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REVIEW
Promotion of Efficient Molecular Breeding Using Chimeric Repressors in Ornamental Flowers

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Abstract
Useful novel floral traits have recently been generated in various ornamental crops by using Chimeric REpressor gene Silencing Technology (CRES-T). CRES-T is a plant-specific gene silencing method that targets transcription factors (TFs), causing a dominant loss-of-function phenotype. In our group project, we applied CRES-T to various ornamental crops and mainly elucidated the following points: 1) CRES-T was effective for the modification of floral traits, 2) it was useful in higher polyploidy crops, and 3) it enabled the creation of commercially valuable flowers. In CRES-T, the attachment of a short repression domain derived from a transcriptional repressor to the C-terminal region of target TFs converts transcriptional activators to dominant chimeric repressors. In the target flower species, some *Arabidopsis* chimeric repressors were effective for the production of new floral traits without cloning the TF genes of interest. Therefore, the use of *Arabidopsis* chimeric repressors could be widely effective for many ornamental crops, even when the genome and/or expressed sequence tag (EST) information of target crops is unavailable or lacking. In addition, the collective transformation of chimeric repressors into ornamental crops is effective in isolating useful and/or target floral traits, such as petal colors, petal shapes, and color patterns. This technology could help to accelerate the developmental period and reduce the cost of developing new floral traits.

Discipline: Biotechnology
Additional key words: CRES-T, floral trait, promoter, transcription factor

Introduction

Genetically modified (GM) flowers such as blue carnations (Tanaka et al. 2009) and blue roses (Katsumoto et al. 2007) were commercialized in Japan from 1997 and 2007, respectively. Thus, GM flowers have almost gained public acceptance in Japan. However, the conventional method of molecular breeding for the development of these GM flowers requires a lot of time (sometimes more than a decade; Ohtsubo 2011), and the costs are very high. Therefore, it is difficult to keep up with consumer preferences that change every year and cope with the high costs of developing even one new variety. At present, there is an urgent need for the next generation of molecular breeding technology to accelerate the developmental period and reduce development costs. Efficient massive screening methods would also be important for reducing costs required for developing a new variety.

Transcription factors (TFs) are master genes that control various floral traits, such as flower colors, flower shape, petal shapes, and fragrance (Petroni & Tonelli 2011, Preston & Hileman 2009, Colquhoun & Clark 2011). Therefore, the modification of TF functions has great potential to dynamically change floral traits. Chimeric REpressor gene Silencing Technology (CRES-T; Hiratsu et al. 2003, 2004) is a plant-specific gene silencing method that dominantly suppresses the functions of TFs. CRES-T has been applied to create target traits in the model plant *Arabidopsis* and in rice (Mitsuda et al. 2006, Mito et al. 2011). In addition, the flower CRES-T project in Japan (Ohtsubo 2011) reported that CRES-T can be applied to various ornamental crops, such as *Torenia*, rose, gentian, lisianthus, cyclamen, chrysanthemum, and morning glory

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(Mitsuda et al. 2008, Mitsuda et al. 2011a), although it was developed for the model plant *Arabidopsis* (Hiratsu et al. 2003, 2004). Therefore, CRES-T may be effective for a large variety of monocot and dicot ornamental crops. In addition, a collective transformation (CT) system (Shikata et al. 2011) would accelerate development to keep pace with changing consumer preferences, while still controlling the costs of commercialization such as in performing massive screening using the CT system.

TFs perform crucial functions in floral traits

In plants, TFs control the expression of their downstream genes by upregulating or downregulating the gene transcriptions. In *Arabidopsis*, there are approximately 2,000 TFs, which account for approximately 5%-10% of their total genes (Riechmann et al. 2000). The number of TF subfamilies identified in several databases ranges from 51-67 (72 in total), depending on the differences in criteria applied to their DNA binding domains (Riechmann et al. 2000, Mitsuda & Ohme-Takagi 2009). TFs bind to the promoters of downstream genes by recognizing a specific DNA sequence — the "*cis*-element." The TFs act as a master switch to upregulate or downregulate the other 90% of the plant genes; therefore, TFs have various important functions in the plant body, such as the floral organs. For example, floral organ identity is explained by the ABCE-model (Ma 1994, Theißen 2001, Ó'Maoiléidigh et al. 2014) in which the A-, B-, C- and E-function genes are TFs having a MADS-box DNA binding domain. Furthermore, floral zygomorphy that is observed in *Antirrhinum majus* and *Lotus japonicus* is also controlled by TFs, such as *CYCLOIDEA*, *DICHOTOMA*, *DIVARICATA*, and *RADIALIS* (Preston & Hileman 2009, Feng et al. 2006).

