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Multi-petal cyclamen flowers produced by AGAMOUS chimeric repressor expression

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Cyclamen persicum **(cyclamen) is a commercially valuable, winter-blooming perennial plant. We cloned two cyclamen orthologues of *AGAMOUS (AG)*, *CpAG1* and *CpAG2*, which are mainly expressed in the stamen and carpel, respectively. Cyclamen flowers have 5 petals, but expression of a chimeric repressor of CpAG1 (CpAG1-SRDX) caused stamens to convert into petals, resulting in a flower with 10 petals. By contrast, CpAG2-SRDX only caused incomplete formation of stamens and carpels. Expression in *Arabidopsis thaliana* showed similar effects on flower organ specification. Simultaneous expression of *CpAG1-SRDX* and *CpAG2-SRDX* in cyclamen induced rose-like, multi-petal flowers, a potentially valuable trait in commercial ornamental varieties. Expression of *CpAG2-SRDX* in a cyclamen mutant lacking expression of *CpAG1* more effectively produced multi-petal flowers. Here, we controlled the number of petals in cyclamen by simple genetic engineering with a chimeric repressor. This strategy may be applicable useful for other ornamental plants with two distinct *AG* orthologues.**

Flower shape and color can enhance the value of ornamental flowers. Therefore, the improvement or creation of novel flower traits can provide substantial commercial value. The organogenesis of flowers has been well studied, especially in *Arabidopsis thaliana* and *Antirrhinum majus*. Extensive studies in these plants have revealed a common molecular mechanism of floral organ formation in seed plants, the "ABC model"[1–3]. In this model, genes encoding MADS-box transcription factors combinatorially specify flower cell fate. Class A genes, such as *APETALA1* in *Arabidopsis*, specify the outer-most floral organs, the sepals. Class B genes such as *APETALA3* and *PISTILLATA* specify petals in concert with class A genes. Class B genes specify male organs in concert with class C genes such as *AGAMOUS* (*AG*). Class C genes specify the inner-most floral organs, the carpels. Mutation of *Arabidopsis* ABC genes produces homeotic transformations of one organ type into another; some of these transformations would increase plant value if they could be recapitulated in commercial ornamental varieties. For example, the flowers of *Arabidopsis ag* mutants have no stamen or carpel and lose the ability to terminate meristematic activity. As a result, *ag* mutants have "multi-petal flowers", which have a repeated structure of sepal-petal-petal including tens of petals[4]. Recapitulating the phenotype of *ag* mutants in commercial ornamental plants would be highly valuable, because this would confer both an interesting appearance and complete sterility, which would prevent the dispersal of transgenic seeds and pollen. Moreover, this trait could be produced in a selected variety with other valuable traits such as perfume or a desirable color.

Cyclamen is a winter-blooming perennial plant and one of the most popular potted flowers in many countries. The genus *Cyclamen* consists of about 15 species, which are distributed in the Mediterranean region. Wild-type cyclamens have five sepals, five petals, five stamens and one pistil with five fused carpels. In traditional breeding, genetic mutation has provided the only means to create multi-petal cyclamen varieties. A homeotic mutant bearing flowers with double (ten) petals and no stamen was discovered[5] and commercialized, but such mutants occur at a low frequency. More interesting mutant cyclamen flowers, such as those that resemble the *Arabidopsis ag* mutants, with tens of petals due to failure of termination of floral meristem, are rarely observed.

We previously isolated 10 putative MADS-box transcription factor genes from cyclamen and analyzed their expression patterns[6]. We found that these MADS-box genes have homologs in model flowering plants such as

Arabidopsis, but have distinct tissue specificities in cyclamen. Among these genes, we identified *CpAG1* and *CpAG2* (AB548889 and AB548890), two closely related class C paralogs similar to *Arabidopsis AG*. CpAG1 and CpAG2 share 90% amino acid identity. In *Arabidopsis*, transcripts of *AG* are expressed in whorls 3 and 4[4,7,8]. In cyclamen, transcripts of *CpAG1* were mainly detected in the stamen, and *CpAG2* expression was mainly detected in the carpel, although the transcripts of each gene were also detected in stamen, carpel and receptacle[6].

