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Multiple *AGAMOUS* Homologs from Cucumber and Petunia Differ in Their Ability to Induce Reproductive Organ Fate

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The C function in Arabidopsis, which specifies stamen and carpel identity, is represented by a single gene called *AGAMOUS* (*AG*). From both petunia and cucumber, two MADS box genes have been isolated. Both share a high degree of amino acid sequence identity with the Arabidopsis AG protein. Their roles in specifying stamen and carpel identity have been studied by ectopic expression in petunia, resulting in plants with different floral phenotypes. *Cucumber MADS box* gene *1* (*CUM1*) induced severe homeotic transformations of sepals into carpelloid structures and petals into stamens, which is similar to ectopic *AG* expression in Arabidopsis plants. Overexpression of the other cucumber *AG* homolog, *CUM10*, resulted in plants with partial transformations of the petals into antheroid structures, indicating that *CUM10* is also able to promote floral organ identity. From the two petunia *AG* homologs *pMADS3* and *Floral Binding Protein* gene *6* (*FBP6*), only *pMADS3* was able to induce homeotic transformations of sepals and petals. Ectopic expression of both *pMADS3* and *FBP6*, as occurrs in the petunia homeotic mutant *blind*, phenocopies the *pMADS3* single overexpresser plants, indicating that there is no additive effect of concerted expression. This study demonstrates that in petunia and cucumber, multiple *AG* homologs exist, although they differ in their ability to induce reproductive organ fate.

INTRODUCTION

Hermaphroditic flowers of most angiosperm plants consist of four concentric whorls of organs. The two outermost whorls contain sterile organs, namely, the sepals (whorl 1) and petals (whorl 2), and the innermost whorls contain the reproductive organs, namely, the stamens (whorl 3) and carpels (whorl 4). The identity of these floral organs is thought to be defined by the action of three distinct classes of floral homeotic genes (A, B, and C). Each is active in two adjacent whorls (reviewed in Coen and Meyerowitz, 1991; Weigel and Meyerowitz, 1994). In the first whorl, only A genes are active and lead to the formation of sepals. In whorl 2, the formation of petals is defined by the combinatorial action of class A and B genes. The expression of a combination of B and C homeotic genes in whorl 3 leads to the development of stamens; in whorl 4, in which only C class genes are expressed, carpels develop. In addition, A and C genes are shown to be mutually antagonistic. This ABC model is based on extensive genetic studies in Arabidopsis and Antirrhinum (Carpenter and Coen, 1990; Schwarz-Sommer et al., 1990; Bowman et al., 1991) and has been confirmed in part for many other species, including petunia (reviewed in Colombo et al., 1997) and tomato (Pnueli et al., 1991).

Genes belonging to the B and C classes of homeotic genes are members of the MADS box gene family. These genes encode transcription factors with a highly conserved DNA binding domain, the MADS box. In addition to the MADS box, these genes contain a moderately conserved domain called the K-box, which is able to form coiled-coil structures that allow dimerization of these transcription factors (Ma et al., 1991). Comparison of the amino acid sequences of MADS box genes from different species has led to the classification of the MADS box gene family in B, C, and other groups of MADS box genes (Purugganan et al., 1995). Ectopic expression or suppression of these organ identity genes by antisense/cosuppression strategies has often confirmed the function of the gene, which was predicted on its sequence.

One of the first isolated MADS box genes is the Arabidopsis *AGAMOUS* (*AG*) gene (Yanofsky et al., 1990), which encodes the C function in Arabidopsis. *ag* mutants have normal sepals and petals, whereas stamens are homeotically converted to petals and the carpels are replaced by another *ag* mutant flower. Overexpression of *AG*, using the 35S cauliflower mosaic virus (CaMV) promoter, phenocopies in part

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the *apetala 2* (*ap2*) mutant, with carpels in the first whorl instead of sepals and stamens replacing the petals in the second whorl (Mizukami and Ma, 1992). The mutant and overexpression phenotypes demonstrate that *AG* is a class C homeotic gene playing a key regulatory role in determining the identity of stamens and carpels.

AG homologs have been isolated from Antirrhinum, petunia, tobacco, tomato, rapeseed, and rice, and in several studies, their function in establishing stamen and carpel identity was demonstrated by ectopic expression (Mandel et al., 1992; Kempin et al., 1993; Tsuchimoto et al., 1993; Pnueli et al., 1994; Saedler and Huijser, 1994; Kang et al., 1995). In most cases, the obtained phenotypes resembled more or less the one obtained by ectopic expression of the *AG* gene in Arabidopsis. However, the petunia *AG* homolog *pMADS3* seems to be an exception, because ectopic expression of this gene in petunia and tobacco resulted in incomplete homeotic transformations (Tsuchimoto et al., 1993). The first floral whorl comprised sepals with some carpelloid characteristics, and the limbs of the second whorl petals were reduced in size and partly converted into antheroid tissue. This *pMADS3* overexpression phenotype resembles the homeotic *blind* mutant (Vallade et al., 1987), which displays incomplete homeotic transformations in the two outer floral whorls as well. To date, the nature of the recessive mutation in the *blind* mutant is not known.

A homeotic mutant resembling an A-type mutant obtained by either abolishing the A function or ectopic expression of a C gene has not yet been identified for petunia. It is not known whether other *AG* homologs, besides *pMADS3*, are required for the complete C function in petunia. In this report, we study the contribution of another petunia *AG* homolog, *Floral Binding Protein* gene *6* (*FBP6*) (Angenent et al., 1993), in establishing the C function. Mutants in which *FBP6* was ectopically expressed, alone or in combination with *pMADS3*, were compared with respect to their ability to induce reproductive organ fate. The possibility of two *AG* homologs regulating the C function is probably not unique, because recently it has been proposed that in maize the C function is orchestrated by two closely related *AG* homologs, *ZAG1* and *ZMM2* (Mena et al., 1996).

In cucumber, we also identified two genes, *Cucumber MADS box* gene *1* (*CUM1*) and *CUM10*, that appeared to have many characteristics in common with *AG.* Cucumber is of particular interest because it is a monoecious species, bearing male and female flowers on the same individual plant. Whether multiple *AG* homologs are somehow connected with the unisexual nature of flowers from cucumber and maize is not known. To gain more information about the role of *CUM1* and *CUM10* in specifying stamen and carpel identity, we ectopically expressed these genes in petunia and compared the results with those obtained from the ectopic expression of the two petunia *AG* homologs. This comparative study provides new information on the conservation of the C function in two evolutionarily diverse species.

