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Original Paper

Arabidopsis chimeric TCP3 repressor produces novel floral traits in *Torenia fournieri* and *Chrysanthemum morifolium*

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Abstract Chimeric REpressor gene-Silencing Technology (CRES-T) is a powerful gene-silencing tool to analyze the function of *Arabidopsis* transcription factors. To investigate whether CRES-T is also applicable to horticultural plants inadequate for genetic engineering because of their limited molecular biological characterization and polyploidy, we applied CRES-T to torenia and the hexaploid chrysanthemum and produced their transgenic plants expressing the chimeric repressor derived from the Arabidopsis TEOSINTE BRANCHED1, CYCLOIDEA, and PCF family transcription factor 3 (TCP3) fused with a plant-specific transcriptional repression domain named SRDX, consisting of 12 amino acids originated from the EAR-motif (TCP3-SRDX). Transgenic torenia and chrysanthemum expressing TCP3-SRDX exhibited fringed leaves and short pistils, while those expressing TCP3 fused with either the mutated repression domain (TCP3-mSRDX) or the overexpressor of TCP3 (TCP3-ox) did not exhibit phenotypic changes. In addition to fringed leaves, TCP3-SRDX transgenic torenia plants exhibited petals with fringed margins, distinctive color patterns, and reduced anthocyanin accumulation. In TCP3-SRDX transgenic chrysanthemum plants, floral organ development was suppressed as compared with the wild type. These results indicate that the *Arabidopsis*-derived TCP3-SRDX induced morphological changes in transgenic torenia and chrysanthemum although the observed phenotypes partially differ from each other. CRES-T may function in various plant species including polyploid species and modify their biological characteristics.

Key words: CRES-T, TCP3-SRDX, chrysanthemum, fringed leaves, torenia.

Shape and color are the most important characteristics of ornamental flowers, and the generation of the novel phenotypes has been pursued in the breeding. Distinct floral morphology such as fringed petals and double flowers has been selected preferentially in the breeding of horticultural crops from wild species.

Transcription factors control the expression of multiple genes and act as the master regulator of various phenotypes. Most of the floral morphological changes in *Arabidopsis* reported until date are attributed to the dysfunction in transcription factors (Ferrario et al. 2004; Jack 2004; Mitsuda and Ohme-Takagi 2009). Knockout or knockdown of transcription factor genes is therefore efficient for the functional analysis of genes and creation of novel plant phenotypes. Although RNA interference (RNAi) effectively knocks down the expression of targeted genes in model plants such as *Arabidopsis* and rice, it is difficult to apply this technology to horticultural plants with limited genomic sequence information and polyploid genomes because of unavailability of target sequences and genetic redundancy which is often accompanied by sequence variability.

CRES-T is a unique gene-silencing method utilizing plant-specific chimeric transcriptional repressors. These chimeric repressors are produced from transcription factors by fusing a transcriptional repression domain SRDX (Hiratsu et al. 2002). This modification converts many transcription factors into strong transcriptional repressors. Chimeric repressors dominantly suppress the expression of respective target genes and confer loss-of-function phenotypes at high frequency, even in the presence of functionally redundant transcriptional activators in *Arabidopsis* and rice (Hiratsu et al. 2003; Koyama et al. 2007, 2010; Matsui et al. 2004, 2005;

Abbreviations: CaMV, cauliflower mosaic virus; CRES-T, chimeric repressor gene-silencing technology; EAR-motif, ETHYLENE RESPONSE FACTOR (ERF)-associated amphiphilic repression (EAR) motif; SEM, scanning electron microscopy; TCP, TEOSINTE BRANCHED1, CYCLOIDEA, and PCF

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Mitsuda et al. 2005, 2006, 2007).

CRES-T has been applied to torenia (*Torenia fournieri* Lind.), a horticultural plant. The transgenic torenia expressing the *Arabidopsis* chimeric AGAMOUS (AG) repressor did not exhibit the double flower phenotype as seen in *ag* mutant and AG-SRDX *Arabidopsis* (Mitsuda et al. 2006). However, AG-SRDX torenia represented distinct petal phenotypes that may arise from partial repression of other *AG*-like activities outside whorls 3 and 4 by the chimeric AG repressor driven by the cauliflower mosaic virus (CaMV) 35S promoter (Narumi et al. 2008). Nonetheless, this may reflect the correct repressive activity because the chimeric repressors of torenia *AG* orthologs *TfFAR-SRDX* and *TfPLE1-SRDX* induced the same phenotype in torenia. In addition, *AG*-overexpressing torenia showed the same phenotype as in *Arabidopsis* transformed with the same construct. Despite the effectiveness of CRES-T in the plant species other than *Arabidopsis*, the applicability of this technology to polyploid horticultural plants has not been reported.

Here, we report the applicability and versatility of CRES-T in horticultural plants such as a diploid torenia and a hexaploid chrysanthemum (*Chrysanthemum morifolium* Ramat.) using the *Arabidopsis* TCP3 chimeric repressor gene (*TCP3-SRDX*). In *Arabidopsis*, the *TCP3* gene belongs to the TCP family, plays a pivotal role in the control of morphogenesis of shoot organs and regulates appropriate development in various organs (Koyama et al. 2007, 2010). TCP3 is controlled by the activity of the microRNA miR319/JAW because *Arabidopsis* expressing the miR319-resistant version of *TCP3* (*mTCP3*) showed abnormal phenotypes with enhanced elongation of hypocotyls, fused cotyledons, and a morphological change in the shoots (Koyama et al. 2007; Palatnik et al. 2003). The expression of TCP3-SRDX in *Arabidopsis* resulted in the induction of wavy leaves and petals, and the expression of TCP3 produced no effect because of the negative regulation by miR319 (Koyama et al. 2007). Because TCP3-SRDX induces wavy and serrated leaf and flower margins in *Arabidopsis*, we introduced the *TCP3-SRDX* gene into torenia and chrysanthemum and investigated whether it modified leaf and flower morphology. The relationship between the effect of TCP3-SRDX and the observed morphological changes in transgenic plants is discussed.

