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Original Paper

Efficient production of novel floral traits in torenia by collective transformation with chimeric repressors of *Arabidopsis* transcription factors

Masahito Shikata[1,†], Takako Narumi[2,†], Hiroyasu Yamaguchi[1], Katsutomo Sasaki[1], Ryutaro Aida[1], Yoshimi Oshima[3], Yuko Takiguchi[3], Masaru Ohme-Takagi[3], Nobutaka Mitsuda[3], Norihiro Ohtsubo[1,*]

[1] National Institute of Floricultural Science, National Agriculture and Food Research Organization, Tsukuba, Ibaraki 305-8519, Japan; [2] Faculty of Agriculture, Kagawa University, Miki, Kagawa 761-0795 Japan, [3] Bioproduction Research Institute, Advanced Industrial Science and Technology, Tsukuba, Ibaraki 305-8562, Japan
* E-mail: nohtsubo@affrc.go.jp Tel & Fax: +81-29-838-6822

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Abstract Molecular breeding with genetic modification enables the production of novel floral traits in floricultural plants that could not be obtained by traditional breeding. To facilitate novel flower production, we collectively introduced 2 sets of 42 and 50 chimeric repressors of *Arabidopsis* transcription factors into *Agrobacterium* and then used these to co-transform torenia (*Torenia fournieri*). We generated 750 transgenic torenias, and identification of the transgenes revealed that more than 80% of the transgenic torenias had a single transgene. A total of 264 plants showed phenotypic modification, and 91.2% displayed modified flower colors and/or shapes, such as altered color patterns, curled petal margins, and wavy petals. These results indicated that the collective transformation system can be applied to molecular breeding of flowers. Detailed analysis of the phenotypes revealed that *PETAL LOSS* could control blotch sizes and that modification of cell shape could change the texture of petals. We found that the chimeric repressors of functionally unknown transcription factors also induced novel floral traits, and therefore, the transgenic torenias provide an understanding of the functions of transcription factors that could not be revealed by previous studies in *Arabidopsis*.

Key words: Collective transformation, CRES-T, flower modification, torenia, transcription factor.

Flower colors and shapes are important commercial characters in floricultural plants. Many flowers with novel floral traits have been generated by cross-fertilization, selective breeding, and mutation breeding (Shibata 2008). However, such traditional breeding methods require enormous time and effort. In contrast, genetic engineering in molecular breeding enables production of novel floral traits in floricultural plants that cannot be obtained by traditional breeding more efficiently and with less effort. For instance, attempts have been made for centuries to produce blue roses by traditional breeding, but this has proved impossible because roses lack a key enzyme, flavonoid 3′,5′-hydoxylase (F3′5′H), for delphinidin biosynthesis necessary for blue pigmentation. Katsumoto et al. (2007) succeeded in producing a delphinidin-accumulating rose with color that was blue-hued because of viola *F3′5′H* gene expression. Flower colors in carnation, petunia, torenia, and gentian have been modified by manipulating flavonoid biosynthesis genes (Nishihara and Nakatsuka 2010; Tanaka et al. 2009). Suppression by antisense gene or RNAi methods and overexpression by sense genes are typical strategies to manipulate genes of interest. However, information on DNA sequences is required for these strategies and genomic or EST analyses are still to be performed for most floricultural plants. On the other hand, sequence information of model plants, including *Arabidopsis*, rice, and tomato, is now available. Application of the genes of such model plants may accelerate genetic engineering of floricultural plants.

Utilization of transcription factors in molecular breeding is appropriate for flower modification because numerous transcription factors play important roles in regulating identification, initiation, and differentiation of flower organs. For example, most genes in the ABC model of flower development, namely *APETALA1* (*AP1*),

Abbreviations: CRES-T: chimeric repressor gene-silencing technology, CT: collective transformation.
† The authors contributed equally to this work.
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APETALA3 (*AP3*), *PISTILLATA* (*PI*), and *AGAMOUS* (*AG*) in *Arabidopsis*, encode MADS-box transcription factors (Goto and Meyerowitz 1994; Jack et al. 1992; Mandel et al. 1992; Yanofsky et al. 1990). AP3/PI negatively regulates *GNC* (*GATA, nitrate-inducible, carbon metabolism-involved*) and *GNL* (*GNC-like*), both of which encode GATA transcription factors (Mara and Irish 2008). *PETAL LOSS* (*PTL*), encoding a trihelix transcription factor, regulates sepal and petal architecture (Brewer et al. 2004; Griffith et al. 1999). In *Antirrhinum majus* flowers, *CYCLOIDEA* (*CYC*), *DICHOTOMA* (*DICH*), *DIVARICATA* (*DIV*), and *RADIALIS* (*RAD*) establish dorsoventral asymmetry. *CYC* and *DICH* encode TCP transcription factors, while *DIV* and *RAD* are MYB genes (Corley et al. 2005; Galego and Almeida 2002; Luo et al. 1996, 1999). In addition to identification, initiation, and differentiation of flowers, transcription factors regulate flower color. A regulatory complex of MYB, bHLH, and WD40 proteins regulate flavonoid biosynthesis and control flower color (Kose et al. 2005; Tanaka et al. 2008). Various families of transcription factors participate in flower development, and therefore, transcription factors are a useful and powerful tool for modification of floral traits.