RESULTS

Isolation of Cucumber cDNAs Homologous to *AG*

To isolate MADS box cDNA clones from cucumber, a female flower–specific cDNA library was screened under reduced stringency conditions. A mixture of DNA fragments containing the 5′ MADS box region of petunia MADS box genes was used as a probe. Ninety-three hybridizing clones were isolated and purified. To identify cucumber cDNAs homologous to *AG*, a second hybridization experiment was performed in which the 3′ gene-specific parts of two petunia *Ag* homologs, *pMADS3* (Tsuchimoto et al., 1993) and *FBP6* (Angenent et al., 1993), were used to screen the 93 purified clones. From this collection of 93 clones, 13 clones hybridized with the *pMADS3*/*FBP6*–specific probe. These 13 clones belong to two independent groups of cDNAs, which were designated *CUM1* and *CUM10.* The clones with the largest inserts were selected for further analysis.

The deduced amino acid sequences of *CUM1* and *CUM10* cDNA clones are shown in Figure 1 and reveal proteins of 262 and 228 amino acid residues, respectively. However, we are not absolutely certain about the size of the CUM1 protein, because five different start codons could be identified preceding the MADS box region. Alignment of CUM1, CUM10, pMADS3, FBP6, and AG clearly shows that the MADS box regions of these five proteins are identical. Figure 1 also shows that CUM10 lacks the N-terminal extension preceding the MADS box, which is present in CUM1, pMADS3, FBP6, and AG. All *AG* orthologs identified to date have this N-terminal extension preceding the MADS box.

To visualize the homology of the CUM1 and CUM10 amino acid sequences with other members belonging to the monophyletic *AG* group (Purugganan et al., 1995), a multiple alignment was performed. This alignment was used to construct a dendrogram, shown in Figure 2. This dendrogram reveals that the CUM1 protein is more closely related to AG, PLENA (PLE), and the solanaceous AG homologs (NAG1, TAG1, and pMADS3) than to CUM10.

Two Petunia *AG* Homologs Expressed in Stamen and Carpel Primordia

The cloning of two petunia *AG* homologs, *FBP6* and *pMADS3*, has been described previously (Angenent et al., 1993; Tsuchimoto et al., 1993). RNA gel blot experiments have demonstrated that both *AG* homologs are exclusively expressed in stamens and carpels. To determine the temporal and spatial expression pattern of these two genes during flower development, we performed RNA in situ hybridization analyses with petunia floral buds. To avoid cross-hybridization with other MADS box genes, the MADS box sequences from the *pMADS3* and *FBP6* probes were removed.

Figure 3 shows the distribution of *pMADS3* and *FBP6*

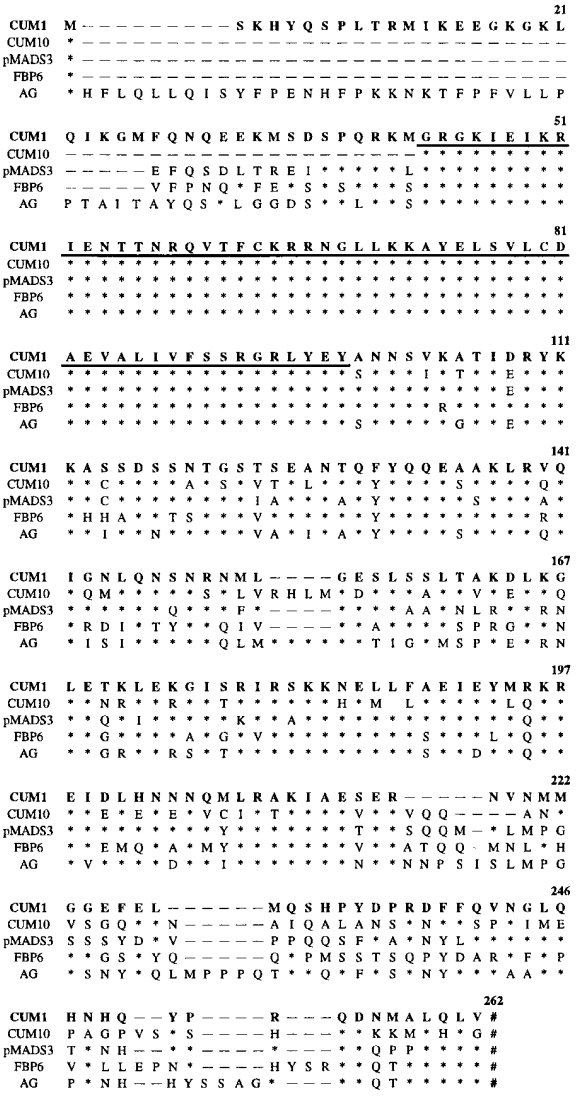

Figure 1. Alignment of the Amino Acid Sequences of AG Homologs of Cucumber (CUM1 and CUM10) and Petunia (pMADS3 and FBP6) and of the Arabidopsis AG Protein.

The CUM1 amino acid sequence is shown in boldface. An asterisk indicates a residue identical to CUM1, and a dash represents a gap introduced to improve alignment. The conserved MADS box region is underlined. Numbers indicate the position of the amino acids in CUM1 starting at the putative translation start site. The GenBank accession numbers for CUM1 and CUM10 are AF035438 and AF035439, respectively.

mRNA at three stages of petunia flower development. At an early stage (Figures 3A and 3B), when the sepal primordia become apparent on the flanks of the floral meristem, *pMADS3* and *FBP6* start to accumulate in the cells that later give rise to the stamen and carpel primordia. When stamen primordia are clearly visible and carpel primordia start to develop (Figures 3D and 3E), *pMADS3* and *FBP6* mRNAs are distributed throughout the stamen primordia and the central part of the floral apex that develops into the pistil. No hybridization signal was detectable in the cells of the sepal and petal primordia. At later stages of pistil development, *FBP6* is highly expressed in the stigma and transmitting tissue of the style (Figure 3G), whereas *pMADS3* transcripts are more abundant in the ovules, vascular tissue (Figure 3F), and the nectaries (3C).

Phenotypic Analyses of Petunia Plants Ectopically Expressing *pMADS3* or *FBP6*

Tsuchimoto et al. (1993) reported that overexpression of *pMADS3* in transgenic petunia plants (line V26) leads to phenocopies of the petunia *blind* mutant, that is, to the formation of antheroid tissue on top of the petal tube and carpelloid tissues in whorl 1. To compare the *pMADS3* overexpression phenotype with phenotypes obtained by ectopically expressing *FBP6* and cucumber *AG* homologs, we

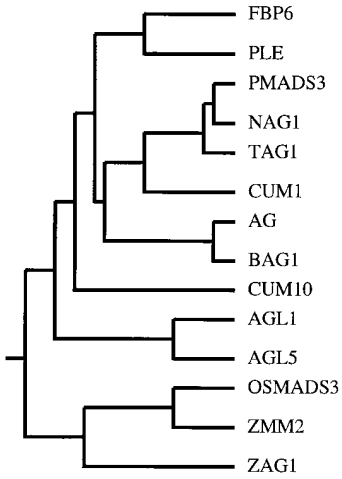

Figure 2. Dendrogram of AG Homologous Proteins.