Difficulty in the functional analysis of TFs due to gene redundancy

The functional analyses of MADS-box and floral zygomorphy genes have been mainly conducted using mutants of *Arabidopsis* and *A. majus* (Ma 1994, Preston & Hileman 2009). However, it is difficult to analyze the function of redundant TFs by a single mutation because other functionally redundant TFs complement this mutation. In some plant species, plant genes frequently show duplication and/or redundancy, including TFs (De Smet & Van de Peer 2012, Moore & Purugganan 2005) and MADS-box TFs (Rijpkema et al. 2007, Soltis et al. 2007, Zhan et al. 2005). Gene-knockout and/or antisense methods specific for a particular TF also sometimes fail to show phenotypic change due to its functional redundancy (Bouché & Bouchez 2001). For example, the mutant phenotype of *Arabidopsis TCP* was not sufficiently observed even in a triple mutant (Koyama et al. 2010). Class B MADS-box genes that play important roles in the formation of petals and stamens are redundantly found in such horticultural crops as *Gerbera hybrida* (Broholm et al. 2010), *Orchis italica* (Salemme et al. 2011), and *Petunia hybrida* (Vandenbussche et al. 2004), but not in *Arabidopsis* and *A. majus*. The analysis of such redundant TFs by a single mutation has limitations, particularly in higher polyploidy plants. Thus, any important functions of floral traits not found in the mutant analyses of *Arabidopsis* and *A. majus* would still be present in such horticultural crops.

Development and application of CRES-T in ornamental crops

The development of floral organs is mainly regulated by TFs, and CRES-T has reportedly been an important breakthrough for the functional analysis of plant TFs (Hiratsu et al. 2003). The strong repression domain (SRDX) derived from plant TFs converts transcriptional activators to chimeric repressors that dominantly repress the function of target TFs, even in the presence of functionally redundant TFs (Hiratsu et al. 2003, Koyama et al. 2007, Mitsuda et al. 2007, Oshima et al. 2013). Figure 1 shows how simple it is to produce a chimeric repressor by attaching SRDX onto the C-terminal regions of target TFs. Although this method was developed in *Arabidopsis* (Hiratsu et al. 2003, 2004), it has also been applied to monocot rice (Mitsuda et al. 2006, Mito et al. 2011, Tanaka et al. 2012). In addition, SRDX is effective for many ornamental crops, such as *Torenia*, rose, gentian, lisianthus, cyclamen, chrysanthemum, and morning glory (Mitsuda et al. 2008, Mitsuda et al. 2011a). Because this system functions dominantly, the chimeric repressors even worked in higher polyploidy plants, such as the hexaploid chrysanthemum (Mitsuda et al. 2011a, Narumi et al. 2011). It is noteworthy that chimeric repressors constructed with *Arabidopsis* TFs frequently worked in other ornamental crops for the functional analysis of TFs and the development of novel floral traits without any further modification (Narumi et al. 2011, Shikata et al. 2011, Gion et al. 2011). Information regarding the floral phenotypes generated in the CRES-T project is available on the FioreDB website: Database for Flower Bio-engineering by CRES-T (Mitsuda et al. 2011a; http://www.cres-t.org/fiore/public_db/index.shtml). The floral phenotypes shown on the web site may provide useful ideas for the development of new flowers in plant species for which it is difficult to isolate new floral traits by using traditional breeding methods.