Chimeric repressor gene-silencing technology (CRES-T) provides a powerful tool for dominant negative genetic modification of a transcription factor. In CRES-T, a short but strong, plant-specific transcriptional repression domain (SRDX) is fused to the coding sequence of transcription factor of interest and expressed in plants[9]. The resulting chimeric repressor can suppress the expression of the transcription factor's target genes and thus induce a dominant negative phenotype of the transcription factor, even in the presence of other functionally redundant transcription factors. For example, introduction of a chimeric repressor of *AG* or its ortholog efficiently induced an *ag*-like multi-petal flower phenotype in *Arabidopsis* and *Pharbitis nil* (morning glory)[10,11]. However, the manipulation of *P. nil* required use of the *P. nil AG* ortholog and expression from an inducible promoter to avoid developmental lethality[11].

In this study, we succeeded in controlling the number of petals (5, 10 and $>$ 40) of cyclamen by using CRES-T constructs for *CpAG1* and *CpAG2*. Furthermore, we showed that the two closely related cyclamen class C genes *CpAG1* and *CpAG2* have divergent functions in cyclamen flower development; *CpAG1* mainly functions to produce stamen and *CpAG2* functions in whorl 4 to produce carpels and terminate the floral meristem. Our findings improve our understanding of flower formation in cyclamen and provide an efficient strategy to control petal number in ornamental plants that show similar subfunctionalization of *AG* orthologues.

Results

Expression profiles of cyclamen class C *AG* homologs. To obtain insights into the functions of *CpAG*s, as part of our strategy to produce multi-petal cyclamen flowers, we analyzed the expression patterns of *CpAG1* and *CpAG2* in wild type floral organs (Fig. 1a). We found that *CpAG1* is predominantly expressed in stamen in addition to low expression in carpel, and *CpAG2* is expressed mainly in carpel but rarely detectable in stamen (Fig. 1b). These data suggest that *CpAG1* and *CpAG2* mainly function in stamen and carpel, respectively.

To evaluate the involvement of *CpAG1* and *CpAG2* in the formation of floral organs, we analyzed their expression by RT-PCR in wild type and mutant varieties. For example, we examined the WP strain, which contains a mutation causing a homeotic transformation resulting in flowers with 10 petals. We detected expression of both genes in wild type, but only *CpAG2* was detected in WP and other similar mutant strains with 10 petals (Fig. 1c). This suggests that *CpAG1* is involved in stamen formation and the lack of *CpAG1* expression causes the homeotic conversion of stamen into petal in WP and other 10- petals varieties.

Expression of CpAG1 and CpAG2 chimeric repressors in *Arabidopsis*. To examine the functions of cyclamen class C genes and determine rapidly which construct is most suitable to induce the desired morphological changes, we first tested CpAG function in transgenic *Arabidopsis*. To create dominant negative mutants, we expressed CpAG1 and CpAG2 chimeric repressors under the control of the CaMV 35S promoter (*35S:CpAG1/2-SRDX*). The *35S:CpAG1-SRDX Arabidopsis* plants showed petaloid stamens but had a less prominent effect on carpels, suggesting that the class C function of *AG* in stamen was impaired (Fig. 2b, f to h). By contrast, *35S:CpAG2-SRDX Arabidopsis* plants had abnormal carpels and