Shown is an UPGMA-based dendrogram detailing the relationship between the amino acid sequences of the Arabidopsis AG protein and proteins homologous to AG. Included in the dendrogram are PLE of Antirrhinum; FBP6 and pMADS3 of petunia; ZMM2 and ZAG1 of maize; OSMADS3 of rice; CUM1 and CUM10 of cucumber; AG, AGL1, and AGL5 of Arabidopsis; BAG1 of rapeseed; NAG1 of tobacco; and TAG1 of tomato. First, the amino acid sequences were aligned by ClustalW (provided by J. Thompson, EMBL, Heidelberg, Germany), and then the dendrogram was obtained by the Protdist and neighbor-joining (UPGMA method) programs of the PHYLIP 3.5c package (provided by J. Felsenstein, Department of Genetics, University of Washington, Seattle, WA).

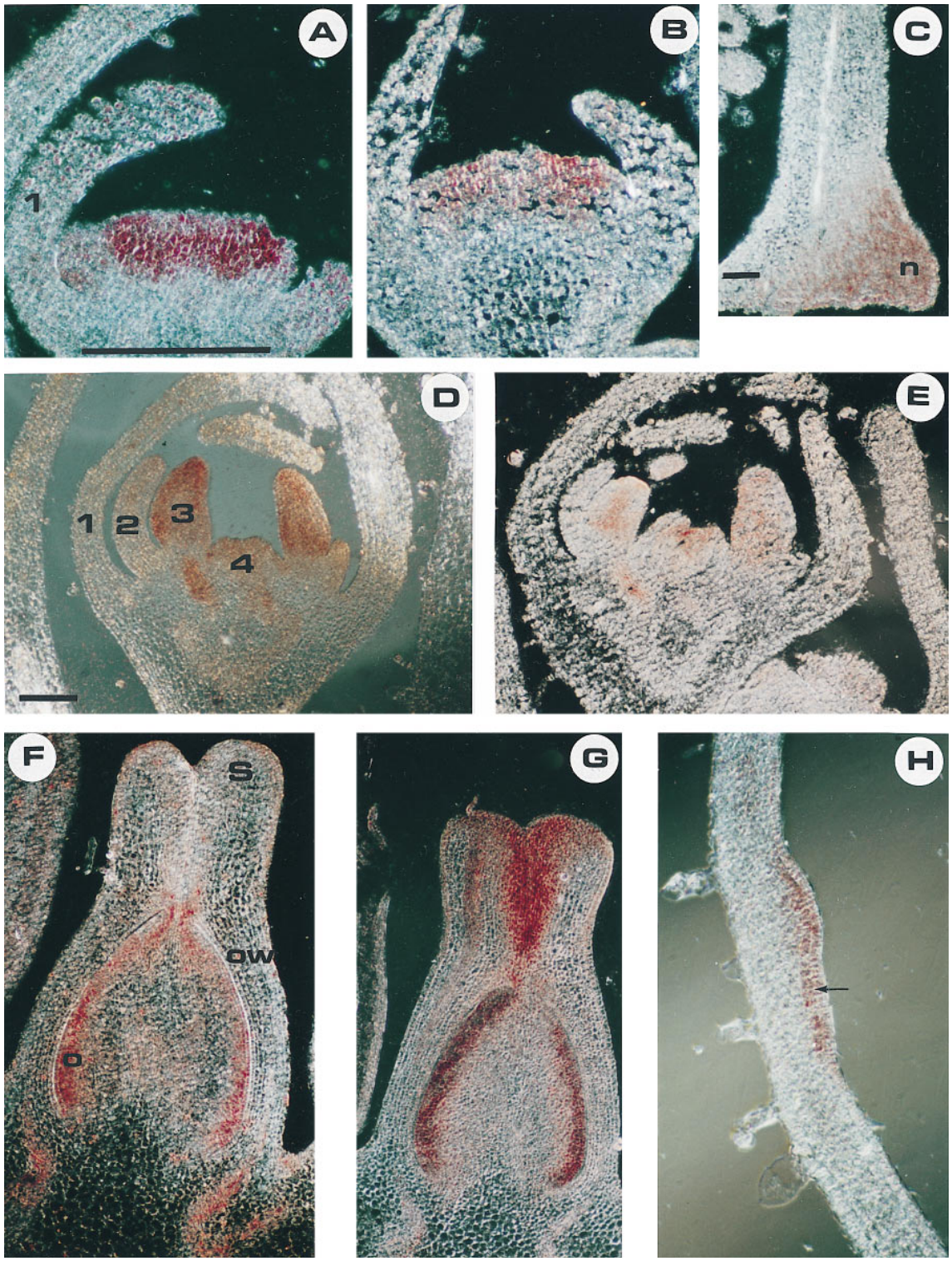

Figure 3. In Situ Localization of *pMADS3* and *FBP6* Transcripts in Wild-Type and Mutant Flowers.

Longitudinal sections were hybridized with digoxigenin-labeled antisense *pMADS3* (**[A]**, **[C]**, **[D]**, and **[F]**) and *FBP6* (**[B]**, **[E]**, **[G]**, and **[H]**) RNA. **(A)** and **(B)** Very early stage floral bud: the two sepal primordia arise.

introduced the *pMADS3* cDNA driven by the CaMV 35S promoter in the petunia line W115.

Fifteen independent kanamycin-resistant plants were generated, of which four had flowers with comparable aberrations. One of these four transformants, T64003, was selected for further analysis. Representative flowers of wild-type plants (W115) and T64003 are shown in Figures 4A and 4B, respectively. The limbs of the petals of T64003 flowers were largely reduced in size, and antheroid tissue developed between the limbs, at the fusion site of the petals (Figure 4C). The part of the petals that forms the tube was not affected. The sepals of T64003 flowers were curled at the tip, and very rarely were stylar and stigmatic structures observed on top of these curled tips (Figures 4D).