Chimeric repressors enable the creation of novel floral traits

Chimeric repressors have produced various novel flowers in many ornamental crops, including commercially

valuable transgenic flowers. Cyclamens have two class *C AGAMOUS* (*AG*) orthologs — *CpAG1* and *CpAG2* (Tanaka et al. 2011) — and the simultaneous overexpression of *CpAG1-SRDX* and *CpAG2-SRDX* produced a multi-petal flower phenotype not previously seen in cyclamen (Fig. 2; Tanaka et al. 2013). The co-overexpression of *CpAG1-SRDX* and *CpAG2-SRDX* caused the stamens and carpels of the transgenic cyclamen to develop petal-like organs, and the multi-petal flowers contained a total of more than 40 petals and petal-like organs, as compared with 5 petals in the wild-type cyclamen. Because the gorgeous rose-like phenotype is one of the most commercially preferred phenotypes, the multi-petal cyclamen would be closest to commercial viability among the GM flowers produced by the chimeric repressors.

To generate various floral traits concurrently, a CT system has been applied in *Torenia* (Fig. 3; Shikata et al. 2011). In this system, *Arabidopsis* TFs were used to produce chimeric repressors, and these mixed chimeric repressor plasmids (42-50 TFs) were co-transformed into

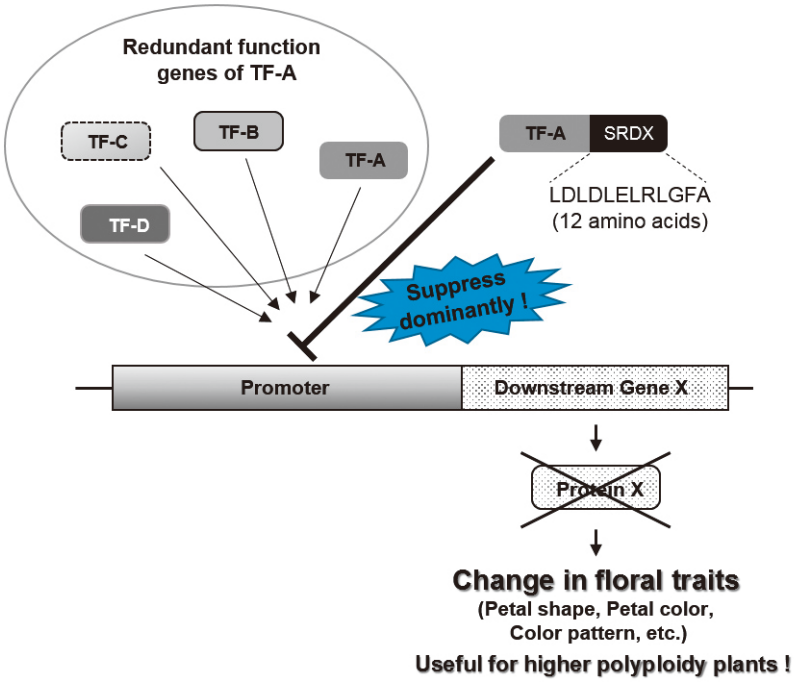

Fig. 1. CRES-T is useful to suppress the function of TFs
CRES-T dominantly suppresses the function of target TFs even in the presence of functionally redundant TFs. This technology is helpful to create novel floral traits in ornamental crops. Detailed protocol for CRES-T was reported by Mitsuda et al. (2011b).

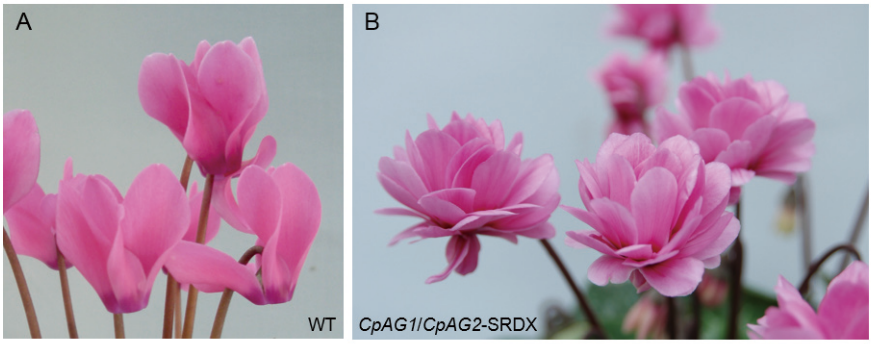

Fig. 2. A multi-petal flower phenotype in cyclamen
A) A wild-type (WT) cyclamen. B) The transgenic cyclamen co-overexpressing *CpAG1-SRDX* and *CpAG2-SRDX* genes with a multi-petal phenotype.

