for the CPPU application are described in Materials and methods. Bars=1 mm

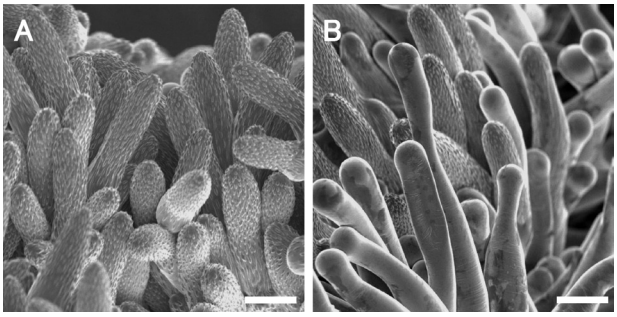

Figure 6. Morphological changes in the stigmatic surface of *AGSRDX* plants. Scanning electron microscopy was performed on freshly-prepared styles. Microscopic images of the stigma surface in wild-type (A) and the *AGSRDX* plant (B) are shown. Bars=28.5 μm

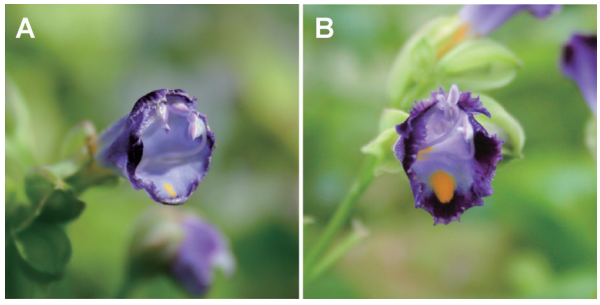

Figure 7. Phenotype of *TfFARSRDX* and *TfPLE1SRDX* transgenic torenia plants. Flower aspects of the transgenic plants, *TfFARSRDX* (A) and *TfPLE1SRDX* (B), are shown. Both showed the same phenotype as in *AGSRDX* plants.

Similarity in the phenotypic change was also observed in the styles of *AGSRDX* plants and CPPU-treated wild-type torenia plants, and both showed extensive formation of extra vascular bundles (Figure 5J, K). Application of CPPU to the styles of *AGSRDX* plants intensified the phenotype as it did in the petals (Figure 5L). Because this alteration appeared to affect the configuration of stigma cells as well, we performed scanning electron microscopy to observe the difference in epidermal cells of wild-type plants and *AGSRDX* plants in detail. As shown in Figure 6B, half of the stigma epidermal cells of the *AGSRDX* plant presented a smooth surface, whereas all the cells in the wild-type plant presented a rough surface (Figure 6A).

Chimeric torenia class C repressors confer the same phenotype as AGSRDX in torenia

It is suspected that the unexpected phenotypes observed in the *AGSRDX* plants are caused by the functional difference in the class C transcription factors between *Arabidopsis* and torenia. To test whether this is the case, we introduced chimeric repressors of *TfFAR* and *TfPLE1* into torenia, and observed their phenotypes. As shown in Figure 7, *TfFARSRDX* and *TfPLE1SRDX* transgenic plants showed almost the same phenotype as the *AGSRDX* plants, including serrated petals and smooth stigma epidermal cells. Therefore, it is probable that the observed morphological change in *AGSRDX* plants is not an *AGSRDX*-specific artificial event, and reflects the native function of the torenia class C genes.

Discussion

In this study, we generated transgenic torenia plants expressing the *Arabidopsis* chimeric *AG* repressor, *AGSRDX*, to confer a double flower phenotype by

repressing the endogenous C-function. Although we could not obtain a plant with redundant petals similar to that in *Arabidopsis*, morphological changes observed in whorl 4 suggested a partial suppression of the C-function. First, the accumulation of anthocyanin and the morphological change in stigma epidermal cells (Figure 1E, 6B) might be explained by the conversion of cell character from stigma to petal. Second, the extensive formation of extra vascular bundles in the style (Figure 5G), in addition to the morphological change in the stigma stated above, may reduce fertility, which suggests the loss of identity as a reproductive organ. In our preliminary experiment, *AGSRDX* plants crossed with wild-type pollen showed a reduction of fertility due to the disruption of pollen germination and pollen tube elongation, thereby producing only 2 seeds per flower on average (data not shown). On the other hand, the *Arabidopsis*-derived *AGAMOUS* gene is assumed to have a C-function in torenia because *AG-ox* transgenic torenia exhibited the same phenotype as *AG*-overexpressing *Arabidopsis* (Mizukami and Ma 1992) as well as the wheat *AGAMOUS* homolog (*WAG*)-expressing torenia (Aida et al. 2003). Thus, the observed phenotypic changes in stigma epidermal cells and styles might be caused by the suppression of torenia endogenous C-function by *AGSRDX*, and the suppression appeared to be partial.

