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Gene Note

Identification and expression analysis of the *Cyclamen persicum* MADS-box gene family

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Abstract Molecular analysis of cyclamen flower architecture has not been widely performed, therefore effective molecular breeding of this plant is not available. The ABCDE model is based on interactions between members of different classes of transcription factors that establish floral organ identity, most of which belong to the MADS-box family. To elucidate the mechanism involved in regulating cyclamen flower development, we isolated genes encoding putative MADS-box transcription factors and analyzed their expression patterns in cyclamen. We cloned full-length cDNAs using homology-based RT-PCR and RACE-PCR, and identified and characterized 10 putative cyclamen MADS-box genes. A phylogenetic tree reveals that these genes are related to the APETALA1, PISTILLATA, APETALA3, AGAMOUS, SEEDSTICK, and SEPALLATA transcription factor subfamilies. Respective genes are expressed in each whorl according to the ABCDE function, but there are slight differences in the expression of several genes in various tissues; class-A homologous gene *CpAP1* is not expressed in petal. One of three identified class-B homologous gene is *TM6*-like but expressed broadly. Two closely related class-C homologous genes are differentially expressed in stamens and carpels. These data suggest that modified ABC model might uniquely evolved in cyclamen.

Key words: ABCDE model, cyclamen, floral organ identity, MADS-box.

Cyclamen persicum Mill. is a major product of Japan's horticultural industry, with tens of millions of potted plants sold each winter. For many years, a number of popular cyclamen cultivars have been produced by cross-hybridization and mutation breeding techniques. In cyclamen, as in many horticultural plants, modification of the flower shape is important for improving the commercial value. However, there is not much natural flower shape variation in cyclamen, and moreover, developing new cyclamen breeds requires immense effort because of its complicated patterns of inheritance. Recently, molecular breeding techniques have been applied to solve this problem. These methods can be used to more directly produce new phenotypes with target characteristics. We have succeeded in demonstrating that the introduction of a chimeric cyclamen *TCP* (*TEOSINTE BRANCHED1*, *CYCLOIDEA* and *PCF*) repressor in the genome of cyclamen produces ruffled flowers with a high ornamental value (Tanaka et al. 2011). To continue to develop this field, it is important to determine genetic information and elucidate the exact mechanisms regulating flower formation in cyclamen.

Cyclamen flowers, like those of many flowering plants, consist of four whorls. In the outermost whorl, there is a calyx consisting of five sepals. In whorl 2, there is a corolla consisting of five petals, with five stamens attached by short filaments in whorl 3. Whorl 4, in the center of the flower, contains one pistil with five fused carpels (Figure 2A; Grey-Wilson 2002). Despite the commercial importance of cyclamen, molecular analysis of its flower architecture has not been completely performed, making effective molecular breeding of this plant impossible. Generally, the specification of floral organ identity is explained according to the classical ABC model (Coen and Meyerowitz 1991, Irish 2010). In this model, the interaction of three transcription factor classes affects the identity of an individual floral organ in each of the four whorls. Class-A genes alone contribute to sepal development in whorl 1, class-A and -B genes together lead to petal formation in whorl 2, class-B and -C genes combine in function to specify stamens in whorl 3, and class-C genes alone specify carpel identities in whorl 4. Following the recognition of class-D and -E genes, this model was extended to become the ABCDE model. In this extended model, class-D function genes required for ovule development were included based on studies of petunias (Angenent and Colombo 1996; Colombo et al. 1995; Theissen et al. 2000; Pinyopich et al. 2003). In addition, class-E genes have been identified

Abbreviations: RACE, rapid amplification of cDNA ends; RT, reverse transcription.
This article can be found at http://www.jspcmb.jp/

as floral organ identity genes, which are involved in the specification of all floral organs in cooperation with other gene classes (Ditta et al. 2004; Honma and Goto 2001; Pelaz et al. 2000; Robles and Pelaz 2005; Theissen 2001). With the exception of *APETALA2* which belongs to the AP2/EREBP family, almost all transcription factors included in the ABCDE model belong to the MADS-box family (Riechmann et al. 1997; Schwarz-Sommer et al. 1990). MADS-box family genes encode transcription factors possessing a DNA-binding region called the MADS domain. MADS-box family genes involved in floral organ identity have been isolated from a wide range of flowering plants. Advances in plant molecular genetics combined with nucleotide sequencing of the Arabidopsis genome in the 1990s revealed that the mechanisms controlling flower development have been highly conserved during evolution (Ng and Yanofsky, 2001).