Male and female sterile flowers have been produced in *Arabidopsis* and rice using a chimeric repressor gene-silencing technology (CRES-T) system in which a transcription factor fused to the repression domain (SRDX) dominantly represses the transcription of its target genes, even in the presence of redundant transcription factors (Hiratsu et al. 2003; Mitsuda et al. 2006). The CRES-T system can be applied to molecular breeding of floricultural plants (Shikata and Ohme-Takagi 2008). We have produced transgenic torenias as a model of genetic engineering using the CRES-T system in floricultural plants (Narumi et al. 2008; Sasaki et al. 2010). The transgenic torenias showed modified flower colors and shapes, indicating that the CRES-T system was effective in the production of novel torenia flowers. For example, TCP3-expressing torenias displayed changes in both flower shape and color patterns (Narumi et al. 2011 in this issue). However, a transgene does not always induce the desired floral traits predicted from phenotypes in *Arabidopsis*. For instance, while a chimeric AG repressor (AG-SRDX) induced double flowers in *Arabidopsis* (Mitsuda et al. 2006), transgenic torenia flowers expressing chimeric repressors of either AG or its torenia homologs (TfFARINELLI and TfPLENA1) did not show double-flower phenotypes (Narumi et al. 2008). Thus, an ideal method to obtain valuable phenotypes for commercial purposes is to produce various transgenic plants. To facilitate this procedure, we developed a system to efficiently produce numerous transgenic plants and evaluate their floral traits. This system will facilitate the molecular breeding of floricultural plants.

Materials and methods

Plant materials and growth conditions

Torenia (*Torenia fournieri* Lind. 'Crown Violet') (Aida et al. 2000) and *Arabidopsis* (*Arabidopsis thaliana*, Col-0 accession) were used as plant materials. Torenia plants were maintained by herbaceous cutting and grown at 25°C under fluorescent lamps with long-day conditions (16-h light and 8-h dark). To observe the floral phenotypic changes, torenia plants were grown in contained greenhouse under natural light. *Arabidopsis* plants were grown at 22°C under long-day conditions.

Selection of bulk mix genes

We prepared 2 sets of genes, namely "the flower bulk" and "the development bulk." For the flower bulk, 42 genes were selected as follows. From the AtGenExpress developmental series (Schmid et al. 2005), we selected 24 genes whose expression was more than 5.0-fold in petals of flower stage 12 (sample ID: ATGE_35) and more than 2.0-fold in petals of flower stage 15 (ATGE_42) compared to the average expression level of all wild-type samples. We also chose 18 genes whose expression was less than 0.2-fold in flower stage 12 of the *lfy-12* mutant (ATGE_54) because *lfy* mutants fail to produce flowers (Huala and Sussex 1992). Genes for the development bulk were selected from literature and database searches. We identified 42 genes that participate in the regulation of organ development and/or cell differentiation. *AGL13* and *MYB21* were added as homologous genes of *AGL6* and *MYB24*, which were used in the flower bulk. Furthermore, 6 functionally unknown genes expressed at a high levels in *Arabidopsis* flowers were included, resulting in a total of 50 genes in the development bulk set.

Plasmid construction

For chimeric repressor constructs, the coding sequences of transcription factors without stop codons were amplified with appropriate primers. Each fragment was cloned into the *Sma*I site of p35SSRDXG entry vectors in frame to the region that encodes SRDX repression domain, resulting in chimeric repressors driven by cauliflower mosaic virus 35S promoter. For the PTL-overexpressor (PTL-ox) construct, the coding sequence with a stop codon was cloned into the *Sma*I site of p35SG entry vector, which is a derivative of p35SSRDXG without SRDX. Each transgene was transferred to pBCKH binary vectors (Mitsuda et al. 2006).

Transformation

The 42 and 50 chimeric repressor constructs for the flower bulk and the development bulk, respectively, were mixed in equal concentrations. The plasmid mixtures were cointroduced into *Agrobacterium* strain EHA105 for torenia and GV3101 for *Arabidopsis* by electroporation. The entire colony suspension was used for transformation. The methods of *Agrobacterium*-mediated transformation were as described previously (Aida and Shibata 1995, 2001; Clough and Bent 1998). Leaf segments of regenerated torenia plants were tested for hygromycin resistance to confirm the transgene.

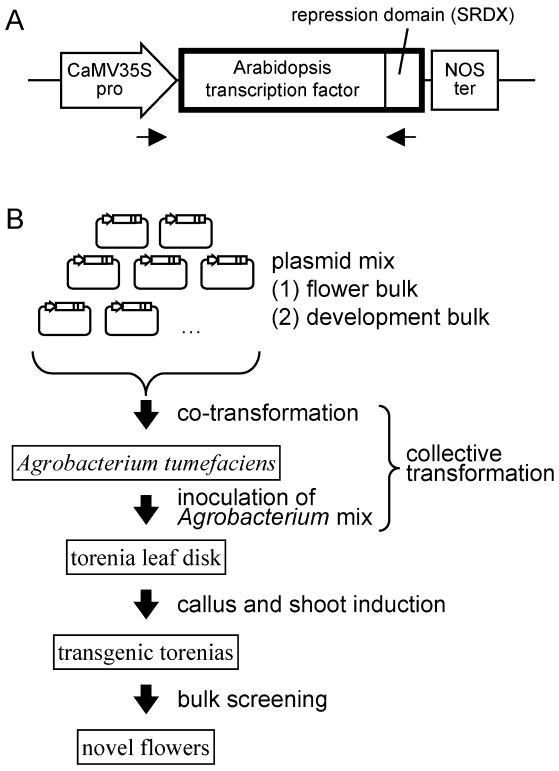

Figure 1. CT system procedure. (A) Schematic representation of the chimeric repressor construct consisting of the cauliflower mosaic virus (CaMV) 35S promoter, a transcription factor from *Arabidopsis* without the stop codon, followed by an in-frame repression domain (SRDX) and a nopaline synthase (NOS) terminator. Arrows indicate the primers used in investigating transgenes. (B) Flowchart of the efficient screening of novel flowers.