Agrobacterium in equal concentrations. *Torenia* was inoculated with a mixture of *Agrobacterium* generating hundreds of transgenic *Torenia* plants. In this system, more than 80% of transgenic *Torenia* plants had a single transgene. The CT system efficiently generated novel and varied floral traits at once, and enabled the selection of desirable traits (Fig. 3B), thereby enabling a reduction of costs required for developing a new variety, while meeting changing customer preferences. Although the CT system was applied in *Torenia* (Shikata et al. 2011), it would also be effective for other ornamental crops with established and highly efficient transformation methods, such as those of *Torenia* (Aida & Shibata 1995) and *Arabidopsis*.

The introduction of chimeric repressors could not only

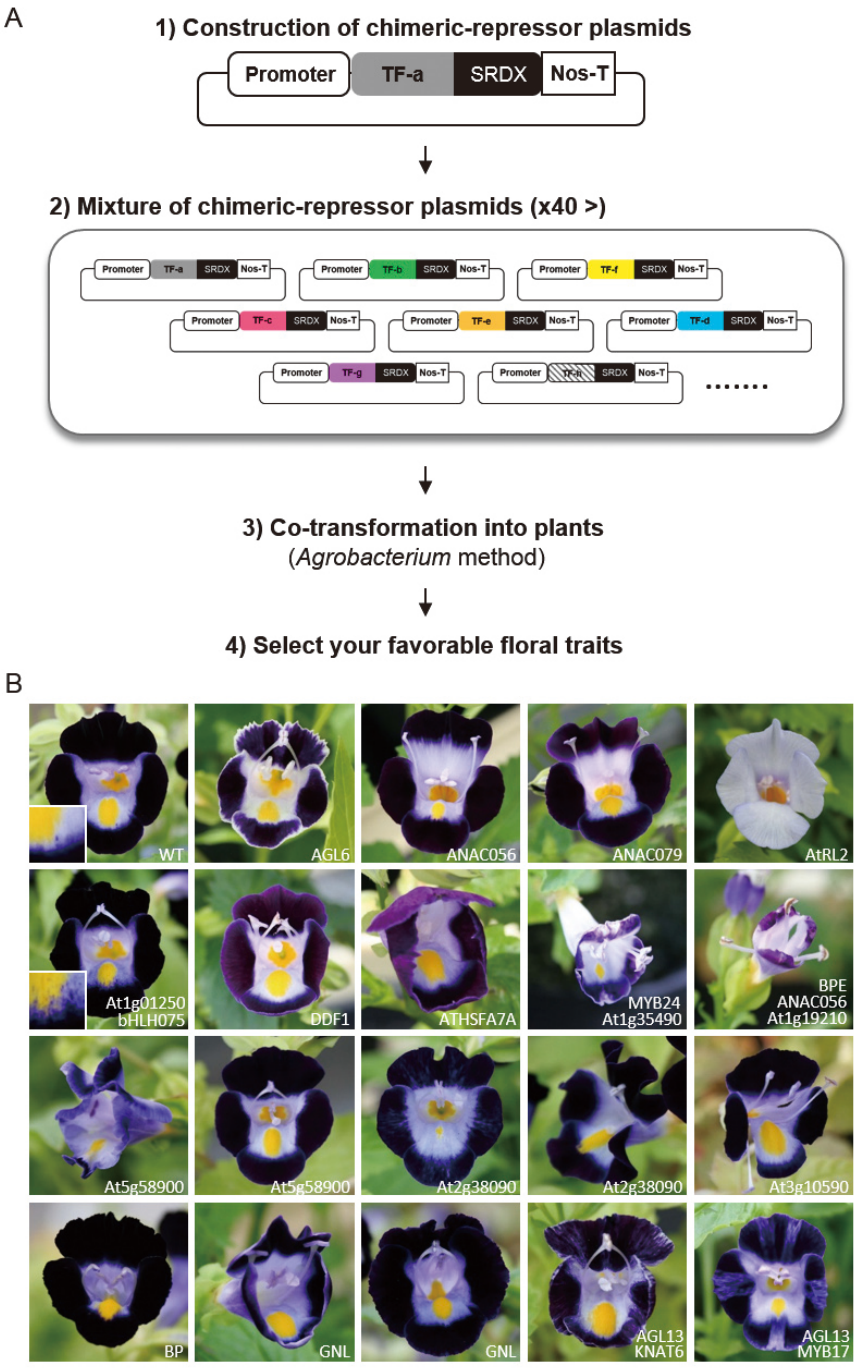

Fig. 3. Simplified flow of the CT system

A) The CT system enabled the selection of target floral traits through massive screening (Shikata et al. 2011).
B) Modified floral traits of transgenic *Torenia* plants generated by the CT system. Floral traits of these transgenic *Torenia* plants were confirmed within approximately six months after the start of transformation.

lead to the functional analysis of TFs but also to the generation of new floral traits. In *Torenia*, there are two orthologs of the class B genes *DEFICIENCE* (*DEF*) and *GLOBOSA* (*GLO*), called *TfDEF* and *TfGLO*, respectively (Sasaki et al. 2010). Simultaneous suppression of these genes by chimeric repressors resulted in sepal-like petals (Sasaki et al. 2014), indicating that *TfDEF* and *TfGLO* synergistically played important roles in the development of petals. However, the expression of single *TfDEF-SRDX* or *TfGLO-SRDX* caused phenotypic changes in petals, resulting in novel floral traits in *Torenia* (Sasaki et al. 2010), unlike the co-suppression of these class B genes. *TfDEF-SRDX* and *TfGLO-SRDX* plants showed partially decolorized