The fertility of plants ectopically expressing *pMADS3* was also affected. The transgenic trait was not transmitted to the progeny through either the male or female parent, suggesting that overexpression of *pMADS3* is either gametophytically or embryonically lethal. To investigate whether overexpression of the petunia *AG* homolog *FBP6* results in homeotic transformations of the perianth organs as well, we ectopically expressed *FBP6* by using the same construct as has been used for the overexpression of *pMADS3.* Five of 14 independent transgenic plants showed flowers with more or less similar modifications. The petal limbs of T66001, which is the transformant showing the most severe modifications, was reduced in size and had the same shape compared with whorl 2 organs of the *pMADS3* overexpression plants (Figure 4E). However, antheroid tissue was never observed on these limbs. The rest of the flower was not affected by the overexpression of this gene. Like the *pMADS3*-overexpressing plants, the T66001 phenotype could not be transmitted to the progeny.

By using a reverse genetics approach with the petunia transposon *dTph1* tagging system as described by Koes et al. (1995), we screened for transposon insertions in *pMADS3.* One of the mutants (M681) from this screen had a transposon insertion in the intron just downstream from the MADS box coding region of *pMADS3.* This insertional mutant, from which a representive flower is shown in Figure 4F, resembles the phenotype of the *blind* mutant (Figure 4G), that is, the corolla limbs are converted into antheroid structures and whorl 1 organs show carpelloid features. The insertion of a transposon in this position of the intron results in ectopic expression of *pMADS3*, causing this aberrant phenotype. A detailed analysis of this insertional mutant will be published elsewhere (L. Colombo and G.C. Angenent, manuscript in preparation). A similar phenomenon has been observed in Antirrhinum, in which a *Tam3* transposon insertion in the second intron of the *AG* homolog *PLE* resulted in ectopic expression of this gene (Bradley et al., 1993).

Ectopic Expression of *CUM1* Results in Severe Homeotic Transformations of Sepals and Petals

To investigate the effect of ectopic expression of a cucumber *AG* homolog in petunia, we fused the *CUM1* cDNA to the CaMV 35S promoter and introduced it in petunia. Three of 17 transgenic plants showed aberrations in the outer floral whorls. Transformant T72010 showed the most severe transformations; a flower with such a transformation is shown in Figure 4H. The petals were completely transformed into five stamens, which in some flowers were indistinguishable from the third whorl stamens. However, often the filaments of the ectopic stamens were thicker and partly fused to each other, forming a tubelike structure.

Whorl 1 organs of transformant T72010 were connately joined, in contrast to the wild-type sepals that normally are separated from one another. The color of the whorl 1 organs is light green rather than the dark green of wild-type sepals, and at their tip, curled filamentous structures developed that resembled style–stigma structures. To demonstrate that these whorl 1 organs possessed real carpel-like features, we performed a detailed scanning electron microscopic analysis of the inner site of these organs. As shown in Figure 5, placenta-like epidermal cells developed on the site where the whorl 1 organs fuse. On these placenta-like areas, style–stigma structures were formed (Figure 5A). The stylar structures occasionally possess trichomes, which are never seen on styles of wild-type plants (Figure 5D), indicating the chimeric nature of these structures (Figure 5B). The stigma-like tissues were covered with papillae, which are characteristic for this kind of tissue. Besides these style–stigma structures, ovules developed on the placenta-like tissue (Figure 5C). The majority of these ovules, which were morphologically indistinguishable from wild-type ovules in the ovary (Figure 5E), developed at the base near the fusion point of the whorl 1 organs.

Overexpression of *CUM1* also affected the morphology of the leaves and the architecture of the inflorescence. The

Figure 3. (continued).

(C) Section through nectary tissue at the bottom of an almost mature ovary.
(D) and **(E)** Early stage floral bud: stamen primordia are clearly visible, and carpel primordia start to develop.
(F) and **(G)** Section through a developing pistil at stage 6, when ovule primordia arise.
(H) Section through a petal with antheroid tissue (indicated by an arrow) of the transgenic petunia plant T64003 overexpressing *pMADS3.*
The floral whorls are indicated with numbers. o, ovule primordia; ow, ovary wall; s, stigma. Bars in **(A)**, **(C)**, and **(D)** = 0.5 mm. Magnifications are the same in **(A)** and **(B)** and in **(D)** to **(H)**.