Identification of transgenes

Genomic DNA was isolated from leaves of regenerated torenia plants, and transgene fragments were amplified with 35S primer (5′-GAAGTTCATTTCATTTGGAGAGG-3′) and SRDX primer (5′-AGCGAAACCCAAACGGAGTTCTAG-3′) (Figure 1A). The PCR products were directly sequenced. Cases in which multiple genes were introduced, PCR products were electrophoresed on agarose gel, recovered from the gel, and used for sequencing.

Statistical analysis

The probability $[q_i(n)]$ of the number of transgenic torenia lines (n) harboring each construct (i) when transformation occurred with equal probability is described by a binomial distribution as follows:

$$q_i(n) = {}_N C_n \, p^n \, (1-p)^{N-n}$$

p is the probability that each construct was equally transformed (p=1/42 for the flower bulk and p=1/50 for the development bulk) and N is the number of transgenic torenias with a single transgene (N=159 for the flower bulk and N=303 for the development bulk).

Results

Development of a collective transformation system for efficient modification of novel floral traits

To produce various transgenic torenias simultaneously, we attempted to simplify the transformation method. First, we used *Arabidopsis* transcription factor genes to produce chimeric repressors (Figure 1A). Second, we mixed approximately 50 types of plasmids in equal concentrations and cotransformed *Agrobacterium*. We then inoculated torenia leaf disks with a mixture of transformed *Agrobacterium*. We called this transformation method the collective transformation (CT) system. Using these procedures, we generated hundreds of transgenic plants and screened novel flower colors and shapes simultaneously and efficiently (Figure 1B).

Since genes regulating flower development, such as *AP1* and *AG* (Mandel et al. 1992; Yanofsky et al. 1990), are expressed in higher level in flowers than in other organs, we selected 42 genes that are highly expressed in *Arabidopsis* flowers from publicly available microarray data (referred to as “flower bulk;” Table 1). On the other hand, transgenic torenias expressing the chimeric repressor of TCP3, which controls the morphology of shoot lateral organs (Koyama et al. 2007), induced serrated petals with various color patterns (Narumi et al. 2011 in this issue). The epidermal cell arrangement of the petals in these transgenic torenias was perturbed, suggesting that genes regulating organ development and/or cell differentiation as well as those controlling the biosynthesis of flower pigments can modify color patterns. Based on the abovementioned hypothesis, we selected 42 genes participating in the regulation of organ development and/or cell differentiation according to a literature and database search. Furthermore, we added 8 genes (see Materials and methods) and referred to the chimeric repressors containing the total 50 genes as the “development bulk” (Table 2). We mixed the 42 and 50 chimeric repressor constructs, respectively, and transformed torenia using the CT system (Figure 1B). After callus induction, adventitious shoot formation, and plant regeneration, we obtained 348 and 402 regenerated plants from the flower bulk and the development bulk, respectively (Table 3).

Simultaneous production of various types of transgenic torenias using the CT system

To investigate whether each chimeric repressor was introduced equally and efficiently, we isolated genomic DNA from transgenic torenias and sequenced the transgenes. We identified transgenes from 196 lines of the flower bulk, and 40 of the 42 introduced genes were found (Table 1). With respect to the transgene number,

Table 1. The flower bulk

Family	AGI code	Common name	Phenotype	1 gene	2 genes	3 genes
AP2/ERF	At1g01250		F	5	1	1
	At1g12610	DDF1	F, O	12**	4	0
	At1g19210		F	3	3	1
	At1g74930	ORA47	F	10*	2	0
B3	At5g18000		F, L, O	8	0	1
	At5g18090		F	4	0	0
	At5g60140		x	2	1	0
bHLH	At1g59640	BPE/ ZCW32/ bHLH031	–	0	0	1
	At1g25330	bHLH075	–	0	2	0
	At2g47270	bHLH151	L	7	0	0
bZIP	At1g35490		x	2	2	0
C2H2ZnF	At1g66500		x	6	0	0
	At4g35700		x	3	1	2
	At2g28200		–	0	0	0
C3HZnF	At5g44260		x	3	1	0
CO	At4g39070		x	1	3	0
GARP	At5g42630	KAN4	x	1	0	0
GRAS	At1g66350	RGL1	–	0	0	0
	At3g03450	RGL2	L	1	1	0
HSF	At2g26150	AtHSFA2	F	5	2	0
	At3g51910	AtHSFA7A	F	4	0	0
LIM	At2g45800		O	7	5	0
	At3g61230		F, O	7	1	0
	At1g01780		x	4	2	1
MADS	At2g45650	AGL6	F	2	2	0
	At3g57390	AGL18	F, O	5	3	0
MYB	At4g09460	AtMYB6/ AtMYB8	F, O	6	3	1
	At5g15310	AtMYB16/ AtMIXTA	F, L	6	1	0
	At2g32460	AtMYB101	F	1	1	1
	At3g06490	AtMYB108/ BOS1	x	1	0	1
	At2g21650	AtRL2/ MEE3	F	11*	8	2
	At5g40350	AtMYB24	L	1	2	1
NAC	At1g52890	ANAC019	F	5	0	0
	At1g61110	ANAC025	x	1	1	1
	At3g15500	ANAC055	x	2	1	0
	At3g15510	ANAC056/ NARS1/ NAC2	F	3	3	1
	At4g28530	ANAC074	F	1	0	0
	At5g07680	ANAC079	F	7	2	0
	At5g61430	ANAC100	L	4	2	0
SBP	At3g15270	SPL5	x	1	1	0
Others	At4g00950	MEE47	x	3	2	0
	At3g24500	MBF1C	x	4	1	0

Phenotypically altered organs in transgenic torenias with a single transgene are represented as F (flowers), L (leaves), O (others), and x (no change). The number of lines with 1, 2, and 3 transgenes are shown. Asterisks indicate values that are significantly different (*$p<0.01$, **$p<0.001$) from the statistical value for equal transformation.