petals and serrated petals, respectively (Fig. 4). In addition, the introduction of these chimeric repressors into blue colored transgenic *Torenia* plants (transgenic line no. 416-20; Fig. 4, right) produced by antisense dihydroflavonol 4-reductase gene (Aida et al. 2000) resulted in differently colored floral traits (Fig. 4). The combination of chimeric repressors with an additional transgene could further produce novel floral traits. On the other hand, heavy-ion beam irradiation has also led to a large variety of colors in *Torenia* flowers (Sasaki et al. 2008). Thus, combination of chimeric repressors and heavy-ion beam irradiation (Ohtsubo et al. 2012) could possibly enable the creation of a wide variety of colorful flowers with new floral traits.

Modification of chimeric repressor constructs

In the flower CRES-T project (Ohtsubo 2011), most ornamental crops used the 35S promoter from cauliflower mosaic virus (CaMV), which usually shows high promoter activity in the whole plant. However, the 35S promoter did not work in certain ornamental crops, and transgenes controlled by the 35S promoter were not expressed in gentian due to the gene silencing of the introduced transgene (Mishiba et al. 2005, Mishiba et al. 2010). Therefore, the *Agrobacterium rhizogenes rolC* promoter and *Arabidopsis Actin2* promoter were used for the transgene expression in gentian instead of the 35S promoter (Nakatsuka et al. 2008, Nakatsuka et al. 2011). For utilizing chimeric repressors, the expression of introduced transgenes should be tested

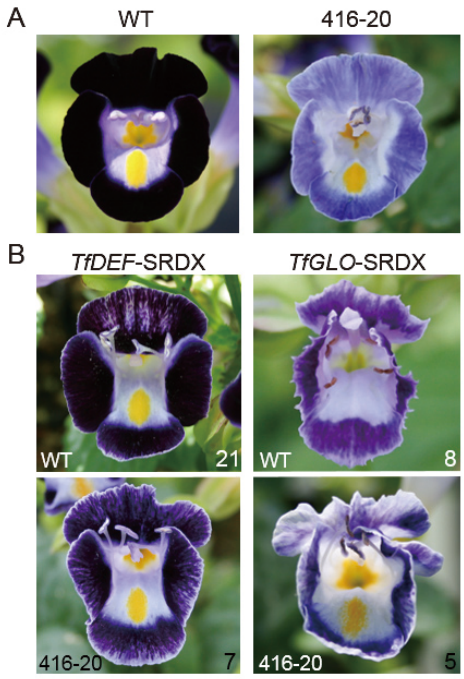

Fig. 4. Floral trait variety generated by CRES-T
A) A wild type (WT; left) and 416-20 (right) transgenic Torenia plants. B) Floral phenotypes generated by class B chimeric repressors. *TfDEF-SRDX* and *TfGLO-SRDX* were introduced into WT (upper) and 416-20 (lower) *Torenia* plants, respectively. The numbers at the lower right in each photo denote the number of transgenic lines.