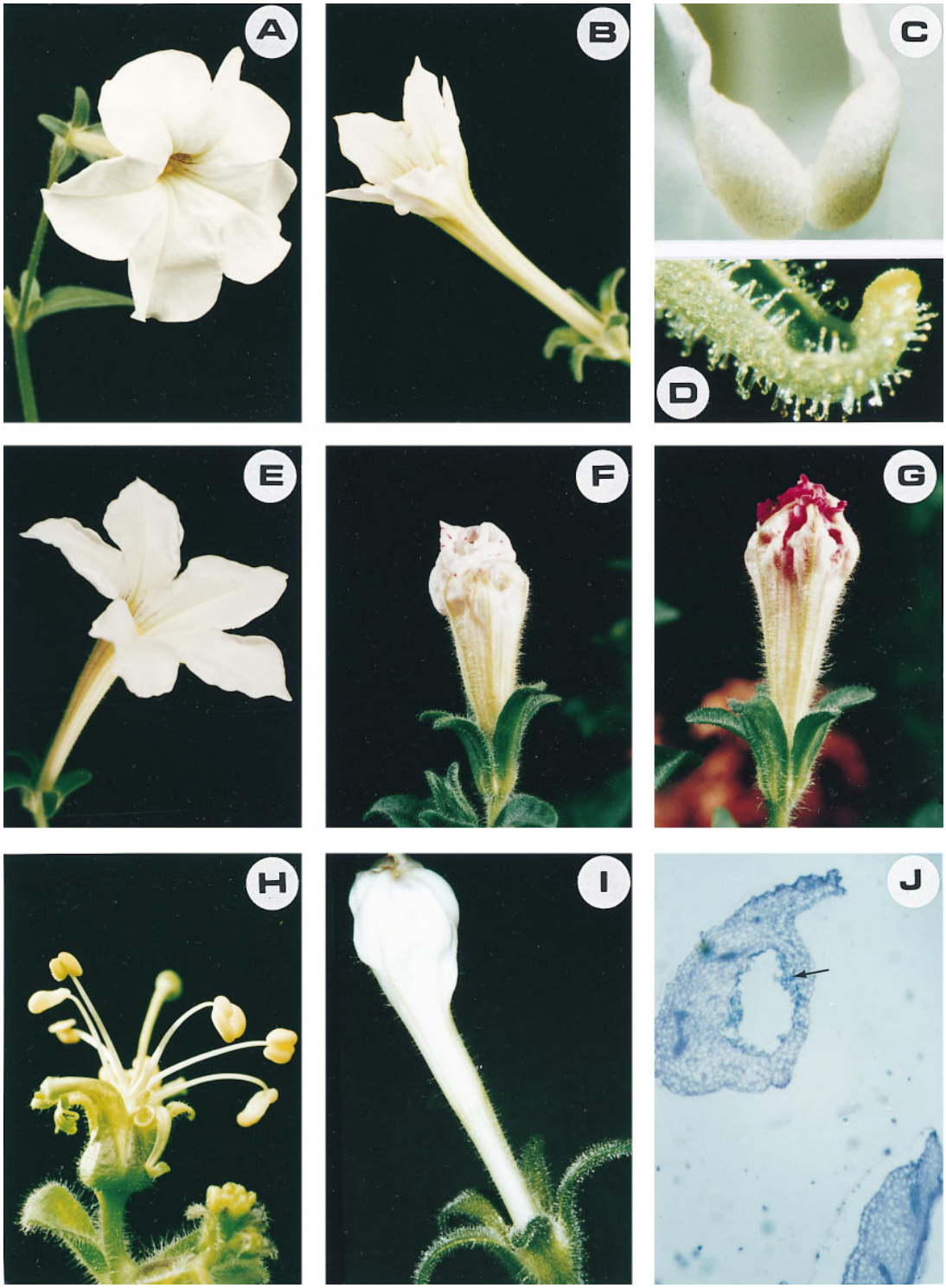

Figure 4. Flower Morphology of Wild-Type Plants, Transgenic Plants Ectopically Expressing *pMADS3*, *FBP6*, *CUM1*, or *CUM10*, and the Mutants M681 and *blind*.

(A) Wild-type petunia flower (W115).
(B) Flower of a *pMADS3* ectopically expressing plant (T64003) showing smaller petals and antheroid tissue developing on the limbs.

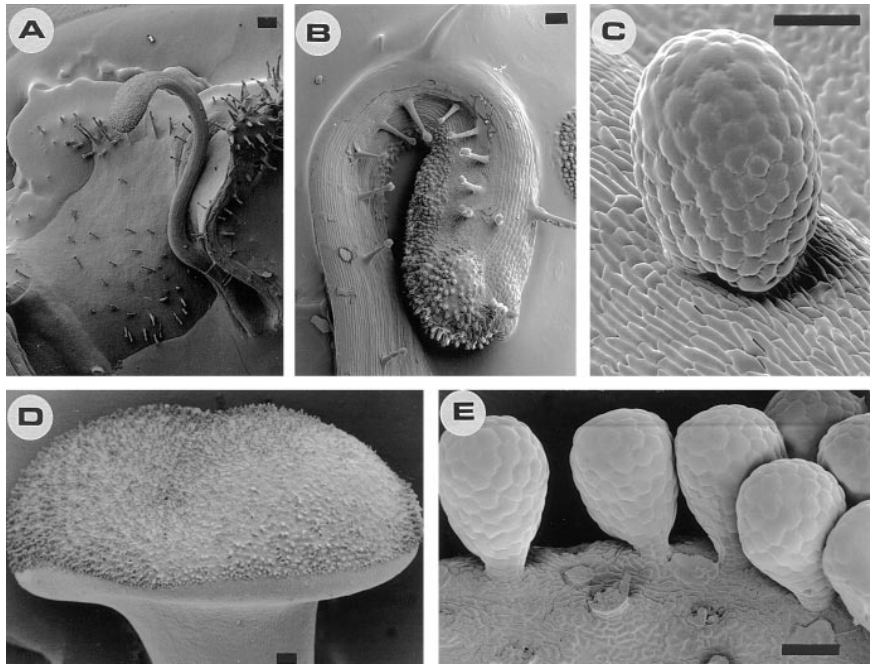

Figure 5. Scanning Electron Microscopy of Wild-Type Pistil Tissues and of the Inner Site of Whorl 1 Organs of a *CUM1* Ectopically Expressing Plant (T72010).

(A) Style–stigma structure on a whorl 1 organ of a T72010 flower.
(B) Style–stigma structure with trichomes (indicating the chimeric nature of the tissue) on a whorl 1 organ of T72010.
(C) Ectopic ovule formation on placenta-like tissue present on the inner side of a whorl 1 organ of T72010.
(D) View of the stigma and part of the style of a wild-type W115 plant.
(E) A wild-type placenta present in the ovary. Some of the ovules have been carefully removed to show the placental cell shape and organization. Bar in **(A)** = 400 μm; bars in **(B)**, **(C)**, **(D)**, and **(E)** = 100 μm.

leaves were partly curled and the inflorescence terminated in a flower, whereas wild-type inflorescences are indeterminate. Similar aberrations were reported for Arabidopsis plants ectopically expressing *AG* (Mizukami and Ma, 1992).

In contrast to *pMADS3* and *FBP6* overexpressers, all petunia plants transformed with the cucumber *CUM1* gene, including transformant T72010, were male and female fertile, although some reduction in fertility was observed. Even the pollen produced by the whorl 2 anthers was fertile. The progeny plants exhibited the same homeotic alterations as the primary transformants.

Overexpression of the Cucumber *AG* Homolog *CUM10* Affects the Shape of Corolla Limbs

To investigate whether the cucumber *AG* homolog *CUM10* is also able to induce homeotic alterations of whorl 1 and 2

Figure 4. (continued)

(C) Close-up of the fusion site of T64003 petals, where antheroid tissue develops.
(D) Curled sepal tip of T64003 with stigmatic tissue.
(E) Flower of a *FBP6* ectopically expressing plant (T66001) showing petals that are reduced in size.
(F) Flower of the M681 transposon insertion mutant.
(G) Flower of the *blind* mutant.
(H) Flower of a *CUM1* ectopically expressing plant (T72010) showing a complete transformation of the petals into stamens and sepals into organs with carpelloid features.
(I) Flower of a *CUM10* ectopically expressing plant (T74001) showing petals that are folded inward.
(J) Longitudinal section through a petal limb of T74001 showing antheroid tissue (indicated by an arrow).

organs, a CaMV 35S–*CUM10* overexpression construct was used to transform petunia. Three transformants of 30 plants showed the same aberrations in sepal and petal development. A typical flower of one of these transformants (T74001) is shown in Figure 4I. The limbs of these flowers were reduced in size and were folded inward instead of outward, thereby completely enclosing the reproductive organs. The tube of the petals was not affected. To investigate the identity of the tissue on top of the tube, we performed histological analysis. The longitudinal section shows the antheroid nature of this yellowish tissue on top of the petals (Figure 4J). The sepals of the T74001 flowers were much bigger in size than those of wild-type flowers; however, no carpelloid features such as style–stigma structures or ovules were found on these sepals.

