The Plant Cell, Vol. 5, 843–853, August 1993

Ectopic Expression of *pMADS3* in Transgenic Petunia Phenocopies the Petunia *blind* Mutant

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We cloned a MADS-box gene, *pMADS3*, from *Petunia hybrida*, which shows high sequence homology to the Arabidopsis *AGAMOUS* and Antirrhinum *PLENA*. *pMADS3* is expressed exclusively in stamens and carpels of wild-type petunia plants. In the petunia mutant *blind*, which shows homeotic conversions of corolla limbs into antheroid structures with pollen grains and small parts of sepals into carpelloid tissue, *pMADS3* is expressed in all floral organs as well as in leaves. Ectopic expression of *pMADS3* in transgenic petunia leads to phenocopies of the *blind* mutant, i.e., the formation of antheroid structures on limbs and carpelloid tissue on sepals. Transgenic tobacco plants that overexpress *pMADS3* exhibit an even more severe phenotype, with the sepals forming a carpel-like structure encasing the interior floral organs. Our results identify *BLIND* as a negative regulator of *pMADS3*, which specifies stamens and carpels during petunia flower development.

INTRODUCTION

Flowers of angiosperms are composed of four organ types (sepals, petals, stamens, and carpels) occupying four whorls, named from the outermost (the first whorl) to the innermost whorl (the fourth whorl). A model has been proposed to explain the determination of floral organ identity, based on the extensive genetic studies in Arabidopsis and Antirrhinum (Carpenter and Coen, 1990; Schwarz-Sommer et al., 1990; Bowman et al., 1991). According to the model, three gene functions, A, B, and C, each expressed in two adjacent whorls, define floral organ identity. In the first whorl, only A is active and leads to sepal formation. In the second whorl, the combination of A and B leads to petal formation, and in the third whorl, both B and C are required for stamen formation. C alone determines carpel formation in the fourth whorl. A and C are mutually antagonistic; the loss of one function will cause the expression of the other function in that particular whorl. According to this ABC model, a mutation in a floral homeotic gene should result in homeotic conversions in two adjacent whorls; for example, a mutation in an A function gene will convert sepals into carpelloid structures in the first whorl and petals into staminoid structures in the second whorl.

In Arabidopsis, A, B, and C correspond to the *APETALA2* (*AP2*) gene, a combination of the *APETALA3* (*AP3*) and the *PISTILLATA* genes, and the *AGAMOUS* (*AG*) gene, respectively; in Antirrhinum, B corresponds to a combination of the *DEFICIENS* (*DEFA*) and the *GLOBOSA* (*GLO*) genes, and C corresponds to the *PLENA* (*PLE*) gene. Among them, *AP3*, *AG*, *DEFA*, *GLO*, and *PLE* have already been cloned and characterized (Sommer et al., 1990; Yanofsky et al., 1990; Jack et al., 1992; Trobner et al., 1992; Bradley et al., 1993). A mutation on each of these genes causes homeotic conversions in two adjacent whorls where it is expressed. Sequence analysis showed that the products of all these genes share a conserved region designated as the MADS box (MCM1, AG, DEFA, and SRF) (Schwarz-Sommer et al., 1990), which is homologous to the DNA binding domain of known transcription factors MCM1 (yeast) and SRF (human) (Dubois et al., 1987; Norman et al., 1988). Indeed, the DEFA and GLO proteins have been shown to bind to specific DNA motifs as a heterodimer by in vitro DNA binding studies (Trobner et al., 1992). These results suggest that floral MADS-box gene products likely act as transcription factors that regulate genes involved in floral organ development.

Petunia hybrida is an important horticultural plant derived from several different ancestral *Petunia* species (Sink, 1984). This plant, which has large flowers, has long served as a model system for investigations on anthocyanin biosynthesis; yet, little is known about the genes that control petunia floral organ identity (de Vlaming et al., 1984). Figure 1 shows schematic diagrams of a petunia wild-type flower. A petunia floral homeotic mutant *green petal* (*gp*) shows a homeotic conversion of petals into sepals, but does not show any conversions of stamens into carpels, unlike Antirrhinum *defA* or Arabidopsis *ap3* mutants. We have recently shown that the *gp* mutant (line PLv) is a null mutant of *pMADS1*, a petunia MADS-box gene that shares sequence homology with the Antirrhinum *DEFA* gene (Kush et al., 1993; van der Krol et al., 1993). The phenotype conferred by *gp* can be restored to wild type by expression of a cauliflower mosaic virus (CaMV) 35S *pMADS1* transgene (van der Krol et al., 1993). *pMADS1* is therefore encoded by