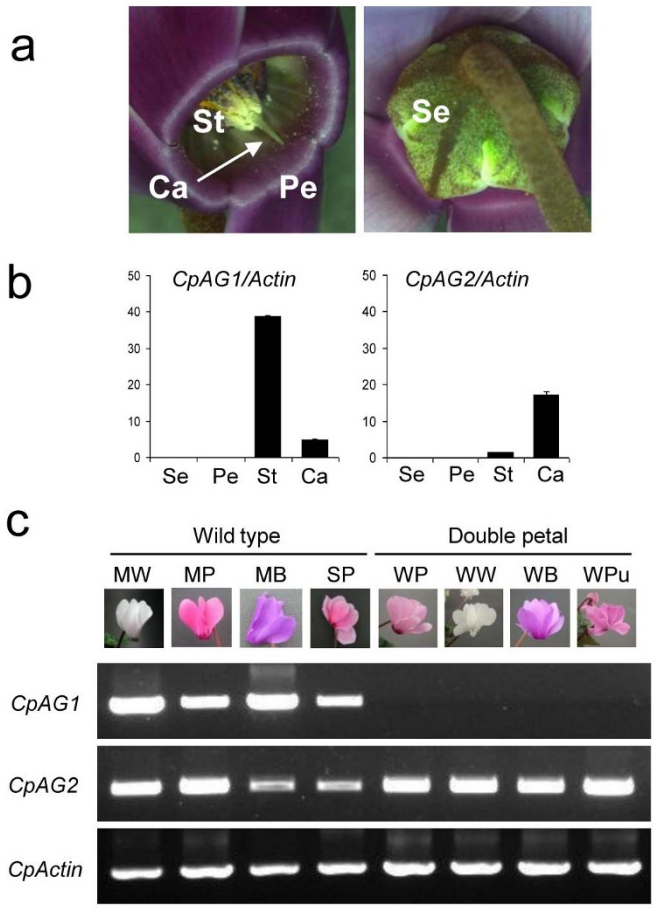

Figure 1 | Expression of *CpAG1* and *CpAG2*. (a) Floral organs of wild-type cyclamen. Se, sepal; Pe, petal; St, stamen; Ca, carpel. (b) Quantitative analysis of *CpAG1* and *CpAG2* expression in floral organs of wild type by RT-qPCR. (c) Expression of *CpAG1* and *CpAG2* in various strains, with five petals; MW: Mini White; MP: Mini Pink; MB: Mini Blue; and SP: Salmon Pink, and with 10 petals; WP: Wink Pink; WW: Wink White; WB: Wink Blue; and WPu: Wink Purple. All photographs were taken by the authors and the figure copyright is reserved by the authors.

ectopic formation of carpels and stamens in addition to abnormal stamens, but at a lower rate than *35S:CpAG1-SRDX* (Fig. 2c, f to h). This phenotype in carpels indicates that another important role of *AG*, termination of meristematic activity, was perturbed. The *Arabidopsis* AG chimeric repressor also induced these phenotypes in mild-phenotype lines of *Arabidopsis* in addition to inducing a complete *ag*-like phenotype in stronger lines[10,12]. Thus, *CpAG1* and *CpAG2* appeared to have at least some of the same roles as *AG* in *Arabidopsis*.

Arabidopsis has only one class C gene in its genome, but cyclamen has at least two class C genes, *CpAG1* and *CpAG2*. To examine whether these two *CpAGs* have a synergetic effect, we introduced *35S:CpAG1-SRDX* and *35S:CpAG2-SRDX* together into *Arabidopsis*. A significant portion of *35S:CpAG1-SRDX 35S:CpAG2-SRDX Arabidopsis* plants showed a multi-petal phenotype similar to the *ag* mutants (Fig. 2d to h), suggesting that *CpAG1* and *CpAG2* might have slightly different functions. Specifically, *CpAG1* acts mainly in stamen formation and *CpAG2* acts mainly in carpel formation and termination of mersitematic activity, but together they add up to complete *AG* function.

Morphologies of *35S:CpAG1-SRDX* and *35S:CpAG2-SRDX* cyclamen. To produce transgenic cyclamen with multi-petal flowers, we first introduced *35S:AG-SRDX* into cyclamen. However, unlike