Materials and methods

Plant material

Laboratory lines derived from *Torenia fournieri* Lind. 'Crown Violet' (Aida et al. 2000) and *Chrysanthemum morifolium* Ramat. 'Sei-Marin' (Seikoen, Hiroshima, Japan) were used as starting materials for the production of transgenic plants. Plants were aseptically maintained in a plant box supplemented with 1/2 Murashige and Skoog (MS) medium containing 0.2% gellan gum at 25°C under fluorescent light (16 h light/8 h dark, 160 μmol m^{-2} s^{-1}) and reproduced vegetatively by herbaceous cutting as reported earlier (Aida and Shibata 2001; Aida et al. 2004).

Generation of transgenic plants

The *TCP3-SRDX*, *TCP3-mSRDX*, and *TCP3-ox* genes have been described earlier (Koyama et al. 2007). Each construct was introduced into the destination vector pBCKK (Narumi et al. 2008). Each transgene vector was then introduced into torenia and chrysanthemum by *Agrobacterium*-mediated transformation (Aida and Shibata 2001; Aida et al. 2004). Transgenic plants regenerated *via* adventitious shoots were grown in the contained greenhouse under natural light.

RNA preparation and real-time PCR

Total RNA was isolated from torenia and chrysanthemum leaves using the SV Total RNA Isolation System (Promega, San Luis Obispo, CA, USA) and from the petals of torenia using the RNeasy Plant Mini Kit (QIAGEN, Hilden, Germany). First-strand cDNA was synthesized using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Life Technologies, Carlsbad, CA, USA). Expression of transgenes such as *TCP3-SRDX*, *TCP3-mSRDX*, and *TCP3-ox* was analyzed by real-time RT-PCR with the SYBR *Premix Ex Taq* (TaKaRa Bio, Ohtsu, Japan). Reactions were performed using the LightCycler ST300 system (Roche Diagnostics, Mannheim, Germany). A plasmid containing each transgene sequence was used for the standard curve assay, and levels of gene transcripts were described as the copy number per 50 ng of total RNA. Gene-specific primers were used for real-time RT-PCR: all transgene forward, 5′-CGAACAAGAGGAGCACGGCGG-3′; all transgene reverse, 5′-AGACCGGCAACAGGATTCAATC-3′; and *TCP3-SRDX* reverse, 5′-AGCGAAACCCAA ACGGAGTTCTAG-3′. The expression level of these genes relative to torenia *Actin3* (*TfACT3*; accession no. AB330989) and chrysanthemum *Actin* (*CmACT*; accession no. AB205087) was calculated for each sample by triplicate experiments. The following primers were used for *TfACT3*: forward, 5′-TGCAGTAAAGTGTATTGTGGAAG-3′; and reverse, 5′-GGAACTATCTGGGTAGGATC-3′. The following primers were used for *CmACT*: forward, 5′-ACATGCTATCTTGCG-TTTGG-3′; and reverse, 5′-CTCTCACAATTTCCCGTTCA-3′.

Anatomical observation of floral organs

Stereomicroscopic observation of torenia and chrysanthemum pistils was performed using Leica MZ16 (Leica, Glattbrugg, Switzerland). Fresh tissues were prepared after the opening of flower buds and examined by scanning electron microscopy (SEM; VE-7000; Keyence Co., Osaka, Japan) without fixing in order to observe petal and stigma surfaces.

Determination of anthocyanin and flavonoid composition by HPLC

HPLC analysis was performed as described by Saito et al. (2006). Fresh torenia petals were divided into lip, boundary, and upper tube regions. Each sample was extracted with 10% acetic acid, and the extract was analyzed using an HP1100

system with a photodiode array detector (Agilent Technologies, CA, USA) and an Inertsil ODS-2 column (4.6×250 mm; GL Science, Tokyo, Japan) at 40°C at a flow rate of 0.8 ml min^{-1}. A linear gradient of 20%–100% solvent B (1.5% H_3PO_4, 20% CH_3CO_2H, 25% CH_3CN) in solvent A (1.5% H_3PO_4) was run for 40 min. Anthocyanins and flavonoids were detected based on their absorption spectra at 200–600 nm, and their concentrations were calculated as the cyanidin 3-rutinoside equivalent by absorption at 530 nm and the quercetin 3-rutinoside equivalent by absorption at 360 nm, respectively.

Results and discussion

Generation of transgenic torenia and chrysanthemum expressing TCP3-SRDX

To investigate whether the *Arabidopsis* chimeric TCP3 repressor (TCP3-SRDX) induces wavy leaves and petals in horticultural plants, we produced transgenic torenia and chrysanthemum expressing TCP3-SRDX. As negative controls, we introduced a TCP3 overexpressor gene (*TCP3-ox*) and a modified TCP3 chimeric repressor gene in which SRDX had been mutated (*TCP3-mSRDX*; Koyama et al. 2007). We obtained more than 10 torenia and 20 chrysanthemum transgenic plants for each transformation (Table S1) and examined their phenotypes in detail.