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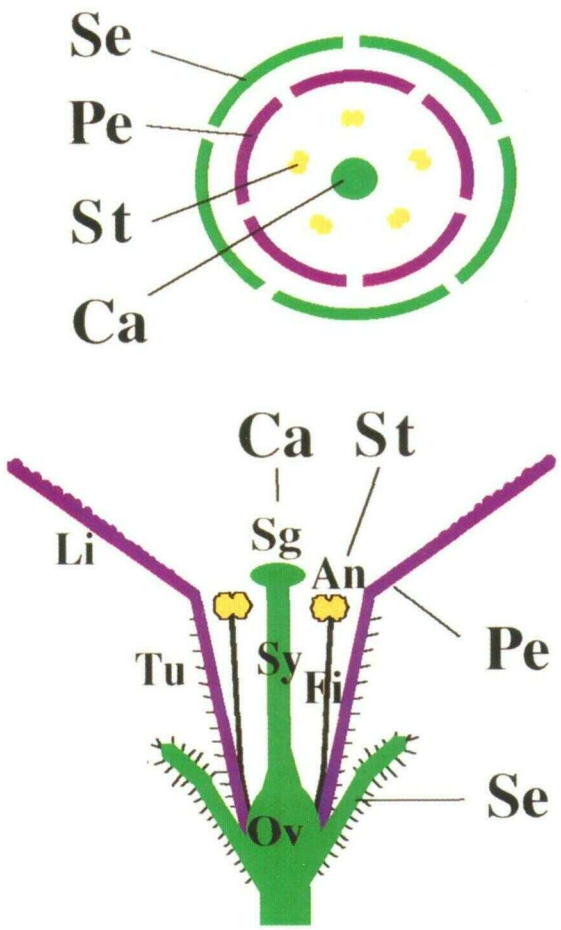


Figure 1. Schematic Diagrams of the *Petunia hybrida* Wild-Type Flower.

Top: A floral diagram showing five sepals, five petals, five stamens, and a bilobed carpel.

Bottom: A longitudinal section of a mature flower. Petals can be divided into corolla limbs and the basal tube. Stamens consist of anthers and filaments fused to the tube at the base. The carpel is composed of the stigma, the style, and the ovary.

Se, sepal; Pe, petal; St, stamen; Ca, carpel; Li, limb; Tu, tube; An, anther; Fi, filament; Sg, stigma; Sy, style; Ov, ovary.

GP, and, accordingly, it will be referred to as *GP* henceforth. Furthermore, ectopic expression of *GP* fused to a CaMV 35S promoter in wild-type plants results in the partial conversion of sepals into petaloid organs, without any conversion of carpels into staminoid organs (U. Halfter, N. Ali, L. Ren, A. Kush, and N.-H. Chua, manuscript submitted). These results obtained with the *GP* gene are not consistent with the model for flower development described above and suggest that the detailed mechanism for floral organ development varies among different plant species.

In this paper, we describe the isolation and characterization of another petunia MADS-box gene, *pMADS3*, which is expressed mainly in stamens and carpels (van der Krol et al., 1993). The petunia *blind* (*bl*) mutant has been previously reported to show a homeotic conversion of corolla limbs into antheroid structures in the second whorl (Vallade et al., 1987) and has long been considered as a homeotic mutant with alterations only in one whorl (de Vlaming et al., 1984; Angenent et al., 1992). We present here that the *bl* mutant, in fact, also shows a homeotic conversion in the first whorl, i.e., sepal apices converted into stigmatoid tissue. The expression of *pMADS3* was observed in all floral organs as well as in leaves of the mutant. The *bl* phenotype can be recapitulated by the ectopic expression of a 35S-*pMADS3* transgene in transgenic petunia. The phenotype of sepals is more prominent in transgenic tobacco plants expressing the same transgene. Our results indicate that *pMADS3* is involved in the development of the third and fourth whorls in petunia flowers and that *BL* is a negative regulator of *pMADS3*. In contrast to *GP*, *pMADS3* and *BL* are likely to have similar functions as C and A function genes of Arabidopsis or Antirrhinum.