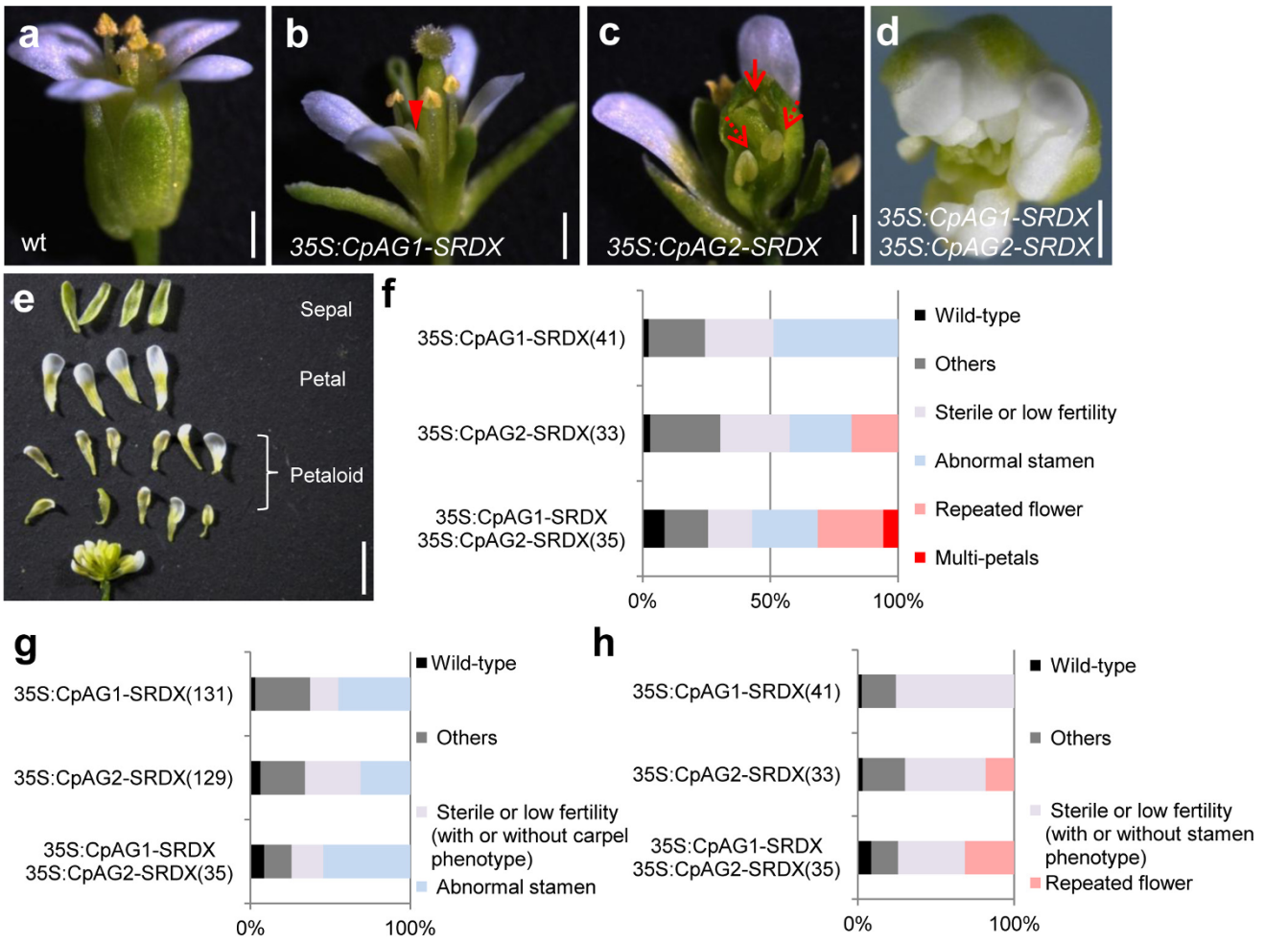

Figure 2 | Phenotypes of *35S:CpAG1-SRDX* and *35S:CpAG2-SRDX Arabidopsis*. (a) A flower of wild-type *Arabidopsis*. (b) A flower of *35S:CpAG1-SRDX Arabidopsis* with petaloid stamens (red arrowhead). (c) A double flower of *35S:CpAG2-SRDX* plant. Inside the carpel is shown by dissection. Solid arrow and dashed arrows indicate ectopically formed carpel and stamen, respectively, in an original carpel. (d) A multi-petal flower of *35S:CpAG1-SRDX 35S:CpAG2-SRDX* plant. (e) The multi-petal flower of (d) has repeated petaloids. (f) The frequency of each phenotype class in *35S:CpAG1-SRDX*, *35S:CpAG2-SRDX* and *35S:CpAG1-SRDX 35S:CpAG2-SRDX* plants. "Abnormal stamen" includes immature-like stamen and petaloid stamen. The abnormal stamen phenotype with carpel alteration is included in "Repeated flower". "Multi-petals" includes triple or more repeated flowers with petaloid stamens. (g) The frequency of stamen alteration in *35S:CpAG1-SRDX*, *35S:CpAG2-SRDX* and *35S:CpAG1-SRDX 35S:CpAG2-SRDX* plants. The frequencies of abnormal stamen were significantly different between *35S:CpAG1-SRDX* and *35S:CpAG2-SRDX* ($p < 0.05$, Fisher's exact test). (h) The frequency of carpel alteration (repeated flower) in *35S:CpAG1-SRDX*, *35S:CpAG2-SRDX* and *35S:CpAG1-SRDX 35S:CpAG2-SRDX* plants. The number of examined plants is given in parentheses in each case. Bars indicate 0.5 mm in (a) to (d) and 2 mm in (e). All photographs were taken by the authors and the figure copyright is reserved by the authors.

