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”. 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 C 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. 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 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. We found that these MADS-box genes have homologs in model flowering plants such as...
**Results**

**Expression profiles of cyclamen class C AG homologs.** To obtain insights into the functions of CPGs, as part of our strategy to produce multi-petal cyclamen flowers, we analyzed the expression patterns of CPG1 and CPG2 in wild type floral organs (Fig. 1a). We found that CPG1 is predominantly expressed in stamen in addition to low expression in carpel, and CPG2 is expressed mainly in carpel but rarely detectable in stamen (Fig. 1b). These data suggest that CPG1 and CPG2 mainly function in stamen and carpel, respectively.

To evaluate the involvement of CPG1 and CPG2 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 CPG2 was detected in WP and other similar mutant strains with 10 petals (Fig. 1c). This suggests that CPG1 is involved in stamen formation and the lack of CPG1 expression causes the homeotic conversion of stamen into petal in WP and other 10-petal varieties.

**Expression of CPG1 and CPG2 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 CPG function in transgenic Arabidopsis. To create dominant negative mutants, we expressed CPG1 and CPG2 chimeric repressors under the control of the CaMV 35S promoter (35S:CPG1/2-SRDX). The 35S:CPG1-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:CPG2-SRDX Arabidopsis plants had abnormal carpels and ectopic formation of carpels and stamens in addition to abnormal stamens, but at a lower rate than 35S:CPG1-SRDX (Fig. 2c, e 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, CPG1 and CPG2 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, CPG1 and CPG2. To examine whether these two CPGs have a synergetic effect, we introduced 35S:CPG1-SRDX and 35S:CPG2-SRDX together into Arabidopsis. A significant portion of 35S:CPG1-SRDX 35S:CPG2-SRDX Arabidopsis plants showed a multi-petal phenotype similar to the ag mutants (Fig. 2d to h), suggesting that CPG1 and CPG2 might have slightly different functions. Specifically, CPG1 acts mainly in stamen formation and CPG2 acts mainly in carpel formation and termination of meristematic activity, but together they add up to complete AG function.

**Morphologies of 35S:CPG1-SRDX and 35S:CPG2-SRDX cyclamen.** To produce transgenic cyclamen with multi-petal flowers, we first introduced 35S:AG-SRDX into cyclamen. However, unlike...
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 CRESTM 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.
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. In core eudicots, C lineage MADS-box genes have separated into euAG and PLENA (PLE) lineages. After duplication, the primary C functions were
subfunctionalized. 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. In Nicotiana benthamiana, euAG- and PLE-like genes redundantly contribute to all class-C functions. 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. This subfunctionalization appears to rely on differences in protein function such as specificity of protein-protein interactions, but not on differences in expression patterns. 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 similar subfunctionalization has been reported in dicots.

In this study, we successfully produced double- and multi-petal cyclamen flowers by regulating the function of CpAGs with CRES-T. The chimeric repressor constructs may save time in breeding novel cyclamen flowers by regulating the function of very recently in evolution, because no other similar subfunctionalization appears to rely on differences in protein function such as specificity of protein-protein interactions, but not on differences in expression patterns. 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 similar subfunctionalization has been reported in dicots.

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. These RNA samples were subjected to RTsqPCR using PrimeScript One Step RT-PCR Kit Ver.2 (Takara Bio Inc., Japan).

For qualitative 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-SDRX and 35S::CpAG2-SDRX constructs were prepared as described previously. To prepare the construct which has both 35S::CpAG1-SDRX and 35S::CpAG2-SDRX, the 35S::CpAG1-SDRX fragment was amplified by PCR using primers with attB4 and attB1R sequence (#4155; 5’-gggaccaactgttataagggatggcGCGCCGGACCACTATTAAGCTT3’; #4156; 5’-gggaccaactgttataagggatggcCTGACTGATTACGATGACAC-3’) and was cloned into pDONR-P4P1R17 by Gateway BP reaction (Life Technologies, USA). The resultant entry clone and 35S::CpAG2-SDRX cloned in regular entry clone were assembled in R4pGW8001 by multi-site Gateway LR reaction (Life Technologies).

Transformation of cyclamen and Arabidopsis. The binary vector plasmids 35S::CpAG1-SDRX and/or 35S::CpAG2-SDRX pBCKH were introduced into Agrobacterium tumefaciens strain GV3101 and LBA4404. Agrobacterium-mediated transformations of Arabidopsis and cyclamen were performed as described. To confirm genetic transformation, total DNA was extracted from the leaf tissue of putative shoots using cetyltrimethylammonium bromide (CTAB). 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’-ATGAAAGACCTGG- AACGCCAACCGGCA-3’ and 5’-TCTCATACACGCAGTTGGCATAC-GAGC-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
Supplementary information accompanies this paper at http://www.nature.com/scientificreports

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