Phenotype of TCP3-SRDX torenia

TCP3-SRDX torenia exhibited a weak dwarf phenotype probably due to reduction in internode length (Figure 1A). In addition, it demonstrated distinct morphological and physiological changes in petals and leaves. As shown in Figure 1B, TCP3-SRDX torenia exhibited fringed margins mainly in the dorsal petal. Moreover, anthocyanin pigmentation, which imparts purple color to the petal lip region of the wild type, was clearly reduced. TCP3-SRDX torenia also exhibited wavy blades and fringed margins in leaves as in TCP3-SRDX *Arabidopsis* (Koyama et al. 2007) and *cincinnata* (*cin*)-756 mutant of *Antirrhinum* (Nath et al. 2003). In contrast, *TCP3-mSRDX* and *TCP3-ox* had no effect when the transgenes were expressed at a certain level in each transgenic plant (Figure S1A). Phenotypes of TCP3-SRDX torenia plants were classified into 3 groups by petal color and shape: type I, normal flower (wild type); type II, flowers with wavy, fringed marginal petals; type III, flowers with pale-colored petals and a wavy, fringed margin (Figure 1C).

In *Arabidopsis*, the pistils of TCP3-SRDX plants were significantly shorter than those of the wild type (Koyama et al. 2007). To confirm whether this held true for TCP3-SRDX torenia, we microscopically observed their pistils. As shown in Figures 1D and S2, TCP3-SRDX torenia showed pistils of reduced length, as in *Arabidopsis*. In addition, stigma width and ovary length of TCP3-SRDX torenia pistils were somewhat greater compared with those of the wild type (Figure S2).

Morphological changes in petal and stigma epidermal cells of TCP3-SRDX torenia

To further characterize the phenotype of TCP3-SRDX torenia petal and stigma, we performed scanning electron microscopy (SEM) of petals and pistils of the wild type and a type III TCP3-SRDX torenia 5010-14. In wild-type plants, the epidermis of the adaxial surface of the petal lip region showed homogenous conical epidermal cells (Figure 2A) and that of the boundary region between the lip and the tube showed several spike-like cells in addition to conical cells (Figure 2B). The epidermis of the upper tube region showed increased numbers of elongated spike-like cells (Figure 2C). Unlike the wild type, TCP3-SRDX torenia exhibited a slightly increased number of spike-like cells not only in the boundary region but also in the petal lip region (Figures 2D, E). There was no difference between these plants with regard to the organization of the upper tube region (Figure 2C, F). These results suggest that the cell identity of the petal lip region in TCP3-SRDX torenia was altered to that of the boundary region; therefore, the petal lip color became paler, as in the tube region. This idea is supported by our finding that the total amount of flavonoids in TCP3-SRDX torenia petals was clearly reduced compared with that in wild-type petals, and the anthocyanin/flavonoids ratio in the whole petals of type III TCP3-SRDX torenia 5010-14 (0.15) was similar to that in the petal tube of wild-type (0.17, Figure S3). Therefore, the phenotype of TCP3-SRDX torenia petals may have arisen through altered regulation of cell identity. Similar results were reported in the *cincinnata* mutant of *Antirrhinum* and transgenic *Arabidopsis* in which a chimeric repressor from TCP5 (TCP5-SRDX), a TCP3-type transcription factor, was expressed (Crawford et al. 2004; T. Koyama, personal communication).

A morphological change in the epidermal cells was observed in type III TCP3-SRDX torenia pistils (Figure 3). Epidermal cells of the stigma surface of TCP3-SRDX torenia showed a smooth surface while those of the wild type showed a rough surface (Figure 3A). Furthermore, in TCP3-SRDX torenia, trichome-like cells on the style surface were observed at the boundary region of style and ovary, and capitate glandular cell-like structures, usually observed in petals, were observed on the lateral area of the style (Figure 3B). Thus, TCP3-SRDX may affect epidermal cell differentiation in both petals and pistils of torenia. In *Antirrhinum*, mutant analyses revealed that MIXTA and AmMYBML1–3 (MYB transcription factors) control petal epidermal cell differentiation and induce trichomes in carpels. MIXTA affects petal shape and size via the developmental control of conical epidermal cells when overexpressed in tobacco (Baumann et al. 2007; Glover et al. 1998; Martin