Expression Analyses of Petunia Plants Overexpressing Cucumber and Petunia *AG*-like Genes

Morphological alterations were observed in floral organs of transgenic plants overexpressing the two cucumber *AG* homologs *CUM1* (T72010) and *CUM10* (T74001) and the two petunia *AG* homologs *FBP6* (T66001) and *pMADS3* (T64003). Similar morphological alterations were observed in the petunia *blind* mutant. RNA gel blot analyses were performed to investigate whether the expression of the endogenous *FBP6* and *pMADS3* genes was changed.

As shown in Figure 6A, high levels of *CUM1* and *CUM10* RNA accumulated in all floral whorls and in leaves of T72010 and T74001 plants, respectively. In contrast to the expression of *FBP6* and *pMADS3* in wild-type flowers, transcripts of these genes accumulated in all four whorls of T72010 flowers (Figure 6B). *FPB6* was expressed at a higher level in whorl 1 than in whorl 2. This is probably due to the fact that in wild-type flowers, *FBP6* transcripts are more abundant in carpels than in stamens. Ectopic expression of *FBP6* and *pMADS3* in the outer two floral whorls of the transgenic plants might be indirect and due to the homeotic transformations into carpels and stamens. The *CUM10*-overexpressing plant T74001 did not ectopically express either *pMADS3* or *FBP6*, indicating that the cucumber gene is not capable of inducing the petunia genes (Figure 6B).

In plant T64003, expressing *pMADS3* in leaves and in all floral whorls (Figure 6B), the expression of *FBP6* was not altered compared with wild-type plants. The homeotic alterations in whorls 1 and 2 were most likely not severe enough to detect *FBP6* expression in these whorls by RNA gel blot analysis. However, RNA in situ hybridization analysis of whorl 2 organs showed that *FBP6* is expressed in the patches of antheroid tissue (Figure 3H). Expression analysis of *pMADS3* in the transgenic plant T66001, overexpressing *FBP6*, revealed that *pMADS3* expression is similar to that in wild-type plants. These results indicate that *FBP6* and *pMADS3* are not activating each other's expression.

The expression of *FBP6* and *pMADS3* in the *blind* mutant

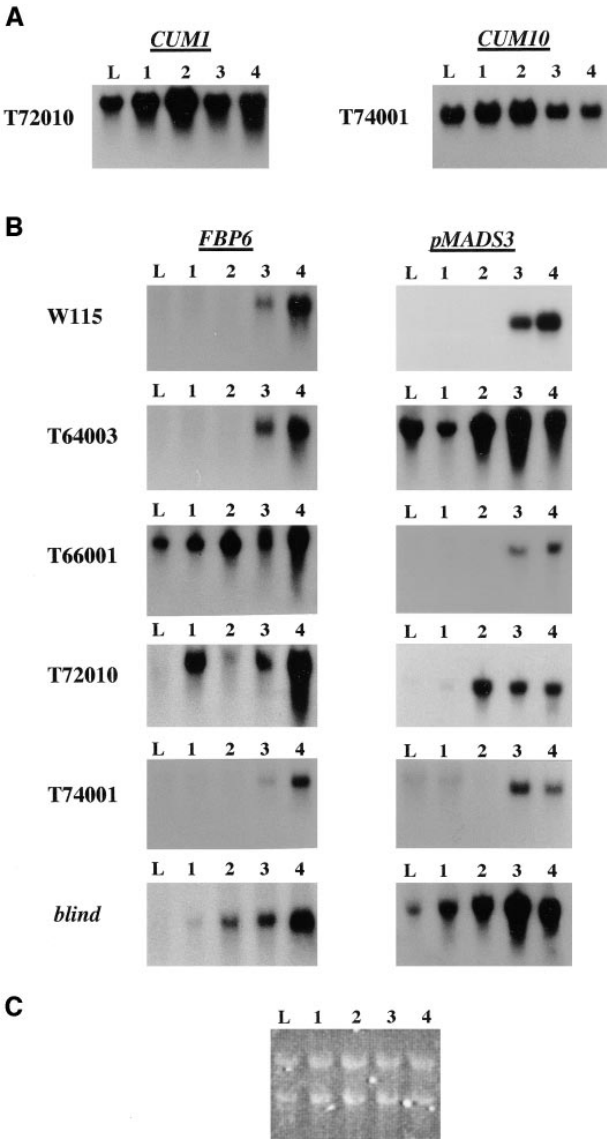

Figure 6. Expression of Cucumber and Petunia *AG* Homologs in Transgenic Petunia Plants Overexpressing These Genes.

(A) mRNA accumulation of *CUM1* and *CUM10* in leaves (L) and the four floral whorls (1 to 4) of *CUM1*- and *CUM10*-overexpressing plants (T72010 and T74001, respectively).

(B) mRNA accumulation of *FBP6* and *pMADS3* in leaves (L) and the four floral whorls (1 to 4) of the wild type (W115), *pMADS3* overexpresser (T64003), *FBP6* overexpresser (T66001), *CUM1* overexpresser (T72010), *CUM10* overexpresser (T74001), and the *blind* mutant.

(C) Representive RNA gel stained with ethidium bromide before blotting and hybridization. Equal amounts of total RNA were loaded. Each lane contains 10 μg of total RNA. Blots were probed with ^{32}P-labeled gene-specific fragments of the *CUM1*, *CUM10*, *FBP6*, or *pMADS3* cDNAs.

was investigated in more detail, because this mutant has homeotic transformations similar to those observed for the transgenic plants ectopically expressing *AG* homologs. As shown in Figure 6B, *FBP6* and *pMADS3* are ectopically expressed in the first two floral whorls of the *blind* mutant. Besides the floral organs, expression of *pMADS3* and *FBP6* was also observed in leaves of this mutant, although the *FBP6* hybridization signal was only detectable after long exposure. These results indicate that the *BLIND* gene product is involved in the suppression of both petunia *AG* homologs in leaves and in the first two floral whorls.