81.1% (159 lines) had a single transgene and 16.3% (32 lines) and 2.6% (5 lines) had 2 and 3 transgenes, respectively (Figure 2, Table 1). This indicates that the CT system is effective for the production of various transgenic plants with a single transgene. In the 159 lines with a single transgene, 38 constructs were found, and 1–12 lines for each construct were obtained (Table 1). Line number distribution was mostly statistically predictable. Therefore, we concluded that transgenes were introduced almost equally and that the CT system efficiently transformed torenia.

In contrast to the statistically predictable result of the flower bulk, the development bulk resulted in biased production of transgenic torenias. We identified transgenes of 329 lines from the development bulk, and the number of introduced chimeric repressors was similar to that of the flower bulk (Figure 2). However, with respect to the transgene number, we found that more than 14 lines of transgenic plants were obtained for 6 constructs (GNL-SRDX, BP-SRDX, AS1-SRDX, ANT-SRDX, MYB106-SRDX, and PTL-SRDX) (Table 2). In particular, the total number of lines with ANT-SRDX, MYB106-SRDX, and PTL-SRDX comprised 47.9% of those with a single transgene (145/303). Furthermore, 16 constructs were not found in 303 transgenic torenias. The number of the transgenic plants harboring the 6 and 16 constructs were significantly ($p<0.01$) larger and smaller, respectively, than the value expected for an equal transformation.

In contrast, transgenic *Arabidopsis* that transformed with the same plasmid mix of the development bulk did not show such a big bias of introduced genes as transgenic torenias (Table 2). Since *Arabidopsis* transformants were not produced via calli but seeds, it is possible that some chimeric repressors used in the development bulk facilitated or suppressed callus formation and/or adventitious shoot initiation. To examine this hypothesis, we compared the adventitious shoot number when chimeric repressors were individually introduced. MYB106-SRDX was selected as a representative of the construct for plants produced with a high frequency, and At5g58900-SRDX and AGL13-SRDX were used as constructs for statistically predictable number plants. In addition, PTL overexpressor (PTL-ox) was examined as a nonrepressor construct. MYB106-SRDX shoots appeared rapidly in almost 100 of 800 leaf disks after 2 months of the infection, while those in other 3 infections just started initiating at that point as usual (Figure 3). The number of shoots from At5g58900-SRDX and AGL13-SRDX calli was less than 40 even 4 months after infection. These results indicate that MYB106-SRDX facilitates adventitious shoot formation. Genes of the development bulk function in organ development and/or cell differentiation, and therefore, the other 21 genes may play roles in callus formation and/or adventitious shoot initiation.

Phenotypic variations of flowers in transgenic torenias

Of a total of 750 transgenic torenias, 71 lines from the flower bulk and 193 lines from the development bulk

Table 2. The development bulk

Family	AGI code	Common name	Phenotype	1 gene	2 genes	3 genes	4 genes	*Arabidopsis*
AP2/ERF	At1g15360	SHN1/WIN1	F	4	1	0	0	1
	At4g37750	ANT	F	36**	1	0	0	7*
AS2/LOB	At2g30130	ASL5/LBD12	–	0*	0	0	0	0
	At1g31320	ASL6/LBD4	–	0*	0	2	0	0
	At2g40470	ASL11/LBD15	–	0*	0	0	0	0
	At3g11090	ASL12/LBD21	x	3	0	0	0	2
	At4g00220	ASL19/LBD30	–	0*	0	0	0	0
	At3g02550	ASL38/LBD41	x	1	0	1	0	2
	At5g67420	ASL39/LBD37	–	0*	0	0	0	0
	At3g49940	ASL40/LBD38	–	0*	1	1	0	3
	At4g37540	ASL41/LBD39	F, L	9	1	1	0	4
BELL	At4g36870	SAW1/BLH2	–	0*	0	0	0	2
	At2g23760	SAW2/BLH4	–	0*	1	0	0	5
bHLH	At4g00870	bHLH014	x	7	0	2	0	14**
	At3g61950	bHLH067	–	0*	0	0	0	0
	At1g59640	BPE	F	3	2	2	1	0
bZIP	At1g75390	bZIP44	F	3	1	0	0	0
C2H2ZnF	At1g68480	JAG	–	0*	0	0	0	0
	At5g06070	RBE	F	2	0	3	0	4
GARP	At5g16560	KAN1	x	1	0	0	0	0
	At1g32240	KAN2	F	1	0	0	0	0
GATA	At5g56860	GNC	–	0*	0	0	0	1
	At3g50870	HAN	F	3	0	1	0	0
	At2g18380	HANL1	x	3	2	2	0	0
	At4g36620	HANL2	L, O	3	1	0	0	0
	At4g26150	GNL	F	14*	1	0	0	2
HD–ZIPIII	At5g60690	REV	F	4	0	0	0	0
	At4g32880	ATHB8	–	0*	0	0	0	2
	At1g52150	ATHB15	F, O	4	0	0	0	0
	At2g34710	PHB	F	7	2	0	0	2
HD-ZIPIV	At1g05230	HDG2	–	0*	0	0	0	0
	At4g04890	PDF2	–	0*	0	0	0	0
	At4g21750	ATML1	–	0*	0	1	0	0
	At4g00730	ANL2	x	1	0	0	0	0
KNOX	At4g08150	BP/KNAT1	F	14*	1	2	0	2
	At1g62360	STM	x	2	1	0	0	0
	At1g70510	KNAT2	F	1	2	0	0	3
	At1g23380	KNAT6	F	8	1	0	1	10**
MADS	At3g61120	AGL13	x	1	2	0	0	3
MYB	At3g61250	MYB17	F, O	3	1	0	0	2
	At3g27810	MYB21	L	9	1	0	0	4
	At3g28910	MYB30	–	0*	0	0	0	0
	At3g01140	MYB106/NOK	F	41**	2	2	0	6
	At2g37630	AS1	F	17**	3	0	1	12**
	At1g75250	AtRL6	F	12	2	2	1	11**
	At5g58900		F	2	1	0	0	2
	At2g38090		F	5	0	0	0	7*
	At3g10590		F, L, O	10	2	1	0	5
NAC	At1g69490	NAP	F	1	0	0	0	0
trihelix	At5g03680	PTL	F	68**	1	1	0	4