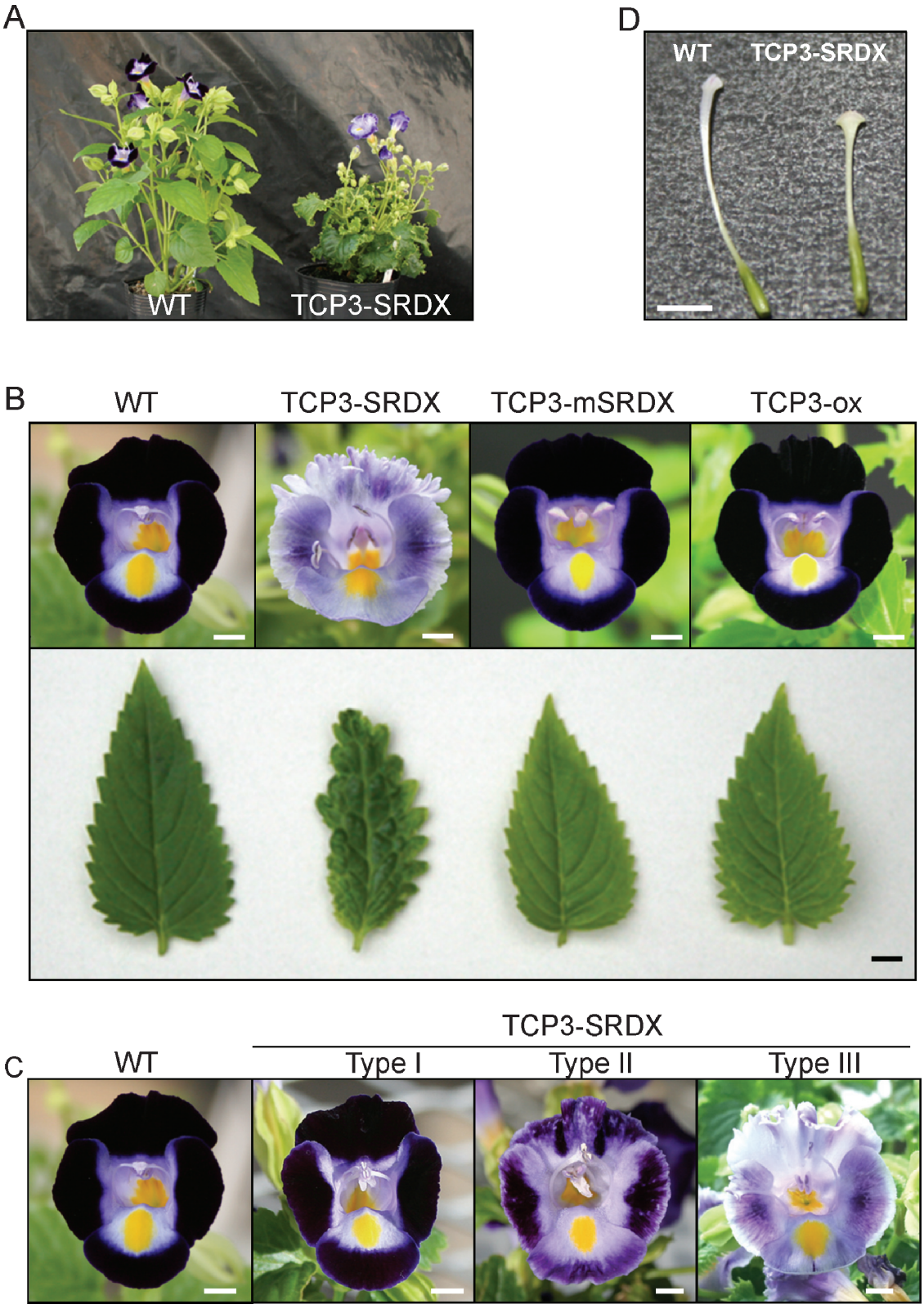

Figure 1. Morphological changes in TCP3-SRDX torenia. (A) Wild-type *Torenia fournieri* 'Crown Violet' (left) and TCP3-SRDX torenia 5010-16 (right). TCP3-SRDX torenia shows wavy blades and fringed margins in leaves and petals. (B) Phenotypic comparison of torenia flowers and leaves. Typical mature flowers of wild-type plants (left), TCP3-SRDX torenia 5010-14, TCP3-mSRDX torenia 5019-8, and TCP3-ox torenia 5018-1 (right) are shown. Upper photographs show comparisons of color pattern, corolla size, divergence, and petal shape; lower photographs show comparison of leaf shape. TCP3-SRDX torenia exhibited fringed leaves and petals as well as reduced anthocyanin accumulation in petals. Bars=5 mm. (C) Comparison of petal color and shape in transgenic torenia plants. Wild-type (left) and TCP3-SRDX torenias with type I (5010-12), type II (5010-52), and type III (5010-22) phenotypes are shown. Bars=5 mm.