DISCUSSION

In this study, we report the functional analysis of *AG* homologs from petunia and cucumber. In both species, two genes were identified that are related with respect to sequence and expression pattern to the class C genes from Arabidopsis and Antirrhinum. To investigate whether the two homologs in petunia and cucumber facilitate the same role in specifying floral organ identity, these genes were ectopically expressed in petunia.

pMADS3 Is the Cognate Homolog of *AG* and *PLE*

Extensive screening for MADS box genes expressed in reproductive organs in petunia yielded two genes that are highly homologous to the class C homeotic genes from Arabidopsis (*AG*) and Antirrhinum (*PLE*). In Arabidopsis and Antirrhinum, only one single homeotic gene facilitates the C function that is the specification of stamen and carpel identity. In several respects, the petunia gene *pMADS3* can be recognized as the cognate homolog of *AG* and *PLE.* The overall amino acid sequence similarity between the deduced proteins is very high, and the spatial and temporal expression patterns of *pMADS3* match exactly with those of *AG* and *PLE* (Figure 3) (Drews et al., 1991; Bradley et al., 1993). Two different types of mutants and transgenic plants in which *pMADS3* was ectopically expressed were used to demonstrate the functional homology between *pMADS3* and *PLE* or *AG.* One of these mutants was obtained from a transposon mutagenesis screening. This mutant (M681) has a transposon insertion in one of the *pMADS3* introns. A detailed analysis of M681 will be described elsewhere (L. Colombo and G.C. Angenent, manuscript in preparation). The phenotype of the M681 mutant resembles the phenotype of the *blind* mutant, which is a mild form of the Antirrhinum *ovulata* mutant (Bradley et al., 1993). As in the *blind* mutant, *pMADS3* is ectopically expressed in all four floral whorls and in the leaves of this insertion mutant. The similarities of these observations to the results obtained with *PLE* are striking and indicate that *pMADS3* is most likely the cognate homolog of *PLE* and *AG.* Bradley et al. (1993) observed that a *Tam3* transposon insertion in the large 4-kb intron of *PLE*, just downstream of the MADS box exon, resulted in a gain-of-function mutation caused by ectopic *PLE* expression. In this mutant showing the *ovulata* phenotype, carpels develop instead of sepals, and stamens are formed in the second whorl in the position normally occupied by petals.

Further support for our hypothesis that *pMADS3* represents the C function in petunia is that ectopic expression of *pMADS3* in transgenic plants resulted in plants having whorls 1 and 2 organs with carpelloid and staminoid features, respectively. This indicates that *pMADS3*, like *AG* and *PLE*, is a class C homeotic gene, controlling the determination of stamen and carpel identity.

In contrast to *AG* and *PLE, pMADS3* is not able to give rise to a complete homeotic conversion of the two types of perianth organs into reproductive organs, in any of the mutants analyzed. In particular, the petal tube is completely unaffected. Because a complete homeotic transformation of a petal into a stamen, by modifying *pMADS3* expression, has never been observed in petunia, one could speculate that this is not possible in petunia. However, the experiments in which we ectopically expressed the cucumber *AG* homolog *CUM1* in petunia clearly demonstrate that a complete homeotic conversion of petals into stamens is possible. In the same transformants, the homeotic transformation of the sepals into carpels in the first whorl was also much more pronounced than in *pMADS3*-overexpressing plants. In conclusion, we analyzed three types of plants (*blind*, M681, and T64003) in which *pMADS3* is ectopically expressed, and all types showed a similar phenotype. These results indicate that *pMADS3* is not able to induce a complete homeotic conversion of the outer perianth organs into reproductive organs. This observation suggests that the C function in petunia may be controlled by two or more class C genes whose functions are combined in one single cucumber gene, *CUM1.*

Multiple *AG* Homologs

In Arabidopsis and Antirrhinum, the C function is represented by a single gene. In contrast, there seem to be more *AG* homologs in other species. It has been described for maize (Mena et al., 1996) that two closely related genes, *ZAG1* and *ZMM2*, appear to orchestrate the C function. A transposon insertion in *ZAG1* did not result in aberrations in the tassel, whereas the fourth whorl in the female flowers still remained carpelloid. Only the determinacy was affected in these *ZAG1* mutant flowers. This phenotype suggests that either there is redundancy with respect to the C function in maize or that both genes contribute to the C function in male and female reproductive organs. *ZMM2* and *ZAG1* have overlapping expression patterns: they are both expressed in the inner two floral whorls, although *ZAG1* expression is higher in carpels, whereas *ZMM2* expression is more restricted to stamens. These expression patterns support the idea that *ZAG1* is more involved in determination of

carpel identity, whereas *ZMM2* may be more involved in stamen formation. However, for a complete C function, both genes are likely to be required.

FBP6 *and* pMADS3*, the Petunia* AG *Homologs*

A candidate for defining the C function in petunia together with *pMADS3* is *FBP6.* Like *pMADS3*, *FBP6* is very similar to *AG* with respect to sequence and expression pattern. Despite these similarities, overexpression of *FBP6* did not result in a homeotic transformation of sepals and petals into carpels and stamens, respectively. Although this result contradicts a possible C function, *FBP6* still might have such a function in combination with *pMADS3* or other factors.

In the *blind* mutant, ectopic expression of *pMADS3* in leaves, sepals, and petals coincides with ectopic expression of *FBP6.* Despite this joint expression, the petal tube was not homeotically transformed, and the sepals also were not severely altered. A similar phenotype was observed for transgenic plants in which only *pMADS3* is ectopically expressed, indicating that *FBP6* does not have an additive effect. These results suggest that there might be another *AG* homolog that plays a role in a combinatorial way with *pMADS3* and/or *FBP6.* We cannot exclude the possibility that there is a single, so far unidentified petunia class C gene that is the key gene to determining the identity of stamens and carpels, as *AG* does in Arabidopsis.

CUM1 *and* CUM10*, the Cucumber* AG *Homologs*

In cucumber, the C function seems to be orchestrated by at least two genes, *CUM1* and *CUM10.* Both of these genes encode proteins that share a high degree of sequence identity with *AG* and *PLE.* Strikingly, *CUM10* does not have the N-terminal extension preceding the MADS box domain that is common to all *AG* homologs. Mizukami et al. (1996) showed that an AG protein that lacks this N-terminal extension is still capable of binding in vitro to the CArG box (CCATTAATGG), which is the consensus target DNA sequence motif for MADS box protein binding (Pollock and Treisman, 1991; Huang et al., 1993). They showed that transgenic Arabidopsis plants overexpressing this truncated AG protein produced *ap2*-like flowers similar to those ectopically expressing AG proteins with the N-terminal region. These results indicate that the N-terminal truncated AG protein still retains its function in vivo and is able to alter the identity of the perianth organs. In addition to determining floral organ identity, *AG* homologs are also responsible for floral determinacy. Whether the N-terminal extension is required for this function is not known.

Furthermore, comparison of the N-terminal extension between various *AG* homologs reveals only limited conservation in both size and sequence. Taking all of these observations together, one may argue that the common ancestral angiosperm possessed this extension, which subsequently rapidly diverged during evolution because of a lack of selective pressure.

The expression of *CUM1* and *CUM10* is restricted to whorl 3 in male and whorl 4 in female cucumber flowers (M.M. Kater and G.C. Angenent, unpublished data), which agrees with the C function of these genes. Overexpression of *CUM1* in petunia resulted in the transformation of sepals into carpel-like structures and petals into stamens, indicating that this gene is the ortholog of *AG.* Overexpression of *CUM10* in petunia resulted in homeotic conversions of parts of the petal limbs into antheroid tissue. These transformations were less severe compared with the changes in *CUM1* overexpression plants.