RESULTS

Isolation and Sequence Analysis of *pMADS3* cDNA

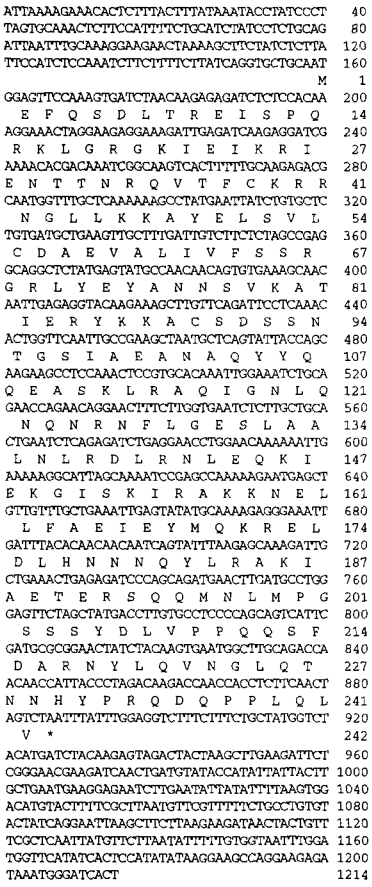
To isolate petunia genes with high sequence homology to the Arabidopsis *AG* (Yanofsky et al., 1990), we screened a petunia floral cDNA library using a polymerase chain reaction probe encompassing the Arabidopsis *AG* coding region. Five independent clones were isolated and sequenced. Sequence analysis showed that they were derived from two different genes, which were designated as *pMADS3* and *pMADS4*. In this paper, we describe the expression pattern and functions of *pMADS3*. The characterization of *pMADS4*, which shows sequence homology to *AGL6* (Ma et al., 1991), will be published elsewhere (S. Tsuchimoto, manuscript in preparation).

Figure 2A shows the nucleotide sequence and deduced amino acid sequence of *pMADS3*. The open reading frame is 242 amino acid residues long and encodes a protein with a molecular weight of 27,900. As shown in Figure 2B, the putative pMADS3 protein shares identical residues with the Arabidopsis AG protein (Yanofsky et al., 1990), the Brassica BAG1 protein (Mandel et al., 1992), and the Antirrhinum PLE protein (Bradley et al., 1993) within the MADS-box region (Ma et al., 1991). Outside of the MADS-box region, the homology between pMADS3 and these three gene products is about 57 to 61%. These results suggest that *pMADS3* may be a homolog of *AG* and *PLE*.

pMADS3 Expression in Wild Type and the *blind* Mutant

To study the function of *pMADS3*, we isolated RNA from each of the floral organs of wild-type petunia plants, shown in Figure 3A, and performed an RNA hybridization experiment with

A



B

Figure 2. Nucleotide and Amino Acid Sequences of *pMADS3* cDNA and Sequence Comparison with Other Floral MADS-Box Genes.

a *pMADS3*-specific probe (see Methods). As a positive control, a probe for the β ATPase gene (Boutry and Chua, 1985), a housekeeping gene, was also used for hybridization to the same blot. Figure 4A shows that *pMADS3* transcript was present in stamens and carpels but not in sepals, the corolla tube, or limbs, confirming previous results that *pMADS3* is exclusively expressed in the third and fourth whorls of wild-type flowers (van der Krol et al., 1993). Moreover, in these two whorls, *pMADS3* expression was detected in anthers, filaments, upper part of carpels (the stigma and the style), and ovary (Figure 4B), suggesting that *pMADS3* may be involved in the development of these organs.

We have previously shown that the expression pattern of *pMADS3* is not altered in the petunia floral homeotic mutant *gp* (van der Krol et al., 1993). To investigate the involvement of *pMADS3* in floral organ identity, we determined the expression pattern of this gene in another petunia homeotic mutant, *bl* (de Vlaming et al., 1984). Figure 3B shows that flowers of the *bl* mutant lack corolla limbs, and instead antheroid structures develop on the top of the tube, which contain fertile pollen grains (Vallade et al., 1987; see Figure 6C). Some plants show a leaky phenotype in which the petal tissue of the limbs also develop together with the antheroid structures, as shown in Figure 3C. We found that in flowers with a severe phenotype, the sepals curl up at their apices (Figure 3B), which contain stigmatoid tissue (Figure 3D) with papillae (Figure 3E). Figures 3F and 3G compare longitudinal sections of the stigmatoid tissue on a *bl* sepal and the wild-type stigmatic tissue. The tube, stamens, and carpels are not affected by the mutation, and the stamen filaments are fused to the tube, as occurs in the wild type (data not shown). Leaves are not affected in the *bl* mutant line Sb1. However, when the *bl* mutant was crossed to *P. violecea* (line S10), one of the presumed ancestral species of *P. hybrida* (Sink, 1984), F_2 plants with the *bl* phenotype showed malformed leaves with glossy surfaces and curled-up edges, as illustrated in Figure 3H. The leaf phenotypes were more severe in upper leaves of flowering branches than in lower leaves of vegetative branches.