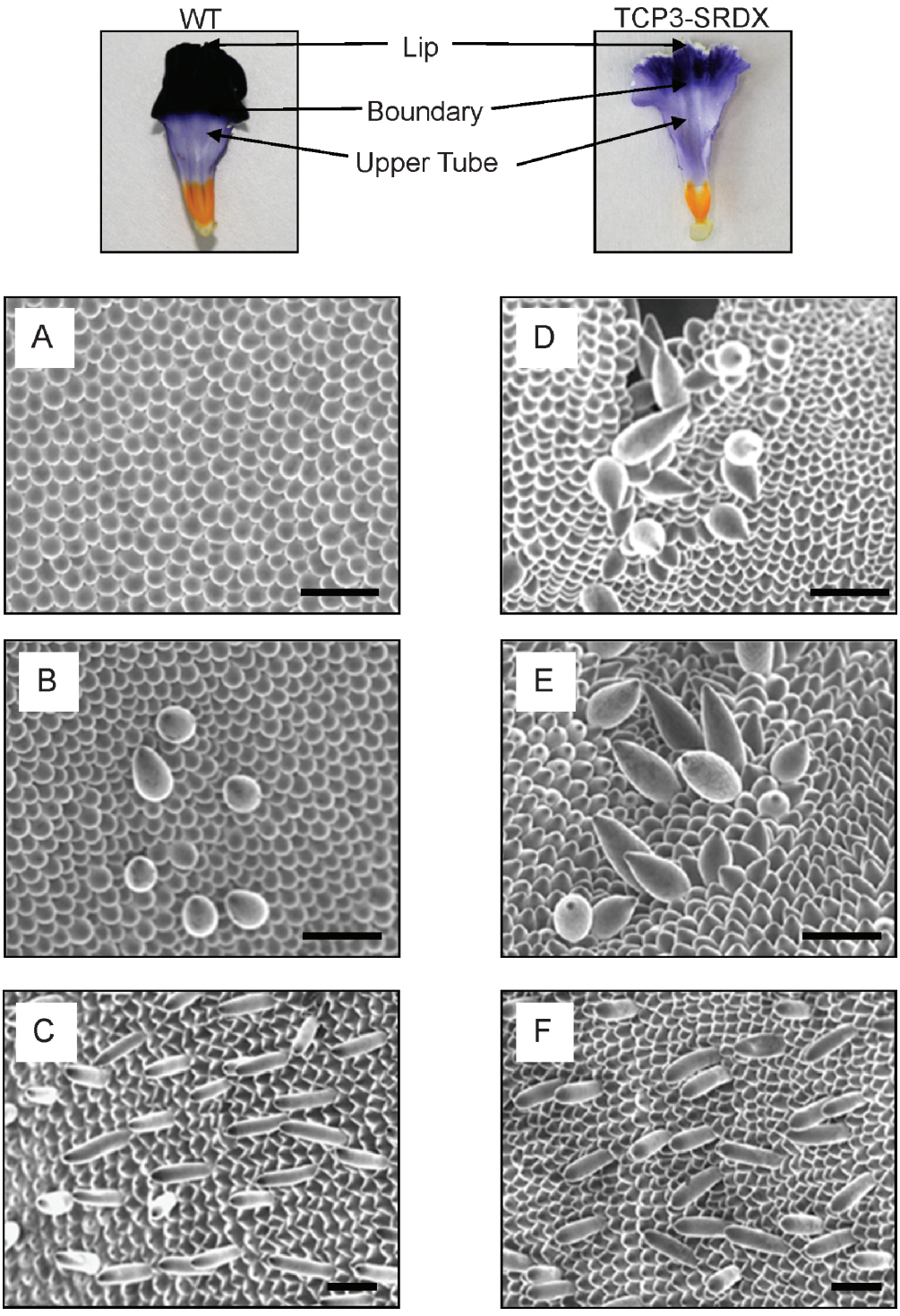

Figure 2. Morphological changes in the epidermal cells of TCP3-SRDX torenia petals. SEM was performed using freshly prepared petals after the opening of flower buds. Petals were divided into 3 zones (lip, boundary, and upper tube) for comparison of petal surface cells in wild-type (A–C) and type III TCP3-SRDX torenia 5010-14 (D–F). (A) and (D) show the petal lip region in each plant. Bars=20 μm. (B) and (E) show the boundary region in each plant. Bars=20 μm. (C) and (F) show the upper tube region. Bars=40 μm.

et al. 2002; Noda et al. 1994; Perez-Rodriguez et al. 2005). Interestingly, TCP3-SRDX reduced the expression of *AtMIXTA* (AT5G15310) in *Arabidopsis* based on microarray data (Koyama et al. 2010; the National Center for Biotechnology Information Gene Expression Omnibus GSE20705). These observations suggest a possible involvement of the MYB gene in the phenotype with which TCP3-SRDX torenia ectopically induced spike-like cells and trichome-like cells in petals and styles, respectively.