Phenotypically altered organs in transgenic torenias with a single transgene are represented as F (flowers), L (leaves), O (others), and x (no change). The number of lines with 1, 2, 3, and 4 transgenes are shown. The results of transformed *Arabidopsis* with the development bulk are represented. Asterisks indicate values that are significantly different (*$p<0.01$, **$p<0.001$) from the statistical value for equal transformation.

Table 3. Production of transgenic torenias.

Bulk name	Flower	Development
Chimeric repressors	42	50
Infected leaf disks	7,000	9,600
Induced calli	not counted	617
Regenerated plants	348	402
Transgene-identified plants	196	329

showed some phenotypic changes in flowers, leaves, and plant posture (Figure 4). Floral phenotypic changes were induced by 19 flower bulk genes and 24 development bulk genes (Tables 1, 2). Phenotypic changes in flower colors and/or shapes were shown in 83.1% of the flower bulk lines and 94.3% of the development bulk lines. We found 48 transgenic plants with modified flower colors in the flower bulk lines (Figure 4A). Of 4 AGL6-SRDX-

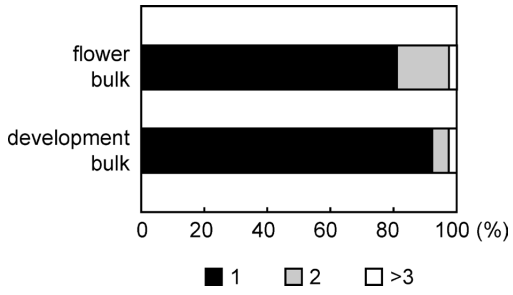

Figure 2. Number of introduced chimeric repressors per plant. Transgenes were identified in 196 and 329 transgenic plants of the flower bulk and the development bulk, respectively.

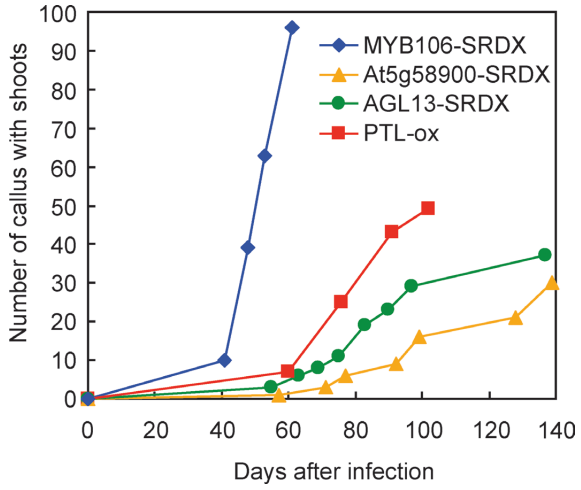

Figure 3. Comparison of the effects of transgenes on adventitious shoot formation. MYB106-SRDX, At5g58900-SRDX, AGL13-SRDX, and PTL-ox were individually introduced into 800 leaf disks, and the number of calli with visible shoots was counted.

containing plants, 3 showed white-margined petals, while the lips of wild-type torenia are uniformly dark violet to the margin (Figure 5A, B). In the ventral petals of 4 of 7 ANAC056-SRDX- and 3 of 9 ANAC079-SRDX-containing plants, the boundary between the 2 colors shifted to the distal position (Figure 5C, D). Similar phenotypes of these plants imply that ANAC056 and ANAC079 function in the same regulatory pathway. Of 11 AtRL2-SRDX plants, 8 showed monotone pale violet corollas, as if the tube color had expanded to the lips (Figure 5E). In contrast to tube color expression, flowers of the transgenic plant harboring bHLH075-SRDX and At1g01250-SRDX had speckles in the boundary region of the ventral petals (Figure 5F). In DDF1-SRDX flowers (9 of 16 plants), pale-colored lips were observed (Figure 5G).