We analyzed whether the expression pattern of *pMADS3* in *bl* differs from that of the wild type; the expression of the *GP* gene was also analyzed for comparison. The β ATPase gene was used as a positive control. The top panel of Figure

(A) cDNA and deduced amino acid sequences of *pMADS3*. Numbers to the right of the cDNA sequence indicate the positions from the 5′ end of the longest cDNA, and those to the right of the amino acid sequence indicate the positions from the putative translational start site.
(B) An asterisk indicates an identical residue with pMADS3, and a hyphen represents a gap introduced to optimize alignment. Numbers indicate the position of the amino acids in pMADS3 from the putative translational start site. The MADS-box region of pMADS3 is underlined. The nucleotide sequence data of *pMADS3* is available in EMBL, GenBank, and DDBJ nucleotide sequence data bases as accession number X72912.

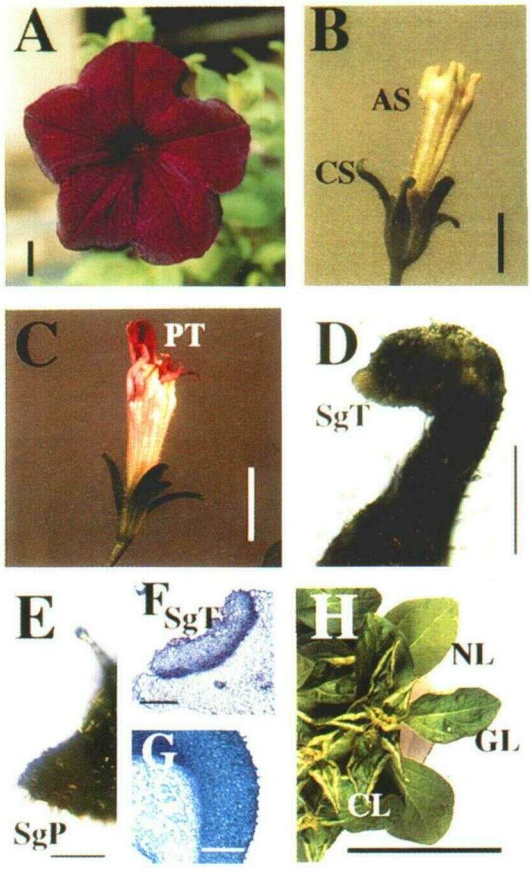


Figure 3. Wild-Type and *bl* Flowers.

(A) A mature wild-type petunia (V26) flower.
(B) A mature *bl* flower with antheroid structures and rolled-up sepals.
(C) A mature *bl* flower with a leaky phenotype.
(D) A curled-up apex of a *bl* sepal with stigmatoid tissue.
(E) A higher magnification of stigmatoid tissue of a *bl* sepal showing stigmatic papillae.
(F) A longitudinal section of stigmatoid tissue of a *bl* mutant.
(G) A longitudinal section of stigmatic tissue of a wild-type (V26) carpel.
(H) Leaves of an F_2 hybrid plant in a cross between the petunia *bl* mutant (line Sb1) and *Petunia violecea* (line S10); the *bl* phenotypes in flowers are shown.
AS, antheroid structure; CS, curled-up apex of sepal; PT, petal tissue; SgT, stigmatoid tissue; SgP, stigmatic papillae; NL, normal leaf; GL, glossy leaf; CL, curled-up glossy leaf. Thick horizontal bar = 10 cm **(H)**; thick vertical bars = 1 cm **(A)**, **(B)**, and **(C)**; thin vertical bar = 1 mm **(D)**; thin horizontal bars = 0.1 mm **(E)**, **(F)**, and **(G)**.