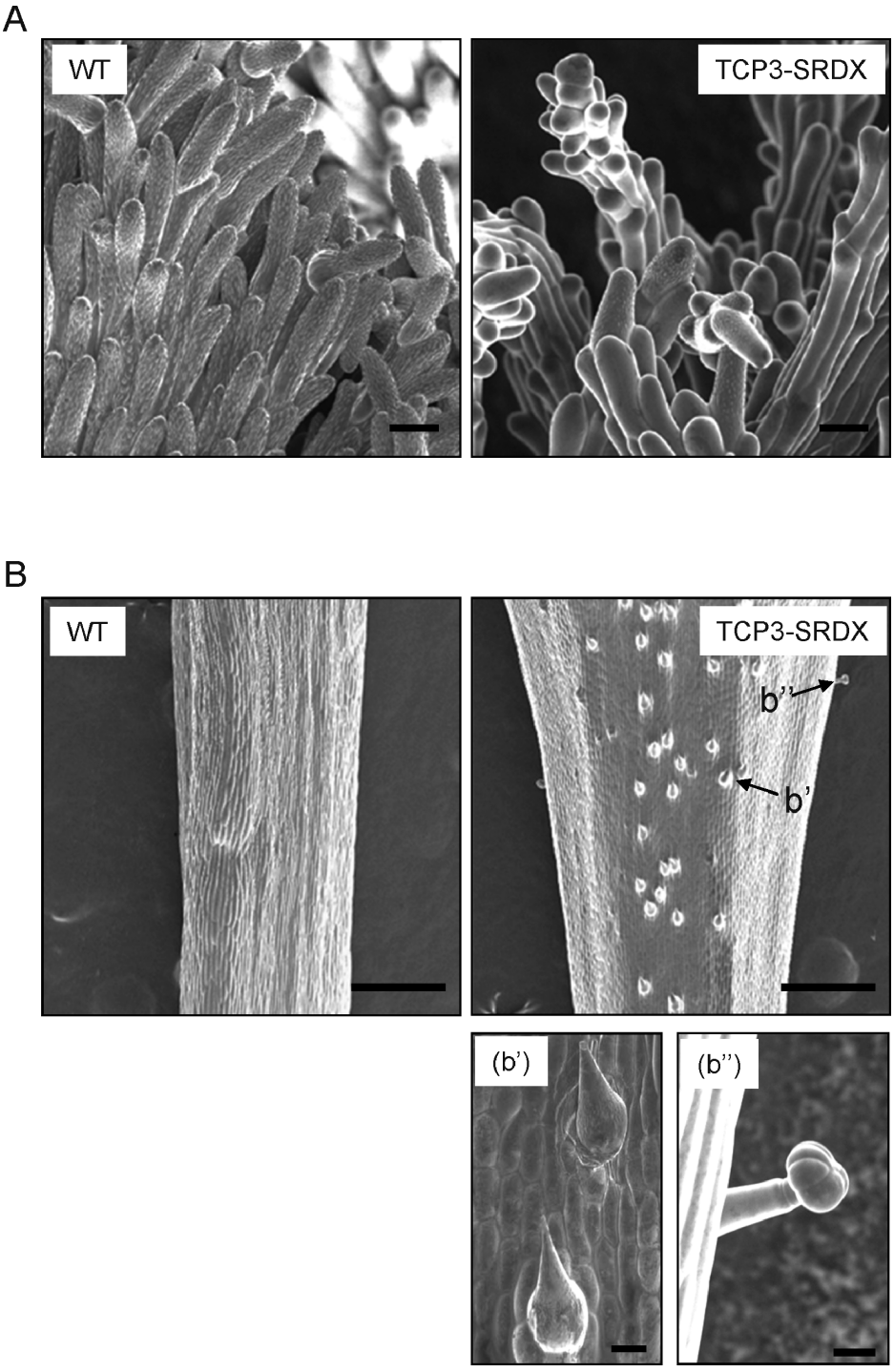

Figure 3. Morphological changes in TCP3-SRDX torenia pistils. SEM was performed using freshly prepared pistils. (A) Microscopic images of the stigma surface in wild-type (left) and type III TCP3-SRDX torenia 5010-14 (right). Bars=20 μm. (B) Microscopic images of the lateral view of style in wild-type (left) and TCP3-SRDX torenia (right). Bars=400 μm. (b′) and (b″) represent enlarged images of trichome-like cells and a capitate glandular cell-like structure, respectively.

Phenotype of TCP3-SRDX chrysanthemum

TCP3-SRDX chrysanthemum had a phenotype similar to TCP3-SRDX torenia with respect to reduced height, fringed leaves, and shortened pistils (Figures 4A, B, C). TCP3-SRDX chrysanthemum could be classified into 2 groups based on leaf morphology: type I, leaves with wavy surfaces and rounded margins and type II, leaves with serrated margins (Figure 4D) similar to those in the *miR319*-overexpressing tomato (Ori et al. 2007). TCP3-mSRDX and TCP3-ox transgenic chrysanthemum exhibited a normal phenotype (Figure 4D), as in the cases of torenia and *Arabidopsis*.