To obtain information about genes and mechanisms related to floral organ formation in cyclamen for molecular breeding applications, we isolated cDNAs corresponding to putative MADS-box homologs and investigated their expression patterns in cyclamen. In this study, we used the wild-type flower shape cultivar 'Fragrance Mini'. Complete floral buds at various stages of development were collected and used for RNA extraction. Total RNA was isolated using the Plant RNA Reagent Kit (Invitrogen/Life Technologies, Carlsbad, CA, USA). Poly(A)$^+$ RNA was isolated from total RNA with the Oligotex-dT30 [Super] mRNA Purification Kit (Takara Bio Inc., Shiga, Japan). From 1 μg of poly(A)$^+$ RNA, first strand cDNAs were synthesized using the SMART RACE cDNA Amplification Kit (Clontech/Takara Bio Inc., Shiga, Japan). To amplify MADS-box gene fragments, combined 3′-RACE and nested degenerate PCR were employed. The forward degenerate primers used were those described by Kramer et al. (2004) and Stellari et al. (2004). Universal Primer A Mix (UPM) of the SMART RACE cDNA Amplification Kit (Clontech/Takara Bio Inc.) was used as the reverse primer. Because we were unable to amplify *APETALA1* (*AP1*), *AGAMOUS* (*AG*), and *SEPALLATA* (*SEP*) subfamily genes using the abovementioned primers, we designed new degenerate primers based on amino acid sequences conserved within each subfamily (Supplemental Table). These PCR reactions were run for 30–40 cycles at annealing temperatures between 50–60°C. To generate cDNAs representing the 5′ or 3′ ends of these genes, we used RACE PCR for each gene-specific primer (Supplemental Table). Phylogenetic analyses included comparisons with representative *Arabidopsis thaliana*, *Antirrhinum majus*, and *Petunia*× *hybrida* MADS-box genes. Deduced amino acid sequences were aligned using the ClustalW program (Thompson et al. 1994), and a phylogenetic tree was constructed with GENETYX ver 9.0 software (Genetyx, Tokyo, Japan). Partial sequences of MADS-box genes were determined after sequencing over 100 RT-PCR clones using each combination of degenerate primers. On the basis of this information, we were able to isolate full-length cDNAs. We finally identified 10 independent MADS-box genes, which were classified according to predicted ABCDE group functions.

Expression patterns of MADS-box genes isolated in this study were examined in various aerial tissues of cyclamen. Total RNA was isolated from sepals, petals, stamens, carpels, receptacles (fused regions at the bottom parts of sepals, petals, stamens, and carpels), flower stalks, leaves, and leaf stems as described above (Figure 2A). Each total RNA sample was DNase-treated using the Turbo-DNA Free Kit (Ambion/Life Technologies, Carlsbad, CA, USA) and reverse transcribed into cDNA with oligo-$(dT)_{20}$ primers (ReverTra Ace-α-®, Toyobo, Osaka, Japan). These cDNAs were subjected to semi-quantitative RT-PCR (RT-sqPCR) using the KOD Plus ver. 2.0 (Toyobo). RT-sqPCR reactions were run for 30 cycles at an annealing temperature of 60°C. Gene-specific primers for RT-sqPCR are shown in the Supplemental Table.