35S:AG-SRDX Arabidopsis, *35S:AG-SRDX* did not alter floral morphology in 7 transgenic cyclamen lines that showed clear expression of the transgene (Fig. S1). This result indicated that *Arabidopsis AG* cannot substitute for *CpAG1* and *CpAG2* in cyclamen in our experiments. This is consistent with reports that *35S:AG-SRDX* did not induce the expected phenotypes in other ornamental plants[13,14].

Next, we introduced *35S:CpAG1-SRDX* and *35S:CpAG2-SRDX* into MW and MR wild-type cyclamen, which have 5 petals in their flowers. Two out of 8 transgenic cyclamen lines expressing *35S:CpAG1-SRDX* produced double-petal (10-petal) flowers, with an additional 5 petals (Fig. 3a, b, d) instead of stamens. Other features such as number of carpel and sepals, petal size, leaf shape were normal (Fig. 3a, b). This phenotype of two independent transgenic lines is similar to that of the cyclamen WP strain in which the expression of *CpAG1* is suppressed. These data suggest that CRES-T efficiently perturbed CpAG1 function and this strategy can produce double-petal flowers in cyclamen. By contrast, 4 out of 11 transgenic cyclamen lines expressing *35S:CpAG2-SRDX* showed abnormal or petaloid stamens (Fig. 3c, d). However, these lines exhibited neither complete conversion of stamen into petal nor indeterminate growth of the floral meristem (Fig. 3c, d).

To obtain multi-petal flowers, as was observed in *Arabidopsis*, we suppressed both CpAG1 and CpAG2 by two strategies. First, we introduced *35S:CpAG2-SRDX* into WP, which lacks *CpAG1* expression. We found that 28 out of 39 *35S:CpAG2-SRDX* WP plants produced multi-petal flowers with a repeated structure of tens of petals (>40 petals) instead of stamens and carpel (Fig. 3e, g–i). Second, we produced transgenic cyclamen lines that harbor *35S:CpAG1-SRDX* and *35S:CpAG2-SRDX* in the MB wild-type background, which has 5 normal petals. We found 3 out of 22 *35S:CpAG1-SRDX 35S:CpAG2-SRDX* cyclamen also produced multi-petal flowers similar to *35S:CpAG2-SRDX* WP plants (Fig. 3f). Thus, we can produce multi-petal flowers by expressing two chimeric AG repressors in wild-type cyclamen.