Torenia C-function genes *TfFAR* and *TfPLE1* are considered to have the same functions in promoting stamen and carpel development because *TfFARSRDX* and *TfPLE1SRDX* transgenic torenia plants exhibited serrated petals similar to that in *AGSRDX* plants, as shown in Figure 7. In *Antirrhinum*, a loss-of-function *ple* mutant leads to the homeotic conversion of reproductive organs to perianth organs and *far* mutants develop normal flowers with partial male sterility. In contrast, the fourth whorl organs of *ple*/*far* double mutants were replaced with well-formed petals. Therefore, the *PLE*, in addition to the redundancy and regulatory interaction with *FAR*, is considered to mainly control reproductive organ development (Davies et al. 1999). On the other hand, Japanese morning glory (*Ipomoea nil*) exhibits double flowers as in the case of *Arabidopsis*, when the floral homeotic gene *DUPLICATED* (*DP*) of the C-function is disrupted (Nitasaka 2003). Thus, functions of class C genes are slightly different among these plant species in the role of floral organ development.

It is also possible that the serrated petal in *TfFARSRDX* and *TfPLE1SRDX* transgenic torenia as well as *AGSRDX* plants were caused by the ectopic expression of the chimeric repressors in other whorls under the control of the CaMV35S promoter. To further understand the C-function in torenia, it would be necessary to express these chimeric repressors under the control of the native promoters. This will probably make it possible to generate double flowers and reveal the cryptic function of *TfFAR* and *TfPLE1*, in addition to the functional conservation of class C genes between *Arabidopsis* and torenia.

These phenotypes with serrated petals closely resembled those of CPPU-treated torenia reported previously by Nishijima and Shima (2006). They showed that the phenotype of the CPPU-treated torenia flower is highly influenced by the timing of CPPU application, and the serrated petal margin is induced when CPPU was applied to the later stage of floral bud formation. Induction of serrated petals in *AGSRDX* plants might be also explained by a specific increase in endogenous cytokinin at this stage or by induction of gene expression involved in the regulation of cytokinin-dependent vascular bundle formation. In our preliminary experiment, expression of a cytokinin oxidase gene in *AGSRDX* petals was slightly reduced as compared to wild-type petals (data not shown). This may lead to the accumulation of active endogenous cytokinins as reported by Bilyeu et al. (2001), thereby inducing the phenotypic change in *AGSRDX* plants.

Cytokinin has been shown to affect floral organ development and related gene expression. The application of N^6-benzylaminopurine (BA) increased the number of petals in carnation (Jeffcoat 1977) and induced an enlargement of the floral meristem in *Arabidopsis* (Venglat and Sawhney 1996). In tobacco, expression of endogenous homeotic genes homologous to *DEFA*, *GLO*, and *PLE* of *Antirrhinum majus* were suppressed when cytokinin content was increased by an *ipt*-overexpressing transgene (Estruch et al. 1993). It is possible that the reduction of *TfDEF* transcripts in the *AGSRDX* plant in Figure 4C is caused by an increase in endogenous cytokinin. This hypothesis is also applicable to explain the greening of petal tips in *AGSRDX* plants (Figure 1F, arrowheads). Suppression of the torenia B-function genes by cytokinin may lead to the transition of organ identity from petals to sepals as in the *Arabidopsis ap3* mutant (Jack et al. 1992; Riechmann and Meyerowitz 1997), *Antirrhinum def* and *glo* mutants (Tröbner et al. 1992; Egea-Cortines et al. 1999), and petunia *phglo1* and *phglo2* mutants (Vandenbussche et al. 2004). Thus, the alteration of vascular pattering in petals and styles of *AGSRDX* plants is probably caused by the effect of the transgene *via* the plant hormone cytokinin. Investigation of cytokinin-responsive gene expressions in *AGSRDX* plants may provide helpful information to understand the mechanism of cytokinin-dependent regulation of petal formation.

The CRES-T system has been shown to be an effective method for facilitating functional analysis of redundant transcription factors (Hiratsu et al. 2003; Matsui et al. 2004, 2005; Mitsuda et al. 2005, 2006, 2007; Koyama et al. 2007), and *Arabidopsis* chimeric repressors are con-

sidered to be effective in a wide variety of plant species because transcription factors are fairly conserved among them (Mitsuda et al. 2006). We have succeeded in conferring the phenotypes observed in the chimeric repressor-expressing *Arabidopsis* by introducing *TCP3SRDX* in torenia and chrysanthemum, as well as *EIN3SRDX* in chrysanthemum (unpublished data). Although *AGSRDX* plants did not exhibit the double flower phenotype as did the *AGSRDX*-expressing *Arabidopsis*, the unexpected phenotypes must be caused by the function of the repression domain because *AG-ox* transgenic torenia did not exhibit the flower shape with serrated petals and anthocyanin accumulation of the stigma surface (Figure 1). From the viewpoint of structural modification, the CRES-T system has the potential to create novel flower phenotypes not only by their ordinary function but also by the cryptic functions of transcription factors, as is the case in this study. We have succeeded to confer the serrated petal phenotype to torenia plants, which has never been reported previously. Ectopic expression of chimeric repressor genes by tissue- or stage-specific heterologous promoters might be a useful tool to increase the horticultural value of ornamental flowers.