Class-A genes: The single gene belonging to this class was termed *CpAP1* (accession number AB600230). The product of CpAP1 is similar to that of Arabidopsis AP1 (60% identity) and was categorized within the AP1/SQUAMOSA (SQUA) subfamily in the phylogenetic tree (Figure 1). Expression analysis in various tissues demonstrated that *CpAP1* gene is expressed in sepals, receptacles, and flower stalks, but only slightly in petals (Figure 2B). *AP1* RNA signals were detected in sepals, petals, and pedicels in mature Arabidopsis buds (Mandel et al. 1992). *SQUA* and many related genes also are expressed at least somewhat in petals (Litt 2007). The expression pattern of *CpAP1* transcripts appears slightly different from other *AP1* homologs. In Arabidopsis, AP1 proteins regulate and specify floral meristems and form floral organ whorls 1 and 2 (Bowman et al. 1993; Irish and Sussex 1990). On the other hand, genes in many other species that are similar in sequence to *AP1* function in specifying floral meristems, but not sepal and petal identities (Litt 2007; Zik and Irish 2003). Our results suggest that CpAP1 does not function mainly in Whorl 2. Moreover, the *CpAP1* gene was expressed earlier than any other gene isolated in this study (Supplemental Figure). The function of CpAP1 has not yet been clarified, but it is likely that this gene specifies floral meristems rather than petals.

Class-B genes: Three class-B genes were isolated. One of the three genes was termed *CpPI* (AB600234), with a predicted amino acid sequence sharing 56% identity with PISTILLATA (PI) of Arabidopsis. Two distinct genes, predicted to encode proteins similar to that of

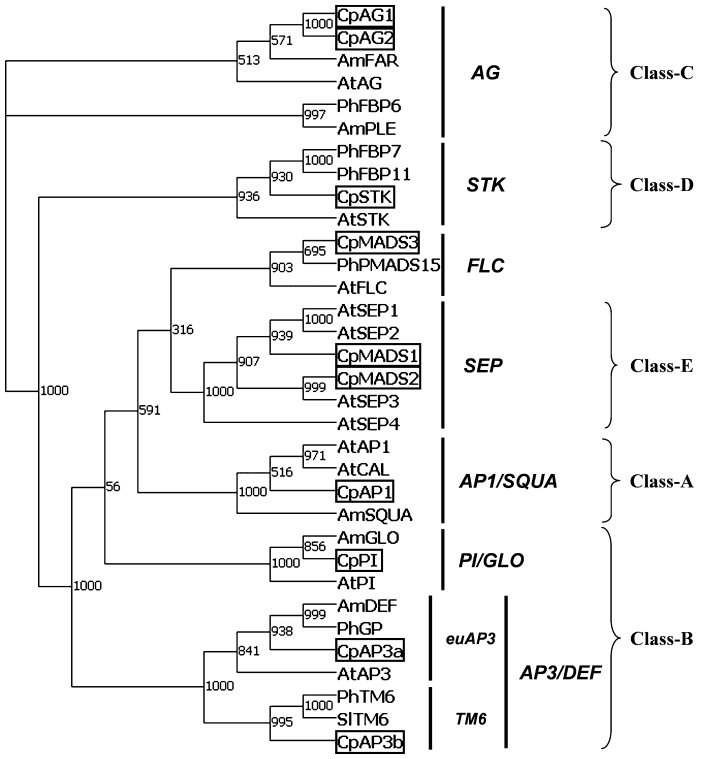

Figure 1. Phylogenetic analysis of full-length amino acid sequences of MADS-box transcription factors from cyclamen, Arabidopsis, *Antirrhinum majus*, petunia, and tomato. Cyclamen genes corresponding to cDNAs isolated in this study are indicated in boxes. The different gene groups and corresponding predicted ABCDE class names are indicated on the right. Previously published plant MADS-box gene sequences were retrieved from GenBank databases. Accession numbers: *Arabidopsis thaliana* (AtAG, NM_118013; AtSTK, NM_117064; AtFLC, NM_121052; AtSEP1, NM_001125758; AtSEP2, NM_111098; AtSEP3, NM_102272; AtSEP4, NM_126418; AtAP1, NM_105581; AtCAL, NM_102395; AtPI, NM_122031; AtAP3, NM_115294), *Antirrhinum majus* (AmFAR, AB516405; AmPLE, S53900; AmSQUA, X63701; AmGLO, AB516403; AmDEF, X62810), *Petunia×hybrida* (PhFBP6, CAA48635; PhFBP7, Q43616; PhFBP11, CAA57445; PhPMADS15, AY370529; PhGP, X69946; PhTM6, DQ539417), *Solanum lycopersicum* (SlTM6, X60759). CAL, CAULIFLOWER.