Several examples have now shown that multiple *AG* homologs exist in monocot and dicot species. To date, two *AG* homologs have been identified in maize, petunia, and cucumber. Also in Arabidopsis, two genes, *AGL1* and *AGL5*, were identified that are highly homologous to *AG* (Figure 2) (Ma et al., 1991). However, their spatial and temporal expression patterns are completely different from those of *AG*, indicating that these genes play a different role (Ma et al., 1991; Savidge et al., 1995). The *AG* homologs found in petunia and cucumber do have an expression pattern comparable with *AG*; it is, however, not clear which of these are the cognate homologs of *AG* and *PLE.* Our results demonstrate that *CUM1* and *pMADS3* are the most likely candidates, although *CUM10* also is able to induce homeotic transformation toward organs with staminoid identity. Presumably, these multiple *AG* homologs did not arise as recent duplications of a single ancestral class C gene, because they vary substantially in sequence, expression pattern, and function. The *CUM10*, *FBP6*, and one of the maize homologs may have evolved into genes with a function that is more diverse than the original C function.

METHODS

Plant Material

Cucumis sativus line GA715, *Petunia hybrida* line W115, and transgenic petunia plants were grown under normal greenhouse conditions.

Screening of cDNA Libraries

A cDNA library was made from poly(A)$^+$ RNA of young female cucumber flowers, using the λZAPII vector (Stratagene, La Jolla, CA). Approximately 100,000 plaques were screened with a mixed MADS box probe containing the 5′ terminal sequences of *Floral Binding Protein* gene *1* (*FBP1*; 362 bp) (Angenent et al., 1992) and *FBP6* (475 bp) (Angenent et al., 1993). Hybridization and washing of the Hybond N$^+$ membranes (Amersham) were done under low-stringency conditions (60°C hybridization and wash with 2 × SSC [1 × SSC is 0.15 M

NaCl, 0.015 M sodium citrate] at 60°C). Ninety-three clones were isolated and purified. To identify which of these 93 clones are homologous to *AGAMOUS* (*AG*), a second hybridization experiment under the same low-stringency conditions was done using a mixed MADS box probe containing the 3′ gene-specific part of *pMADS3* (600 bp) (Tsuchimoto et al., 1993) and *FBP6* (660 bp). Thirteen hybridizing clones were identified belonging to two independent groups of cDNAs that were designated *Cucumber MADS box* gene *1* (*CUM1*) and *CUM10.* The clones with the largest inserts were selected, and the pBluescript SK− plasmids containing these inserts were excised in vivo from the λZAP vector, according to the protocol of Stratagene. The GenBank accession numbers of *CUM1* and *CUM10* cDNA sequences are AF035438 and AF035439, respectively.

Construction of Binary Vectors and Plant Transformation

To facilitate the cloning of *FBP6*, *pMADS3*, and *CUM10*, cDNAs under the control of the double cauliflower mosaic virus (CaMV) 35S promoter were used to generate new restriction sides by polymerase chain reaction (PCR). *FBP6*, *pMADS3*, and *CUM10* cDNAs were amplified using the following 5′ primers: *FBP6*, 5′-GCTCTAGACCATGGTGTTTCCTAATCAAGAATTTGAG-3′; *pMADS3*, 5′-GCTCTAGACCATGGAGTTCCAAAGTGATCTAACAAGAG-3′; and *CUM10*, 5′-GGCCATGGGGAGAGGAAAGATAGAG-3′ (Isogene Science, Maarssen, The Netherlands), which correspond to the cDNA sequence surrounding the ATG translation start site, and the following 3′ primers: *FBP6*, 5′-CGGGATCCATCAGACAAGCTGTAGAGCAG-3′; *pMADS3*, 5′-CGGGATCCATCAGTTGATCTTGCTTCCCG-3′; and *CUM10*, 5′-CCGGATCCGTCCCCATCATTTTGGTCTCC-3′ (Isogene Science), corresponding to the cDNA sequence just downstream of the translation stop site. The 5′ primers contain an NcoI recognition site, and the 3′ primers contain a BamHI recognition site. The amplified fragments were inserted as a NcoI-BamHI fragment into the binary vector pCPO31 (Florack et al., 1994).

To facilitate cloning of the *CUM1* cDNA, restriction sites were generated by PCR. *CUM1* was amplified using the 5′ primer 5′-CTCGAGAATTTGAGATGCCATTGTAATGTCC-3′ and 3′ primer 5′-CCGGATCCAACTTCCTGTTTGAGTACCTTTC-3′. The 5′ primer corresponds to the cDNA sequence surrounding the first translation start site, which is 126 bp upstream of the MADS box. The PCR-amplified fragment was inserted as a XhoI-BamHI fragment into the binary vector pCPO31. Transformation was performed as described previously (Colombo et al., 1995).

RNA Gel Blot Analyses

Total RNA was isolated from petunia leaves or mature floral tissues, according to Verwoerd et al. (1989). Subsequently, 10 μg of glyoxal (1.5 M) denaturated total RNA was electrophorized and blotted onto Hybond N^+ membranes (Amersham). The *pMADS3*, *FBP6*, *CUM1*, and *CUM10* 3′ gene-specific fragments were labeled by random oligonucleotide priming (Feinberg and Vogelstein, 1984). Blots were hybridized as described by Angenent et al. (1992).

In Situ RNA Hybridizations

Floral buds of wild-type plants (W115) and petal tissue of T64003 were fixed and embedded in paraffin, and 10-μm sections were prepared as described by Cañas et al. (1994). Digoxigenin-labeled RNA probes were synthesized by in vitro transcription using the pSPT18/19 vectors (Boehringer Mannheim). For the synthesis of antisense RNA, 3′ terminal cDNA fragments of *pMADS3* (600 bp) and *FBP6* (660 bp) were introduced into pSPT18 or pSPT19 as BglII-XhoI and HindIII-XhoI fragments, respectively. Transcripts were partially hydrolyzed by incubation at 60°C in 0.1 M Na_2CO_3/$NaHCO_3$ buffer, pH 10.2, for 45 min. Hybridization and immunological detection were performed as described by Cañas et al. (1994).

Microscopy

For light microscopic analysis, the material was fixed, sectioned, and stained according to Angenent et al. (1993). For cryoscanning electron microscopy, samples were mounted on a stub, frozen in liquid nitrogen, coated, and observed as described by Angenent et al. (1995).

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