In addition to modification in color, some transgenic torenias showed modification in flower shapes, such as serrated petals, elongated or bent dorsal petals, wide ventral petals, and half-opened flowers (Figure 5B, C, E, H). Furthermore, a transgenic torenia containing both At1g35490-SRDX and MYB24-SRDX had small corolla

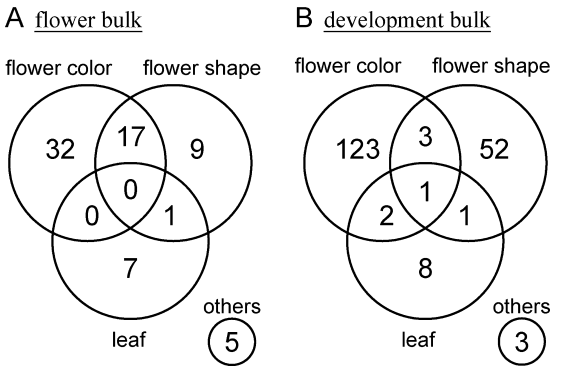

Figure 4. Venn diagram of modified traits. The total number of plants with some phenotypic change was 71 from the flower bulk (A) and 193 from the development bulk (B). "Others" includes dwarf and bushy phenotypes with no change in both flowers and leaves.

(Figure 5I). A deep split of corolla with a small lip area was produced in a transgenic plant containing 3 chimeric repressors of ANAC056, BPE, and At1g19210 (Figure 5J). Thus, the flower bulk genes efficiently induced modification of flower colors and shapes in torenia.

In addition to novel floral traits from the flower bulk, we found 182 plants with altered flower color and/or shape in the 402 transgenic plants from the development bulk (Figure 4B). MYB transcription factors, including At5g58900, At2g38090, and At3g10590 as well as AtRL2 in the flower bulk induced various flower colors and shapes. One At5g58900-SRDX plant displayed wavy petals with a narrow dark violet region (Figure 5K). Another At5g58900-SRDX flower showed a paler tube color than that of wild-type flowers (Figure 5L). At5g58900 is homologous to *Antirrhinum* DIV, which controls the dorsoventral asymmetry of flowers (Almeida et al. 1997; Corley et al. 2005; Galego and Almeida 2002). A chimeric repressor of At2g38900, which showed similarity to DIV and At5g58900, also induced pale tube color (data not shown), and another 2 At2g38900-SRDX lines displayed splash pattern and wavy petals, respectively (Figure 5M, N). These results suggest that transcription factors of the DIV subfamily have similar functions in controlling color patterns and configurations of petals with some phenotypic variation, although the floral asymmetry of the transgenic torenias was not affected. At3g10590 is another MYB protein, and 1 of 13 At3g10590-SRDX plants showed deeply split corollas (Figure 5O).

Besides the MYB family, 10 of 17 lines expressing the chimeric repressor of BP, a KNOX transcription factor, showed expansion of the violet-colored region of the lips (Figure 5P). Of 14 chimeric repressors of GATA transcription factor GNL, 2 lines induced inward curled flowers and 1 showed serrated petals (Figure 5Q, R), although *GNL* has not been shown to control organ margin development. Other notable phenotypes were derived from the chimeric repression of AGL13, a

Figure 5. Modified flower phenotypes of transgenic torenias. (A) Wild-type torenia flower. (B–T) Transgenic torenia flowers from the flower bulk (B–J) and the development bulk (K–T). Integrated transgenes without SRDX are represented in the pictures. Inset in (A) and (F) shows boundary region of colors in the ventral petals. At5g58900-SRDX (K, L), At2g38090-SRDX (M, N), and GNL-SRDX (Q, R) induced phenotypic variations.

MADS-box transcription factor. Two lines contained AGL13-SRDX with other chimeric repressors, KNAT6-SRDX and MYB17-SRDX, and they showed wrinkled petals with radial white lines and 5 white sectors in the lips, respectively (Figure 5S, T). The sectors were in the center of the lateral and ventral petals, and 2 sectors in the dorsal petal were probably attributable to the fusion of 2 petals on the dorsal side (Nishijima and Shima 2006). The various phenotypes from the development bulk indicate that genes regulating organ development and/or cell differentiation can be utilized to modify flower colors and shapes.

Morphological alteration in leaves

Of 750 transgenic torenias, we found phenotypic modification in the leaves of 20 (Figure 4). Deep serrated leaves were produced in a transgenic torenia harboring 3 chimeric repressors, ATML1-SRDX, PTL-SRDX, and bHLH014-SRDX (Figure 6B). One of 4 HANL2-SRDX plants had a dwarf phenotype with small and uneven leaves (Figure 6C). A similar phenotype was observed in transgenic *Arabidopsis* overexpressing *HAN*, which is a close homolog of *HANL2* (Zhao et al. 2004). One of 2 RBE-SRDX plants as well as 1 MYB24-SRDX plant and 6 of 9 MYB21-SRDX plants had epinastic leaves (Figure 6D, E, F). MYB24 and MYB21 belong to subgroup 19 of the MYB family proteins, which include *Antirrhinum* AmMYB305 and AmMYB340, and genes in this subgroup are specifically expressed in flowers (Jackson et al. 1991; Shin et al. 2002; Yang et al. 2007). Thus, the CT system with chimeric repressors can be also utilized to modify leaf morphology.