Acknowledgments

We thank Hiroyasu Yamaguchi and Katsutomo Sasaki for valuable discussions and critical reading of the manuscript. We also thank Satoko Ohtawa, Yasuko Taniji, and Yoshiko Kashiwagi for their excellent technical assistance. This work was supported by the Grant-in-Aid "Research Project for Utilizing Advanced Technologies in Agriculture, Forestry and Fisheries" from the Research Council, Ministry of Agriculture, Forestry and Fisheries of Japan (grant No. 1782).

References

Aida R, Shibata M (2001) Transgenic *Torenia fournieri* Lind. (torenia). in: Bajaj YPS (ed) Transgenic Crops III vol 48, *Biotechnology in Agriculture and Forestry*. Springer Verlag, Berlin, pp 294–305

Aida R, Murai K, Kishimoto S, Ohmiya A (2003) Introduction of *WAG*, a wheat *AGAMOUS* homolog, reduces corolla size in torenia. *Bull Natl Inst Flor Sci* 3: 21–27

Angenent GC, Franken J, Busscher M, van Dijken A, van Went JL, Dons HJM, van Tunen AJ (1995) A novel class of MADS box genes is involved in ovule development in Petunia. *Plant Cell* 7: 1569–1582

Becker D (1990) Binary vectors which allow the exchange of plant selectable markers and reporter genes. *Nucleic Acids Res* 18: 203

Bilyeu KD, Cole JL, Laskey JG, Riekhof WR, Esparza TJ, Kramer MD, Morris RO (2001) Molecular and biochemical characterization of a cytokinin oxidase from maize. *Plant Physiol* 125: 378–386

Bowman JL, Smyth DR, Meyerowitz EM (1991) Genetic interactions among floral homeotic genes of *Arabidopsis*. *Development* 112: 1–20

Coen ES, Meyerowitz EM (1991) The war of the whorls: genetic interactions controlling flower development. *Nature* 353: 31–37

Davies B, Motte P, Keck E, Saedler H, Sommer H, Schwarz-Sommer Z (1999) *PLENA* and *FARINELLI*: redundancy and regulatory interactions between two *Antirrhinum* MADS-box factors controlling flower development. *EMBO J* 18: 4023–4034

de Mesa MC, Santiago-Doménech N, Pliego-Alfaro F, Quesada MA, Mercado JA (2004) The CaMV 35S promoter is highly active on floral organs and pollen of transgenic strawberry plants. *Plant Cell Rep* 23: 32–38

Ditta G, Pinyopich A, Robles P, Pelaz S, Yanofsky MF (2004) The *SEP4* gene of *Arabidopsis thaliana* functions in floral organ and meristem identity. *Curr Biol* 14: 1935–1940

Doyle JJ, Dickson EE (1987) Preservation of plant samples for DNA restriction endonuclease analysis. *Taxon* 36: 715–722

Egea-Cortines M, Saedler H, Sommer H (1999) Ternary complex formation between the MADS-box proteins SQUAMOSA, DEFICIENS and GLOBOSA is involved in the control of floral architecture in *Antirrhinum majus*. *EMBO J* 18: 5370–5379

Estruch J, Granell A, Hansen G, Prinsen E, Redig P, Onckelen HV, Schwarz-Sommer Z, Sommer H, Spena A (1993) Floral development and expression of floral homeotic genes are influenced by cytokinins. *Plant J* 4: 379–384

Gómez-Mena C, De Folter S, Costa MMR, Angenent GC, Sablowski R (2005) Transcriptional program controlled by the floral homeotic gene *AGAMOUS* during early organogenesis. *Development* 132: 429–438

Hiratsu K, Matsui K, Koyama T, Ohme-Takagi M (2003) Dominant repression of target genes by chimeric repressors that include the EAR motif, a repression domain, in *Arabidopsis*. *Plant J* 34: 733–739

Honma T, Goto K (2001) Complexes of MADS-box proteins are sufficient to convert leaves into floral organs. *Nature* 409: 525–529

Huijser P, Klein J, Lönnig WE, Meijer H, Saedler H, Sommer H (1992) Bractomania, an inflorescence anomaly, is caused by the loss of function of the MADS-box gene squamosa in *Antirrhinum majus*. *EMBO J* 11: 1239–1249