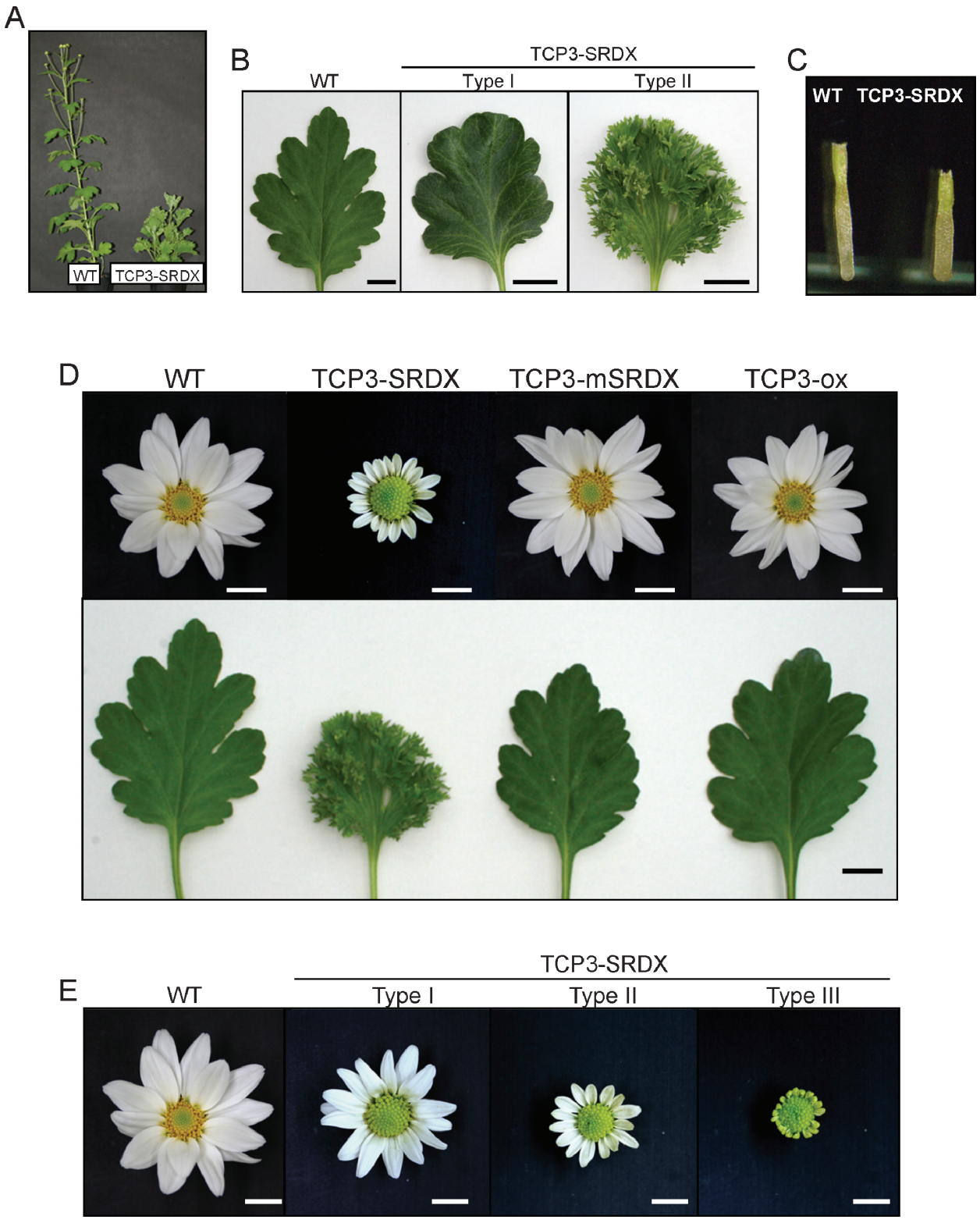

Figure 4. Morphological changes in TCP3-SRDX chrysanthemum. (A) Wild-type *Chrysanthemum morifolium* 'Sei-Marin' (left) and TCP3-SRDX chrysanthemum 1160-33 (right). (B) Comparison of leaf shape in transgenic chrysanthemum plants. Wild-type (left) and TCP3-SRDX chrysanthemum of type I (center) and type II phenotypes (right) are shown. Type I; 1160-27, Type II; 1160-33. Bars is=1 cm. (C) Stereomicroscopic image of pistils in wild-type (left) and TCP3-SRDX chrysanthemum 1160-36 (right). Bars=0.5 mm. (D) Phenotypic comparisons of chrysanthemum flowers and leaves. Typical mature flowers of wild-type plants (left), TCP3-SRDX 1160-33, TCP3-mSRDX 1185-9, and TCP3-ox 1187-15 (right) are shown. Bars=1 cm. (E) Comparison of corolla size, petal shape, and disk floret maturity in transgenic chrysanthemum plants. Wild-type (left) and TCP3-SRDX chrysanthemum with type I (1160-17), type II (1160-36), and type III (1160-34) phenotypes are shown. Bars=1 cm.

Unlike the leaf phenotype, TCP3-SRDX chrysanthemum exhibited specific flower phenotypes differing from those of TCP3-SRDX torenia. TCP3-SRDX chrysanthemum had a smaller ligulate corolla with immature ray florets (Figure 4D). TCP3-SRDX chrysanthemum can be classified into 3 groups based on corolla morphology and size: type I, flowers with undersized corolla compared with those of the wild type; type II, flowers with ligulate corolla smaller than those of type I; and type III, flowers with an immature ligulate corolla (Figure 4E). Corolla phenotypes were not closely related to leaf morphology in TCP3-SRDX