Further analysis of blotch size and petal texture

Some phenotypes of transgenic torenias have not been identified in *Arabidopsis* research, such as enlarged blotch size in the ventral petals of PTL-SRDX plants (40 of 68 lines) (Figure 7A). Blotches in PTL-SRDX extended in width rather than in length (Figure 7D). *PTL* acts as a growth repressor in the inter-sepal zone and is

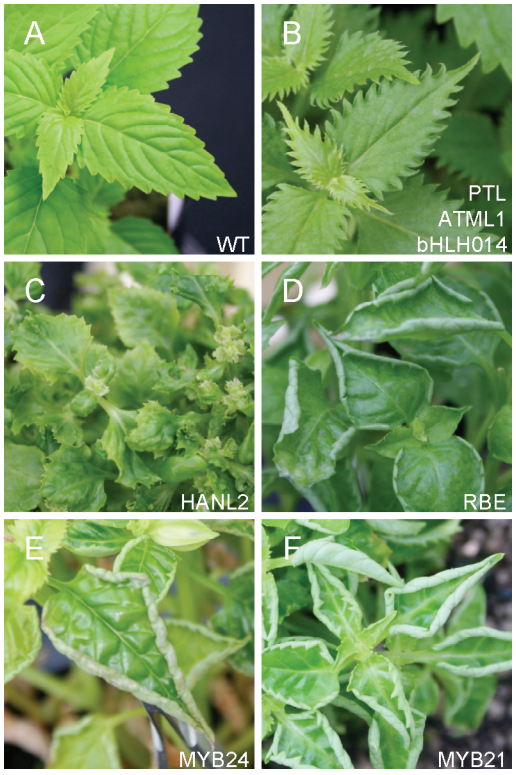

Figure 6. Modified leaf phenotypes of transgenic torenias. (A) Wild-type leaf. (B–F) Transgenic torenia leaves. Integrated transgenes without SRDX are represented in the pictures.

important in boundary formation to separate sepals in *Arabidopsis* (Brewer et al. 2004). To confirm that *PTL* reduces blotch area, we generated overexpressing torenias. Of 37 PTL-ox torenias, 14 displayed small blotch size (Figure 7C, D), indicating that *PTL* controls the blotch size in torenia flowers.

Another notable trait observed was lusterless flowers in transgenic plants harboring both KNAT2-SRDX and bZIP44-SRDX, while wild-type flowers were velvety (Figure 7E, G). Observation with scanning electron microscopy revealed that the epidermal cells of the lusterless flowers showed distinctive shapes with enlarged bases, while those of the wild-type were cone shaped (Figure 7F, H). Flower color intensity has been shown to be dependent on epidermal cell shape (Noda et al. 1994), and ectopic *KNAT2* expression affects cell shape in *Arabidopsis* leaves (Pautot et al. 2001). The lusterless flowers might be due to the modified cell shape by the chimeric KNAT2 repressor.

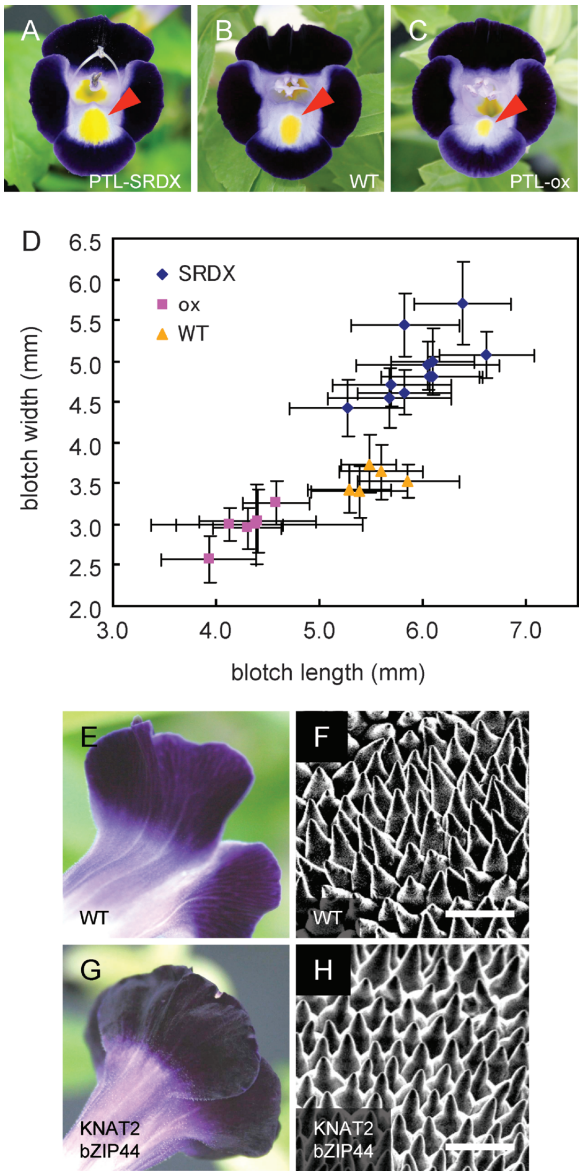

Figure 7. Detailed analysis of transgenic torenias with big blotches and lusterless flowers. (A) PTL-SRDX flower. (B) Wild-type flower. (C) PTL-ox flower. Red arrow heads indicate blotches. (D) Blotch length and width. The data are average ±SD of 10 flowers. (E–H) Flowers of wild-type (E) and KNAT2-SRDX- and bZIP44-SRDX-harboring (G) plants. Scanning electron microscopy observation of petal epidermal cell in wild-type (F) and KNAT2-SRDX- and bZIP44-SRDX-harboring (H) plants. Scale bars in (F) and (H) represent 50 μm.