Arabidopsis APETALA3 (AP3), were also among the cyclamen genes isolated in this study. These genes were termed *CpAP3a* and *CpAP3b* (AB600231 and AB600232). Deduced amino acid sequences of CpAP3a and CpAP3b share 48% and 45% identity with AP3, respectively. The *CpPI* gene products belong to the clade including *PI* and *GLOBOSA* (*GLO*) of *A. majus* (Figure 1). The *CpAP3a* gene products fall into the clade including AP3 and DEFICIENS (DEF) of *A. majus* and GREEN PETAL (GP) of petunia. The sequences of *CpPI* and *CpAP3a* are significantly related to two partial sequences of known *C. persicum* MADS-domain transcription factors: *CpPI* is 95% identical to *CpGLO* (ACY91927) and *CpAP3a* is 93% identical to *CpDEF* (ACY08905). On the other hand, the *CpAP3b* gene product clusters with petunia PhTM6 in the AP3/DEF subfamily. In core eudicots, a duplication event in the *AP3* lineage produced two lineages, euAP3 and TM6, derived from *TOMATO MADS-BOX GENE6* (*TM6*) of tomato (de Martino et al. 2006; Kramer and Irish 1999; Kramer et al. 1998). *AP3*, *DEF,* and *GP* belong to the euAP3 lineage, while *PhTM6* belongs to the TM6 lineage (Rijpkema et al. 2006a). We think that cyclamen *CpAP3a* and *CpAP3b* belong to the euAP3 and TM6 lineages, respectively. Expression analysis in various tissues demonstrated that *CpPI* and *CpAP3a* genes are highly expressed in petals and stamens (Figure 2). These expression patterns are consistent with those of *PI* and *AP3* (Goto and Meyerowitz 1994; Schwarz-Sommer et al. 1992). Also, tissues expressing *CpPI* were consistent with those previously reported for *CpGLO*, but *CpAP3a* shows a slightly different expression pattern than *CpDEF* in that *CpDEF* is expressed in carpels (Viaene et al. 2009). Thus, *CpAP3a* and *CpDEF* might be two different cyclamen genes. On the other hand, the *CpAP3b* gene is expressed in all floral tissues, including sepals, carpels, receptacles, and flower stalks. This expression differs from that of *TM6* and *PhTM6*, which are hardly expressed in sepals (de Martino et al. 2006; Rijpkema et al. 2006a). In Arabidopsis, the TM6 protein lineage has been lost (Rijpkema et al. 2006b; Causier et al. 2010), and PI and AP3 together confer B function in whorls 2 and 3. It has been suggested that TM6 lineage genes function mainly in stamen development in petunia and tomato (de Martino et al. 2006; Rijpkema et al. 2006a). It is possible that CpAP3b has a similar function to TM6,