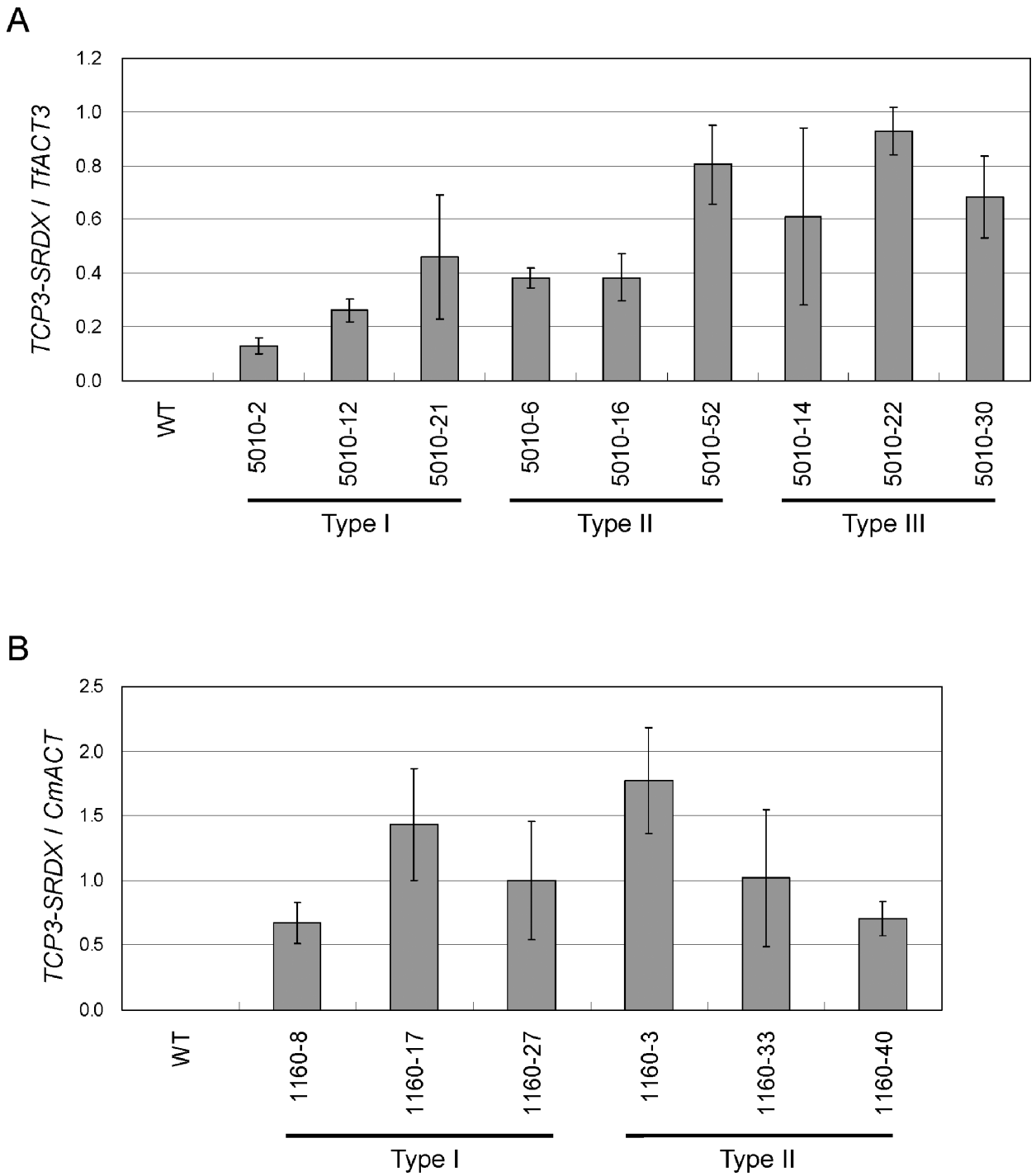

Figure 5. Expression of the *TCP3-SRDX* transgene in torenia and chrysanthemum. (A) Total RNAs were prepared from petals of wild-type and TCP3-SRDX torenias with type I (5010-2, 5010-12, 5010-21), type II (5010-6, 5010-16, 5010-52), and type III (5010-14, 5010-22, 5010-30) phenotypes. (B) Total RNAs were prepared from the leaves of wild-type and TCP3-SRDX chrysanthemums with type I (1160-8, 1160-17, 1160-27) and type II (1160-3, 1160-33, 1160-40) phenotypes. RNA preparation and quantitative real-time RT-PCR reactions were performed as described in materials and methods. Data are expressed as mean±SE.

chrysanthemum (data not shown). The disk floret in all corolla types of TCP3-SRDX chrysanthemum remained green, while that of the wild type turned yellow with corolla development and flowering. The immature ligulate corolla of TCP3-SRDX chrysanthemum may have been caused by the suppression of floral organ development, as epidermal cell expansion was suppressed in transgenic plants with type III phenotype (Figure S4). This difference in the floral phenotypes of torenia and chrysanthemum may be due to the difference in floral structures; the floral structure of chrysanthemum is a pseudanthium composed of large numbers of disk florets and ray florets, while that of torenia is a single flower. In addition, it is possible that the functions of endogenous TCP3 counterparts in these plants differ during floral development.

Relationship between levels of TCP3-SRDX transcript and phenotype

To investigate whether the phenotypic intensity of TCP3-SRDX plants depends on the expression level of the transgene, we analyzed the mRNA levels of *TCP3-SRDX* using real-time PCR. Total RNA was extracted from TCP3-SRDX torenia petals and TCP3-SRDX chrysanthemum leaves of some typical lines in each phenotypic group. As shown in Figure 5, expression of the *TCP3-SRDX* gene was observed in all transgenic plants. On the other hand, the *TCP3-SRDX* mRNA level in each line did not reflect phenotypic intensity. The reason for variation in the phenotypes of these plants is presently not clear, but it may be controlled by other mechanisms such as protein dose and its regulation (Gygi et al. 1999), or may be due to a slight difference in the timing and localization of *TCP3-SRDX* gene expression (Koyama et al. 2007). Such mRNA dose-independent phenotypic change has also been reported for *KNAT1*-overexpressing *Arabidopsis* (Chuck et al. 1996). Endogenous microRNAs corresponding to miR319 may, in part, determine this delicate balance because they may also target *TCP3-SRDX* transcripts in addition to the transcripts from the endogenous counterpart of the *TCP3* gene. In this case, controlling *TCP3-SRDX* gene expression by tissue- and/or stage-specific promoters may be useful in modifying such balance and creating greater variations in flower color patterns and petal shapes.

Chimeric repressors of development- and differentiation-related transcription factors have the ability to modify floral traits

In the present study, we demonstrated through the phenotypic analyses of transgenic plants that the *Arabidopsis*-derived TCP3 chimeric repressor, TCP3-SRDX, functions beyond plant species in diploid torenia and hexaploid chrysanthemum. TCP transcription factors belong to ancient proteins are highly conserved within the broad range of plant species such as pluricellular green algae, gymnosperms, and angiosperms (Floyd and Bowman 2007; Navaud et al. 2007). Recently, more than 20 members have been identified by whole-genome search in *Arabidopsis*, rice, poplar, and grapevine (Martin-Trillo and Cubas 2010). Our results indicate that we have succeeded in suppressing endogenous TCP-related activities in torenia and chrysanthemum, without isolating and identifying their sequences or target genes. In addition, TCP3 function may be highly conserved within these plant species because the expression of TCP3-SRDX altered leaf morphology in a similar manner. This supports the general perception of the conserved function of TCP family proteins beyond plant species.

In the present study, we demonstrated that CRES-T has the ability to modify biological characters even in the polyploid species, probably by knocking down the endogenous counterpart of their target genes. Our results suggest that CRES-T is a powerful tool for the functional analysis of transcription factors, in addition to increasing the horticultural values of torenia, chrysanthemum, and other horticultural crops irrespective of their ploidy. As in the case of TCP3-SRDX, chimeric repressors of development- and differentiation-related transcription factors may have the ability to modify petal colors and patterns in addition to petal size and shape via the modification of celllar identity or positional information within the petal. The efficient transformation and screening system for chimeric repressors (CT system; Shikata et al. 2011 in this issue) may be effective to categorize such cryptic functions of transcription factors.

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