Discussion

Efficient transformation using the CT system

We developed the CT system to produce transgenic torenias efficiently. In our results, 80–90% transgenic torenias had a single transgene (Figure 2). This indicates that the CT system is more efficient and convenient than individual transformation in production of various transgenic plants. Meanwhile, almost half of the transgenic plants from the development bulk comprised PTL-SRDX, MYB106-SRDX, and ANT-SRDX plants (Table 2), suggesting that these transgenic torenias were generated preferentially through the transformation process for some reason. The bias in the development bulk and relatively equal transformation in the flower bulk implied that modification of organ development and cell differentiation affected the process of transformation. The process of transformation consists of 5 steps:

(i) plasmid introduction into *Agrobacterium*, (ii) *Agrobacterium* infection of torenia leaf disks, (iii) callus induction, (iv) adventitious shoot initiation, and (v) plant regeneration. We introduced the same plasmid mix into *Arabidopsis*, and no obvious bias was seen as in torenia (Table 2). This result excluded the possibility of (i) and maybe (ii) affecting transformation because these processes in torenia are similar to those in *Arabidopsis*. We obtain transgenic torenias via callus and not via seed as in *Arabidopsis*, indicating that the biased production of transgenic torenias was probably because of some chimeric repressors facilitating callus formation and/or adventitious shoot initiation. Rapid adventitious shoot induction of MYB106-SRDX also supports this idea (Figure 3). In addition, it has been reported that *PTL* reduced the outgrowth of lateral organ margins (Brewer et al. 2004), implying that PTL-SRDX stimulated the outgrowth of adventitious shoots from the callus. On the other hand, callus formation and adventitious shoot initiation require an appropriate auxin/cytokinin ratio. *PTL* overexpressing mutant in *Arabidopsis* affected auxin homeostasis (Li et al. 2008), and *ANT* probably acts downstream of auxin to regulate growth (Krizek 2009). PTL-SRDX and ANT-SRDX may perturb the auxin/cytokinin balance facilitating callus formation and adventitious shoot initiation. Interestingly, *ANT*, *PTL*, and *MYB106* expression levels in *Arabidopsis* calli were lower than those in seedlings (GEO accession No. GSE21631; Iwase et al. 2011). Callus formation and adventitious shoot initiation may require down-regulation of these transcription factors. Functional analysis of *ANT*, *PTL*, and *MYB106* in calli will provide a novel technique for controlling callus formation and adventitious shoot initiation.

In contrast, 12 of 50 chimeric repressors of the development bulk were not found in the 329 transgenic torenias. From the viewpoint of transcription factor family, few or no transgenic plants with chimeric repressors of the AS2/LOB, BELL, and HD-ZIP IV family proteins were present (Table 2). Most AS2/LOB family proteins are functionally unknown, while *ASL1*/*LBD36* controls proximal–distal patterning in petals and *ASL9*/*LBD3* is induced by cytokinins (Chalfun-Junior et al. 2005; Naito et al. 2007). *SAW1*/*BLH2* and *SAW2*/*BLH4* function as negative regulators of growth (Kumar et al. 2007). The expression of HD-ZIP IV family genes is restricted in the outer cell layer of plant organs (Ariel et al. 2007). These functions are probably concerned with the suppression of callus formation and/or adventitious shoot initiation. Because we used a constitutive promoter, cauliflower mosaic virus 35S, in this research, using alternative promoters such as a flower-specific promoter and an inducible promoter could exclude the effect of chimeric repressors on development and differentiation.

Modification of flower colors and shapes by transcription factors controlling development

We obtained many more phenotypes in flowers than in leaves from the flower bulk and the development bulk (Figure 4). It was noted that transgenic plants affected in both flower color and shape were 28.8% and 2.2% of the flower-modified plants in the flower bulk and the development bulk, respectively. These results indicate that genes in the development bulk specifically affect flower color or shape, for example, by controlling cell shapes relating to petal texture (Figure 7G, H). In *Arabidopsi*s, conical and flat cells are on the distal and proximal position on the petals, respectively (Chalfun-Junior et al. 2005). Modification of cell type and arrangement can be utilized as a novel for controlling the color pattern of petals without engineering pigment biosynthesis.

One of the most impressive flowers was derived from the chimeric repressors of AtRL2 and At5g58900. The phenotypes of the colored pattern were beyond the predicted function of the homologous genes, *Antirrhinum RAD* and *DIV*, both of which control dorsoventral asymmetry of flowers (Almeida et al. 1997; Baxter et al. 2007; Corley et al. 2005; Galego and Almeida 2000). In addition to well-characterized transcription factors, functionally unknown factors, such as ANAC079 and At3g10590, modified floral traits. These results indicate that we can utilize a broad range of genes for flower modification beyond that expected from the putative gene function in *Arabidopsis* and other model plants. Furthermore, analysis of transgenic torenias will provide the first clue in understanding the functions of transcription factors that have not been identified in previous studies on *Arabidopsis*.

Possibility of application to other ornamental flowers

The CT system with chimeric repressors of *Arabidopsis* transcription factors enables the efficient production of novel floricultural plants with valuable floral traits, without increasing effort, time, and space required for molecular breeding. We used torenia as a plant material, which is a possible model plant for the examination of the molecular biology of floricultural plants (Sasaki et al. 2008, 2010). The CRES-T system can be applied to other floricultural plants such as gentian, cyclamen, and morning glory, lisianthus, and even polyploid floricultural plants such as tetraploid rose and hexaploid chrysanthemum (Gion et al. 2011 in this issue; Mitsuda et al. 2008, 2011 in this issue; Nakatsuka et al. 2011 in this issue; Sage-Ono et al. 2011 in this issue; Tanaka et al. 2011 in this issue). The phenotypes of transgenic torenias in our studies are available from "FioreDB," which is a database of phenotypic information induced by the CRES-T system in *Arabidopsis* and floricultural

plants (http://www.cres-t.org/fiore/public_db/) (Mitsuda et al 2008, 2011 in this issue). Selection of the gene of interest from this database and the application of the CT system to any floricultural plants will facilitate acquisition of valuable flowers for commercial use.

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