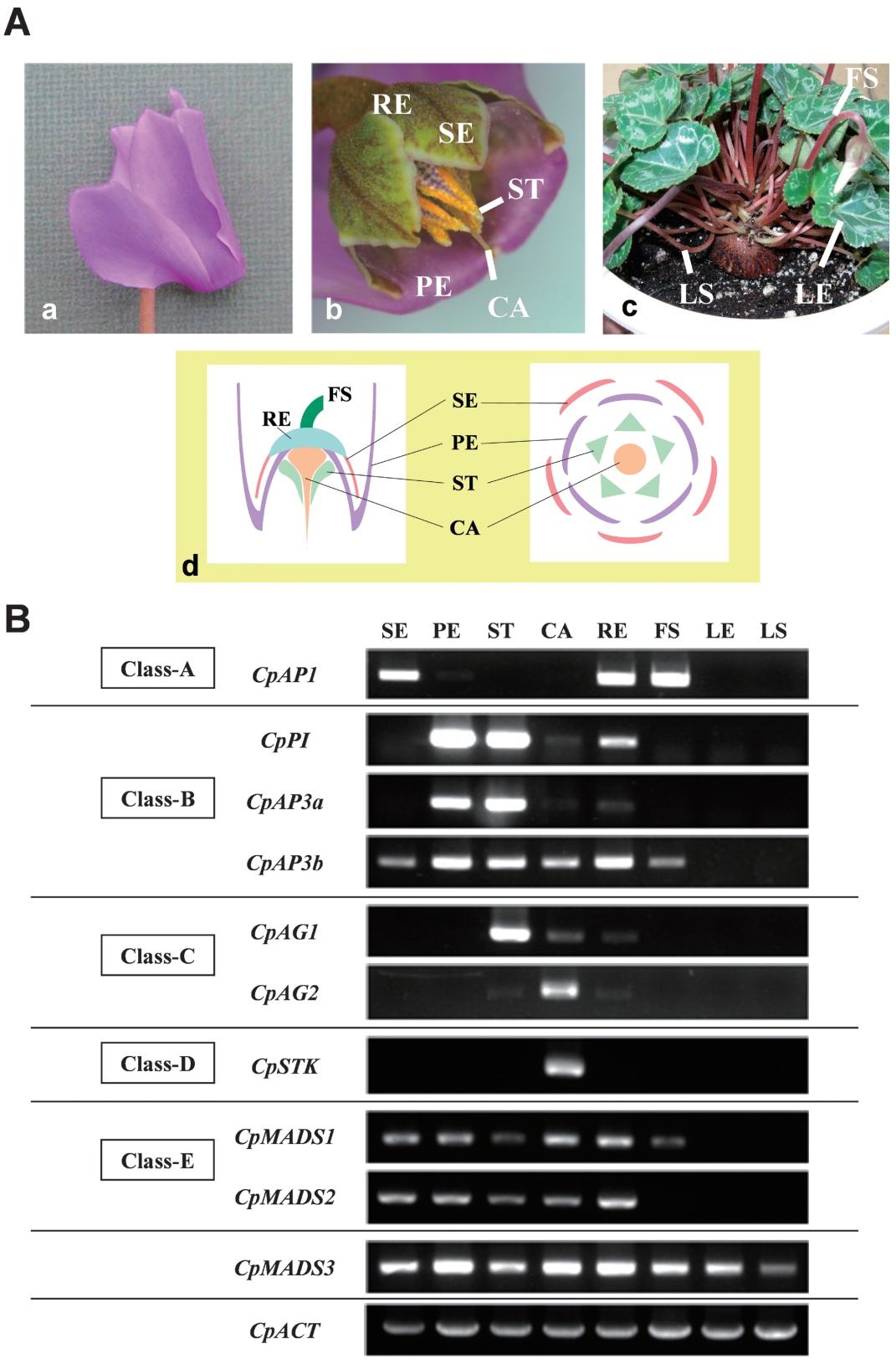

Figure 2. Expression patterns of cyclamen MADS-box genes in various tissues. (A) Flower morphology of *Cyclamen persicum*. Lines and characters show each tissue. a, cyclamen flower; b, cyclamen flower with three petals removed from the front side; c, potted cyclamen plant; d, scheme of lateral side (left) and floral diagrams (right) showing the relative placement of floral organs. (B) RT-sqPCR analysis of cyclamen MADS-box gene expression patterns in various tissues. Total RNA from each tissue was extracted from a 2-cm bud (Stage 9 in Supplemental Figure A) and used for RT-sqPCR. The names of corresponding genes and predicted ABCDE classes are indicated at the left. The actin gene (*CpACT*) was amplified from the same cDNA as a standard control to normalize the cDNA amount used in RT-sqPCR. SE, Sepal; PE, Petal; ST, Stamen; CA, Carpel; RE, Receptacle; FS, Flower Stalk; LE, Leaf; LS, Leaf Stem.

although expresssion pattern of *CpAP3b* is slightly different from that of *TM6*.