4C shows that in contrast to the wild type, *pMADS3* transcript was detected in the first and second whorls of *bl* flowers. In the second whorl, the expression was higher in the antheroid structures compared to the tube. On the other hand, the middle panel of Figure 4C shows that *GP* is expressed only in the second and third whorls of *bl* flowers, which is identical to the expression pattern in wild-type flowers (van der Krol et al., 1993). Interestingly, we also detected *pMADS3* expression in leaves (Figure 4C). We collected leaves from flowering branches (inflorescence leaves) as well as leaves from vegetative branches (vegetative leaves) of the same *bl* or wild-type plant, and the samples were analyzed separately. The *pMADS3*

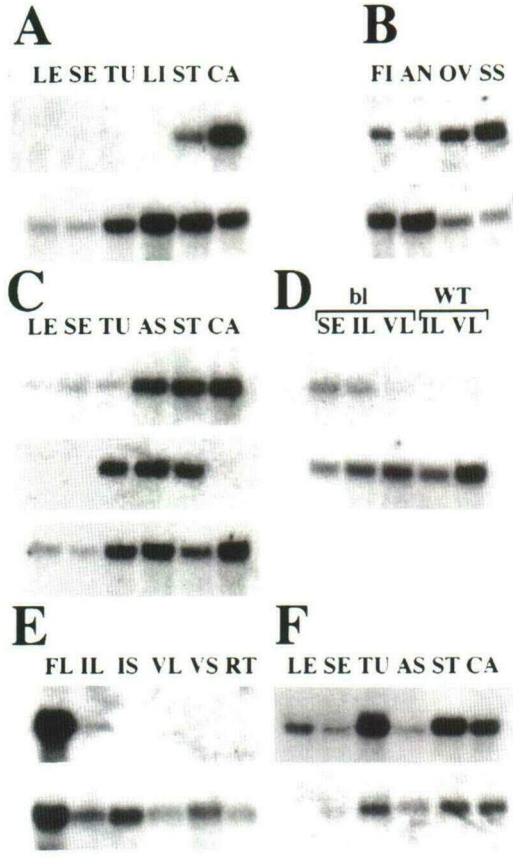


Figure 4. Expression of *pMADS3* and *GP* in Wild-Type (V26), *bl* (line Sb1), and Transgenic Plants.

RNA gel blots with a *pMADS3*-specific probe and a *GP*-specific probe are shown. Equal amounts [15 μg for **(A)** to **(D)**, 20 μg for **(E)**, and 10 μg for **(F)** and **(G)**] of total RNA were analyzed on each blot. A β ATPase-specific probe was used as a positive control [**(A)** to **(E)**].
(A) and **(B)** Expression of *pMADS3* (upper panel) and β ATPase (lower panel) in wild-type plants.
(C) Expression of *pMADS3* (top panel), *GP* (middle panel), and β ATPase (bottom panel) in *bl* plants.
(D) Expression of *pMADS3* (upper panel) and β ATPase (lower panel) in green tissue of *bl* and wild-type plants.
(E) Expression of *pMADS3* (upper panel) and β ATPase (lower panel) in *bl* plants.
(F) Expression of *pMADS3* in transgenic petunia plants DMP5 (upper panel) and DMP12 (lower panel).
LE, leaf; SE, sepal; TU, tube; LI, limb; ST, stamen; CA, carpel; FI, filament; AN, anther; OV, ovary; SS, stigma and style; AS, antheroid structure; FL, flower; IL, inflorescence leaf; VL, vegetative leaf; IS, inflorescence stem; VS, vegetative stem; RT, root; WT, wild type.

expression was detected in inflorescence leaves but was very slightly or not detectable in vegetative leaves of the *bl* mutant, whereas it was not detected at all in either type of leaves of the wild type (Figure 4D). The difference in the expression level between inflorescence leaves and vegetative leaves may correlate with the difference in the severity of the leaf phenotype described above (Figure 3H). The *pMADS3* expression was not detected either in stems or roots of the *bl* mutant, as shown in Figure 4E.

These results indicate that *BL* encodes a specific negative regulator of *pMADS3*, which is likely to be expressed in the first and second whorls of the flower as well as in leaves, especially those on inflorescences. The absence of the *BL* gene product allows the ectopic expression of *pMADS3* in the tissue where this gene is normally not expressed. The expression of *pMADS3* in the first and second floral whorls may indicate its involvement in the homeotic conversions of sepals into stigmatoid tissue and of limbs into antheroid structures, and the expression of *pMADS3* may implicate it in the morphological alternation in leaves in the genetic background of *P. violecea.*