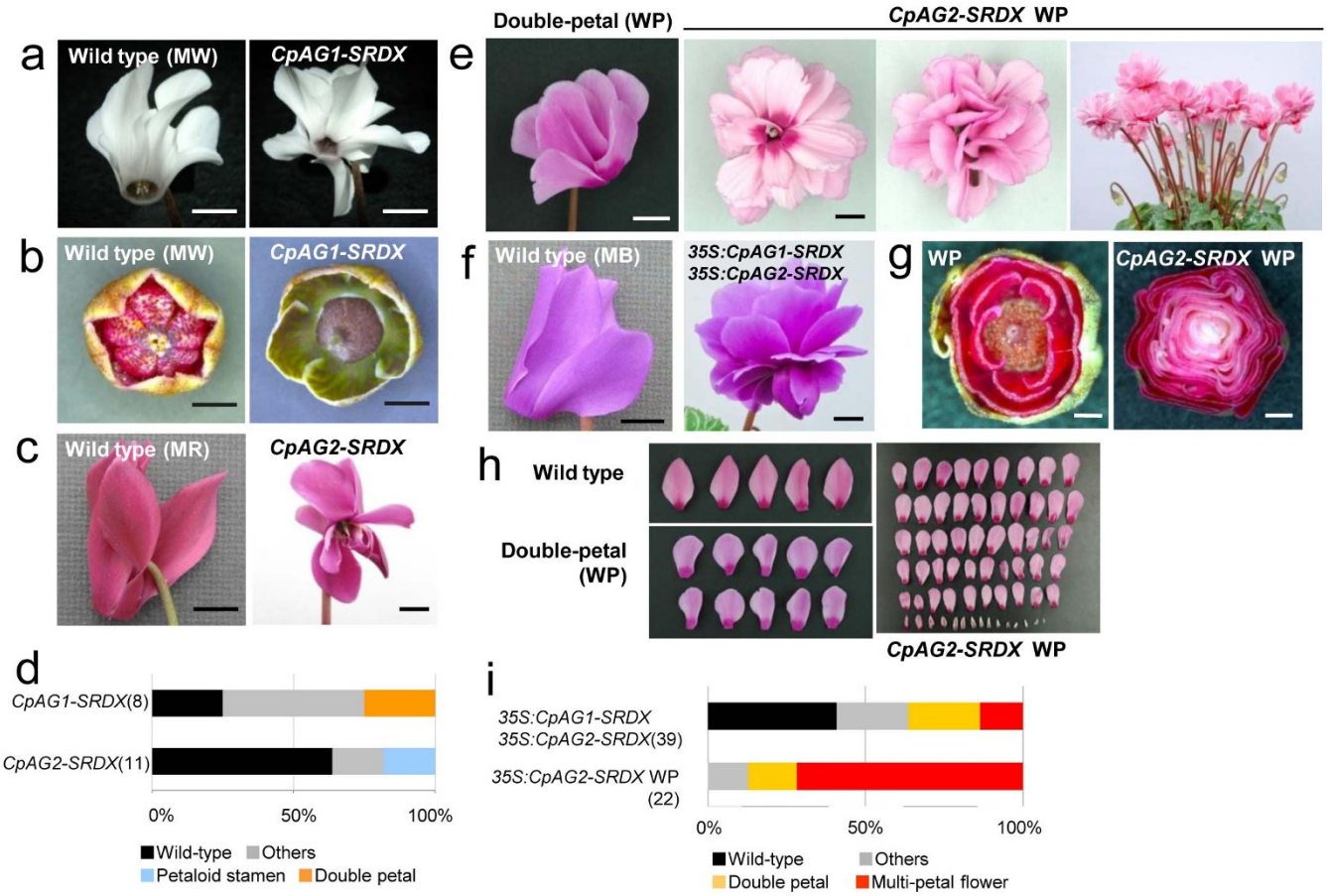

Figure 3 | Phenotypes of *35S:CpAG1-SRDX* and *35S:CpAG2-SRDX* cyclamen. (a) A flower of a MW wild-type plant (left) and a double-petal flower of a *35S:CpAG1-SRDX* plant (right). (b) A flower of MW wild-type (left) and *35S:CpAG1-SRDX* (right) plants, with the petals removed. The *35S:CpAG1-SRDX* plant has no stamen. (c) A flower of an MR wild type plant (left) and a flower of a *35S:CpAG2-SRDX* plant with abnormal petaloid stamens (right). (d) The frequency of each phenotype in *35S:CpAG1-SRDX* and *35S:CpAG2-SRDX* plants. The number of examined plants is given in parentheses in each case. (e) A double-petal flower of WP and a multi-petal flower of a *35S:CpAG2-SRDX* WP plant. The view from top (left), bottom (middle), and the whole plant (right). (f) A flower of a *35S:CpAG1-SRDX 35S:CpAG2-SRDX* plant (MB background). (g) Horizontal sections of flowers of WP (left) and *35S:CpAG2-SRDX* WP (right). The *35S:CpAG2-SRDX* WP plant has neither stamen nor carpel and produced tens of petals. (h) The petals of wild type, a double-petal mutant and *35S:CpAG2-SRDX* WP plants. (i) The frequency of each phenotype in *35S:CpAG1-SRDX 35S:CpAG2-SRDX* (MB background) and *35S:CpAG2-SRDX* WP plants. Bars indicate 10 mm in (a), (c), (e), (f) and 2 mm in (b), (g). All photographs were taken by the authors and the figure copyright is reserved by the authors.