Jack T, Brockman LL, Meyerowitz EM (1992) The homeotic gene *APETALA3* of Arabidopsis thaliana encodes a MADS box and is expressed in petals and stamens. *Cell* 68: 683–697

Jeffcoat B (1977) Influence of the cytokinin, 6-benzylamino-9-(tetrahydropyran-2-yl)-9H-purine, on the growth and development of some ornamental crops. *J Hortic Sci* 52: 143–153

Koyama T, Furutani M, Tasaka M, Ohme-Takagi M (2007) TCP transcription factors control the morphology of shoot lateral organs via negative regulation of the expression of boundary-specific genes in *Arabidopsis*. *Plant Cell* 19: 473–482

Matsui K, Tanaka H, Ohme-Takagi M (2004) Suppression of the biosynthesis of proanthocyanidin in *Arabidopsis* by a chimeric PAP1 repressor. *Plant Biotechnol J* 2: 487–494

Matsui K, Hiratsu K, Koyama T, Tanaka H, Ohme-Takagi M (2005) A chimeric AtMYB23 repressor induces hairy roots, elongation of leaves and stems, and inhibition of the deposition of mucilage on seed coats in *Arabidopsis*. *Plant Cell Physiol* 46: 147–155

Mitsuda N, Seki M, Shinozaki K, Ohme-Takagi M (2005) The NAC transcription factors NST1 and NST2 of *Arabidopsis* regulate secondary wall thickenings and are required for anther dehiscence. *Plant Cell* 17: 2993–3006

Mitsuda N, Hiratsu K, Todaka D, Nakashima K, Yamaguchi-Shinozaki K, Ohme-Takagi M (2006) Efficient production of male and female sterile plants by expression of a chimeric repressor in *Arabidopsis* and rice. *Plant Biotechnol J* 4: 325–332

Mitsuda N, Iwase A, Yamamoto H, Yoshida M, Seki M, Shinozaki K, Ohme-Takagi M (2007) NAC transcription factors, NST1 and NST3, are key regulators of the formation of secondary walls in woody tissues of *Arabidopsis*. *Plant Cell* 19: 270–280

Mizukami Y, Ma H (1992) Ectopic expression of the floral homeotic gene *AGAMOUS* in transgenic Arabidopsis plants alters floral organ identity. *Cell* 71: 119–131

Nitasaka E (2003) Insertion of an *En*/*Spm*-related transposable element into a floral homeotic gene *DUPLICATED* causes a double flower phenotype in the Japanese morning glory. *Plant J* 36: 522–531

Nishijima T, Shima K (2006) Change in flower morphology of *Torenia fournieri* Lind. induced by forchlorfenuron application. *Sci Hort* 109: 254–261

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

Pelaz S, Gustafson-Brown C, Kohalmi SE, Crosby WL, Yanofsky MF (2001) *APETALA1* and *SEPALLATA3* interact to promote flower development. *Plant J* 26: 385–394

Pinyopich A, Ditta DS, Savidge B, Liljergren SJ, Baumann E, Wisman E, Yanofsky MF (2003) Assessing the redundancy of MADS-box genes during carpel and ovule development. *Nature* 424: 85–88

Riechmann J, Meyerowitz E (1997) Determination of floral organ identity by *Arabidopsis* MADS domain homeotic proteins AP1, AP3, PI and AG is independent of their DNA-binding specificity. *Mol Biol Cell* 8: 1243–1259

Tröbner W, Ramirez L, Motte P, Hue I, Huijser P, Lönnig WE, Saedler H, Sommer H, Schwarz-Sommer Z (1992) *GLOBOSA*: a homeotic gene which interacts with *DEFICIENS* in the control of *Antirrhinum* floral organogenesis. *EMBO J* 11: 4693–4704

Vandenbussche M, Zethof J, Royaert S, Weterings K, Gerats T (2004) The duplicated B-class heterodimer model: whorl-specific effects and complex genetic interactions in *Petunia hybrida* flower development. *Plant Cell* 16: 741–754

Venglat SP, Sawhney VK (1996) Benzylaminopurine induces phenocopies of floral meristem and organ identity mutants in wild-type *Arabidopsis* plants. *Planta* 198: 480–487

Weigel D, Meyerowitz EM (1994) The ABCs of floral homeotic genes. *Cell* 78: 203–209

Yanofsky MF, Ma H, Bowman JL, Drews GN, Feldmann KA, Meyerowitz EM (1990) The protein encoded by the *Arabidopsis* homeotic gene agamous resembles transcription factors. *Nature* 346: 35–39

Zahn LM, Leebens-Mack J, Arringtonas JM, Hu Y, Landherr L, dePamphilis CW, Becker A, Theissen G, Ma H (2006) Conservation and divergence in the *AGAMOUS* subfamily of MADS-box genes: evidence of independent sub- and neofunctionalization events. *Evolution and Development* 8: 30–45