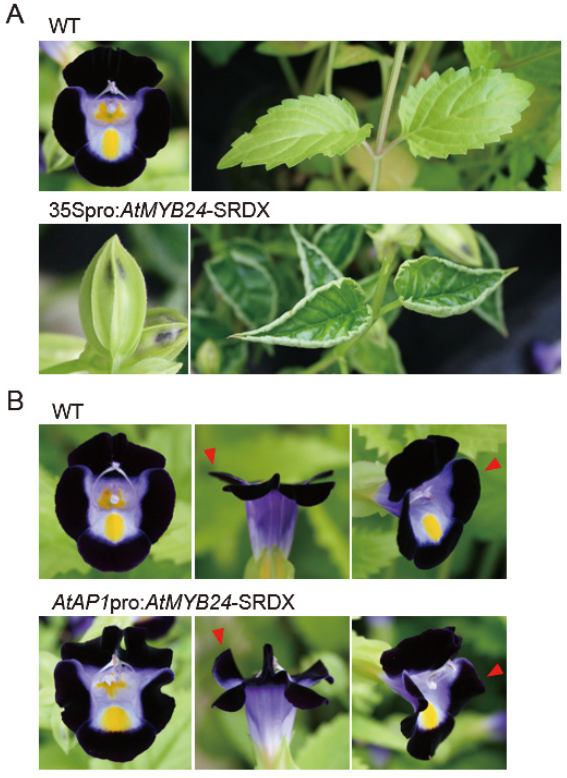

Fig. 5. Changing the promoter leads to the modification of floral traits
A) WT *Torenia* plants (upper) and transgenic *Torenia* plants with 35S pro:*AtMYB24-SRDX* (lower). B) A floral organ specific promoter was used for the expression of *AtMYB24-SRDX*. WT *Torenia* plants (upper) and transgenic *Torenia* plants with *AtAP1* pro:*AtMYB24-SRDX* (lower). Red arrowheads indicate the altered points.

in target plant species. It is desirable to check promoter activity in a target ornamental crop before use by using the particle bombardment method.

Among the chimeric repressors, the 35S promoter sometimes caused an unfavorable phenotype in unexpected organs due to the ectopic expression derived from promoter activity. Overexpression of the *Arabidopsis MYB24-SRDX* (*AtMYB24-SRDX*) resulted in unopened flower buds and glossy dark green leaves with curled margins in *Torenia* (Sasaki et al. 2011, Fig. 5A). Because the petals inside the flower buds exhibited a different coloration pattern than that of wild-type plants, a floral organ-specific promoter was used to express *AtMYB24-SRDX* for exhibiting the concealed petal phenotype in *Torenia*. Floral organ-specific expression of *AtMYB24-SRDX* from the *Arabidopsis APETALA1* (*AtAP1*) promoter resulted in wavy petals (Fig. 5B). Use of the floral organ-specific promoter also prevented unfavorable phenotypes caused by the 35S promoter other than in floral organs; hence, the plants exhibited the normal leaf phenotype. The generation of a new floral phenotype without changes in non-targeted organs is a favorable feature because cultivation conditions for the new variety may not differ from those for the parental variety. It is important to culture the new variety in the already established manner, so as to ensure a stable supply of this commodity. Thus, changing the promoter can solve problems that arise when transgenic plants have unfavorable phenotypes in non-targeted organs.

Perspectives

In this review, we have introduced the utilization of CRES-T for changing floral traits in ornamental crops. However, the commercialization of transgenic ornamental crops requires an evaluation of their impact on biodiversity, in accordance with the Cartagena Protocol on Biosafety and the laws of each country. Blue carnations and roses have been commercialized through specific processes of evaluation in Japan. CRES-T could be applicable for further uses, such as the regulation of flowering, disease resistance, environmental tolerance, and improvement of growth characteristics. The combined use of the methods introduced in this review would have high potential for accelerating the development of ornamental crops. CRES-T is also expected to contribute to the commercialization of useful GM flowers in the near future.

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