Class-C genes: Two genes encode proteins related to Arabidopsis AG. These were termed *CpAG1* and *CpAG2* (AB548889 and AB548890). The deduced amino acid sequences of these genes are similar to that of AG (CpAG1: 71%, CpAG2: 68% identity). The *CpAG1* and *CpAG2* gene products are 90% identical and form a single cluster in the AG subfamily (Figure 1). Arabidopsis possesses only one class-C gene, *AG*. In *A. majus*, *FARINELLI* (*FAR*) and *PLENA* (*PLE*) represent the two types of known class-C homologous genes (Davies et al. 1999). The products of these genes are about 70% identical to each other. The *CpAG1* and *CpAG2* gene products belong to the same clade as *FAR*. We did not isolate any genes belonging to the PLE clade in this study. Transcripts of *AG*, *FAR,* and *PLE* are expressed in Whorls 3 and 4 (Davies et al. 1999; Riechmann et al. 1997; Yanofsky et al. 1990). *CpAG1* and *CpAG2* transcripts were detected in stamens, carpels, and receptacles, but *CpAG1* was mainly detected in stamens and *CpAG2* mainly in carpels (Figure 2). AG plays a pivotal role in the development of the floral meristem, the repression of AP1 in whorls 3 and 4, and the specification of the floral organ in whorls 3 and 4. On the other hand, class-C function is retained by PLE and FAR functions mainly in pollen development (Davies et al. 1999; Pinyopich et al. 2003; Kramer et al. 2004). In cyclamen, this suggests that two *AG* homologous genes have been recently duplicated. It is not clear whether CpAG1 and CpAG2 have distinct functions; however, the differences in transcription regulation of these genes are interesting in light of their high sequence similarity.

Class-D genes: The gene product of CpSTK (AB600237) is 60% identical to that of Arabidopsis SEEDSTICK (STK). Phylogenetic analysis revealed that the *CpSTK* gene product belongs to the same clade as STK, Floral Binding Protein (FBP) 7, and FBP11 of petunia (Figure 1). Class-D members are known to specify the ovule that develops within the carpels of whorl 4 (Colombo et al. 1995; Pinyopich et al. 2003), and STK specifically to function in funiculus growth and seed abscission in Arabidopsis ovule development (Pinyopich et al. 2003). We found *CpSTK* expression only in whorl 4, a finding that is consistent with Class-D function (Figure 2).

Class-E: Two genes were found to belong to the *SEP* subfamily (Figure 1). The deduced amino acid sequence of one gene is 66% identical to SEP2. This gene was named *CpMADS1* (AB600235). Another gene, named *CpMADS2* (AB600236), encodes a protein that is 72% identical to that encoded by SEP3. *CpMADS1* transcriptional products were detected in all floral tissues that were examined, and *CpMADS2* transcriptional products were found in same tissues except flower stalks (Figure 2). In Arabidopsis, *SEP2* and *SEP3* are expressed in petals, stamens, and carpels (Mandel and Yanofsky 1998). Class-E genes are known to interact with ABC-class genes in each whorl. The two cyclamen *SEP* homologs showing different expression pattern in sepals, might interact slightly differently with other ABC-class genes.

Other gene: cDNA was found encoding a protein 52% identical to FLOWERING LOCUS C (FLC), which was named *CpMADS3* (AB600233). This gene encodes polypeptides clustering within FLC (Figure 1) and was transcribed in all tissues analyzed in this study (Figure 2). FLC in Arabidopsis has been identified as a repressor of flowering and does not express inflorescence tissue (Michaels et al. 1999). These results suggest a novel function for CpMADS3 in cyclamen.

In this study, we isolated 10 distinct cyclamen MADS-box genes. Their predicted amino acid sequences place them in the AP1, PI, AP3, AG, STK, SEP2, SEP3, and FLC subfamilies. We revealed that these MADS-box homologous genes, which have been well analyzed in other flowering plants, are highly conserved in cyclamen, but differ in tissue expression patterns. For example, we found two closely related class-C homologous genes to be differentially expressed in stamens and carpels. In cyclamen, rare homeotic mutant flower varieties sometimes appear with double petals and no stamens (Grey-Wilson 2002). This suggests that, different from Arabidopsis and *A.majus*, the formation of stamen and carpel of cyclamen might be independently regulated by two closely related class-C genes. Such mutants might be useful in analysis of cyclamen MADS-box gene functions. The expression of all cyclamen MADS-box genes isolated in this study was found to be associated with the formation of the floral organs. These results enhance our understanding of floral organ identity and flower development in cyclamen, and form a basis for molecular genetics-based cyclamen breeding.

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