Discussion

Here we describe the production of new transgenic cyclamen varieties with double-petal (10-petals) and multi-petal (>40 petals) flowers by perturbing the functions of CpAG1 and CpAG2 with CRES-T. Even though the amino acid sequences of CpAG1 and CpAG2 are quite similar, the effects of CpAG1-SRDX and CpAG2-SRDX are quite different and synergistic. This may be due to the following reasons: First, our evidence indicates that CpAG1 and CpAG2 have slightly different functions. For example, constitutive expression of *CpAG1-SRDX* induced petals instead of stamens, but expression of *CpAG2-SRDX* did not induce an increase in petals in cyclamen. *CpAG2-SRDX* was also less effective than *CpAG1-SRDX* in inducing abnormal stamens in *Arabidopsis*. Therefore, we conclude that CpAG1 and CpAG2 have similar protein functions but have their own distinct roles in whorl 3 and whorl 4, respectively. The examination of how such paralogous transcription factors exert their own distinct functions, probably with slight different binding-site sequence specificities, will provide an interesting topic for future research.

In addition to differences in protein function, differences in gene expression may also affect *CpAG1* and *CpAG2* functions. For example *CpAG1* and *CpAG2* were detected in carpels, but *CpAG1* is mainly expressed in stamens. Thus, we hypothesized that *CpAG1* regulates stamen (whorl 3) development and *CpAG1* and *CpAG2* redundantly regulate the whorl 4 development because: First, the expression of *CpAG1* is preferably detected in stamens and not detected in WP, which has 10 petals and no stamen. Second, no mutant showing defects only in whorl 4 has been reported to date in cyclamen. Third, introduction of *CpAG2-SRDX* into WP, which lacks expression of *CpAG1*, effectively induced an increased number of petals instead of carpels and indeterminate growth of the floral meristem; this was more effective than the simultaneous or sole introduction of *CpAG1-SRDX* and *CpAG2-SRDX* into wild type. We consider that the genetic redundancy of *CpAG1* and *CpAG2* in whorl 4 was not fully overcome by the introduction of chimeric repressor(s) into wild type. By contrast, the introduction of *CpAG1-SRDX* into wild-type was effective enough to induce the same phenotype observed in WP, probably because of a lack of genetic redundancy in the stamen.

During the evolution of flowering plants, duplication of MADS-box genes was followed by gene loss, neofunctionalization and subfunctionalization by changes in transcriptional regulation and protein sequence in different lineages[15,16]. In core eudicots, C lineage MADS-box genes have separated into *euAG* and *PLENA (PLE)* lineages[17]. After duplication, the primary C functions were

subfunctionalized[15]. For example, in *Antirrhinum majus*, *PLE* has a main role in C functions, including termination of floral meristem and establishment of stamen and carpel identities, but the *euAG* sub-lineage gene *FARINERRI (FAR)* contributes only to male fertility. The differences in these genes reside not only in their expression patterns but also in their protein functions[18]. In *Nicotiana benthamiana*, *euAG*- and *PLE*-like genes redundantly contribute to all class-C functions[19]. The class-C function in monocots is also divided between two genes, but in a distinct manner. Rice *OsMADS3* and *OsMADS58* play a more predominant role in stamen and carpel identity and meristem termination, respectively[20]. This subfunctionalization appears to rely on differences in protein function such as specificity of protein-protein interactions, but not on differences in expression patterns[20]. The situation in cyclamen is somewhat similar to that of rice where the genes that mainly function in stamen and carpel are different. However, this subfunctionalization of *CpAG1* and *CpAG2*, both of which belong to the *euAG* sub-lineage, occurred very recently in evolution, because no other similar subfunctionalization has been reported in dicots.

In this study, we successfully produced double- and multi-petal cyclamen flowers by regulating the function of *CpAG*s with CRES-T. The chimeric repressor constructs may save time in breeding novel double- and multi-petal cyclamen varieties based on preexisting cultivars with valuable characteristics such as perfume, ruffled petals and attractive colors. Our strategy to create multi-petal flowers may be able to be implemented in other ornamental plants with similar class C subfunctionalization.

Methods

Plant materials. *Cyclamen persicum* variety Mini White (MW), Mini Red (MR), Mini Purple (MP) and Mini Blue (MB) were used for wild-type control plants. The varieties of Wink Pink (WP) and Wink White (WW) were homeotic mutants which have flowers with 10 petals. For the experiments in *Arabidopsis*, *Arabidopsis thaliana* ecotype Columbia-0 (Col-0) was grown in Petri dishes or on soil under 16/8 hr light/dark cycle at 23°C.

Expression analysis of *CpAG1* and *CpAG2*. Floral buds of wild-type cyclamen and homeotic mutant were collected and used for RNA extraction and semi-quantitative RT-PCR (RTsqPCR). Total RNA was extracted and treated with DNase as described previously[6]. These RNA samples were subjected to RTsqPCR using PrimeScript One Step RT-PCR Kit Ver.2 (Takara Bio Inc., Japan).

For quantitative analysis, total RNAs were isolated from sepals, petals, stamens and carpels of wild-type flowers and were treated with DNase as described above. These RNA samples were used for reverse transcription by using High Capacity cDNA Reverse Transcription Kit (Life Technologies Inc., USA) according to the manufacturer's instructions. Quantitative real-time RT-PCR with three biological replicates was carried out using Applied Biosystems 7300 real-time PCR system and Power SYBR Green PCR Master Mix (Life Technologies Inc., USA). Since the coding sequences of *CpAG1* and *CpAG2* gene are very similar, gene-specific primers were designed in their 3′UTRs.

Plasmid construction. The full-length coding regions of *CpAG1* and *CpAG2* were cloned from *C. persicum* cvs. MP and used in this study. The *35S:CpAG1-SRDX* and *35S:CpAG2-SRDX* constructs were prepared as described previously[10]. To prepare the construct which has both *35S:CpAG1-SRDX* and *35S:CpAG2-SRDX*, the *35S:CpAG1-SRDX* fragment was amplified by PCR using primers with attB4 and attB1R sequence (#4155; 5′-ggggacaactttgtatagaaaagttgaaGGCGCCGGAACCAATTAAGCTT-3′, #4156; 5′-ggggactgcttttttgtacaaacttggCGATCTAGTAACATAGATGACAC-3′) and was cloned into pDONRG-P4P1R[12] by Gateway BP reaction (Life Technologies, USA). The resultant entry clone and *35S:CpAG2-SRDX* cloned in regular entry clone were assembled in R4pGWB501[21] by multi-site Gateway LR reaction (Life Technologies).

Transformation of cyclamen and *Arabidopsis*. The binary vector plasmids *35S:CpAG1-SRDX* and/or *35S:CpAG2-SRDX* pBCKH were introduced into *Agrobacterium tumefaciens* strain GV3101 and LBA4404. *Agrobacterium*-mediated transformations of *Arabidopsis* and cyclamen were performed as described[22,23]. To confirm genetic transformation, total DNA was extracted from the leaf tissue of putative shoots using cetyltrimethylammonium bromide (CTAB)[24]. Approximately 10 mg of leaf tissues were homogenized with 200 µl of CTAB buffer. Integration of *HPT* was confirmed by PCR with the specific primers: 5'-ATGAAAAAGCCTG-AACTCACCGCGA-3' and 5'-TCCATCACAGTTTGCCAGTGATACA-3'. The plantlets were transplanted into pots containing growing soil with vermiculite and perlite, and were grown in a growth chamber at 20°C with 16 h of light per day. For flowering, transgenic plants were grown in a closed, special-netted greenhouse.

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Author contributions

Y.T. and Y.O. performed all experiments in cyclamen and *Arabidopsis*, respectively. T.Y. and M.S. helped experiments in cyclamen. Y.T., Y.O., N.M. and T.T. analyzed all data. Y.T., Y.O., N.M., M.O.T., N.O. and T.T. wrote the paper. M.O.T., N.O. and T.T. supervised the entire project.

Additional information

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