Ectopic Expression of *pMADS3* in Petunia Plants

If the ectopic expression of *pMADS3* is indeed responsible for the conversions of sepals into stigmatoid tissue and of corolla limbs into antheroid structures, it should be possible to recapitulate this phenotype in wild-type transgenic plants by overexpression of *pMADS3.* To investigate this point, we transformed *P. hybrida* (V26) plants with a modified CaMV 35S-*pMADS3* chimeric gene (see Methods). Eight of 14 independent transgenic plants showed the *bl* phenotype in the second whorl, i.e., antheroid structures at the position of limbs as shown in Figure 5A. The carpels, stamens, and the tube were not morphologically affected, and the number of organs remained the same. Figure 5B shows the stamen filaments fused to the base of the tube, as occurred in the wild type or the *bl* mutant. The petal tissue of the limbs developed together with the antheroid structures, similar to the leaky phenotype of the *bl* mutant. We selected two transgenic lines, DMP5 and DMP12, with severe phenotypes for further analyses.

The relative positions between the antheroid structures and the limbs varied among different transgenic lines. In DMP5 (Figure 5C left; also see Figures 5D to 5F), the antheroid structures developed along the edges of the limbs, which made the latter separate from one another. In DMP12 (Figure 5C right; also see Figures 5G to 5I), the antheroid structures developed between the edges of the limbs and the main vein, thus allowing the limbs to fuse.

The severity of the conversion of the limbs into antheroid structures varied even within a single transgenic plant. In DMP12, the limbs of some flowers were almost completely converted into antheroid structures and only the main veins of the limbs remained at the top of the antheroid structures, as shown in Figures 5A and 5G. This is the most severe phenotype observed among all of the transgenic plants. By contrast, in other flowers of the same plant, the antheroid structures were found as small patches of thickened white tissue on the limbs (Figure 5I). Figure 5H shows flowers with intermediate phenotypes that were also observed on the same plant. Similar morphological variations were observed in flowers of line DMP5 (Figures 5D to 5F). It has been reported that transgenic Arabidopsis plants ectopically expressing *AG* (Mizukami and Ma, 1992) and transgenic tobacco plants expressing *BAG1* (Mandel et al., 1992) also exhibited variable phenotypes within the same line.

Figures 6A and 6B show transverse sections of antheroid structures on the limbs of transgenic plants. Figure 6A shows the most severe phenotype flower of DMP5, and Figure 6B shows an intermediate phenotype flower of DMP12. There were pollen grains in the antheroid structures of both plants. The petal tissue with the characteristic epidermal cell layer remained in the middle of the limb surrounding the main vein. DMP12 also had the petal tissue between antheroid limbs, allowing the latter to fuse to each other (Figure 6B). Figure 6C shows the antheroid structures of the *bl* mutant. There were pollen grains inside the antheroid structures, and petal tissue also remained in the middle of the limbs. Figure 6D shows the limbs of a wild-type flower for comparison.

Flowers with severe or intermediate phenotypes in DMP5, as well as flowers with severe phenotypes in DMP12, possessed sepals that were curled-up along their edges. Figure 7A shows that the tissue appeared less green and contained a smaller number of trichomes along the edges. The pale-colored tissue sometimes formed extra curly filamentous structures that had stigmatoid tissue with papillae on the top (Figures 7C and 7D). The surface of other parts of the curly structures consisted of elongated cells like those of the wild-type style (Figure 7E). Note that the position of the pale-colored tissue on the sepals of DMP5 corresponds to the position of antheroid structures on the limbs of the same plant (Figures 5C, 5E, and 5F). DMP5 but not DMP12 sometimes had curled leaves with glossy surfaces, which are similar to those observed in the *bl* mutant (Figure 3H). Figures 7B and 7G show sepals and leaves, respectively, of the wild-type plant.

Although all of the transgenic plants produced pollen grains in their anthers, they were male sterile. Seeds were obtained when pollen of the wild-type plant was used for fertilization, but their germination rate was less than 10% of that of wild-type seeds. The phenotype of the transgenic plant was inherited to the F_1 progeny.

The *pMADS3* gene was expressed in all floral organs of lines DMP5 and DMP12, as shown in Figure 4F. In both plants, the expression level in the tube was even higher than that in the limbs with antheroid structures, although no morphological changes were observed on the tube as mentioned above. *pMADS3* expression was detected also in the leaves of DMP5, which were curled along the edges.

Our results showed that ectopic expression of the *pMADS3* gene can lead to the homeotic conversion of corolla limbs into antheroid structures with pollen grains, which is the phenotype exhibited by the *bl* mutant. In addition, we also observed

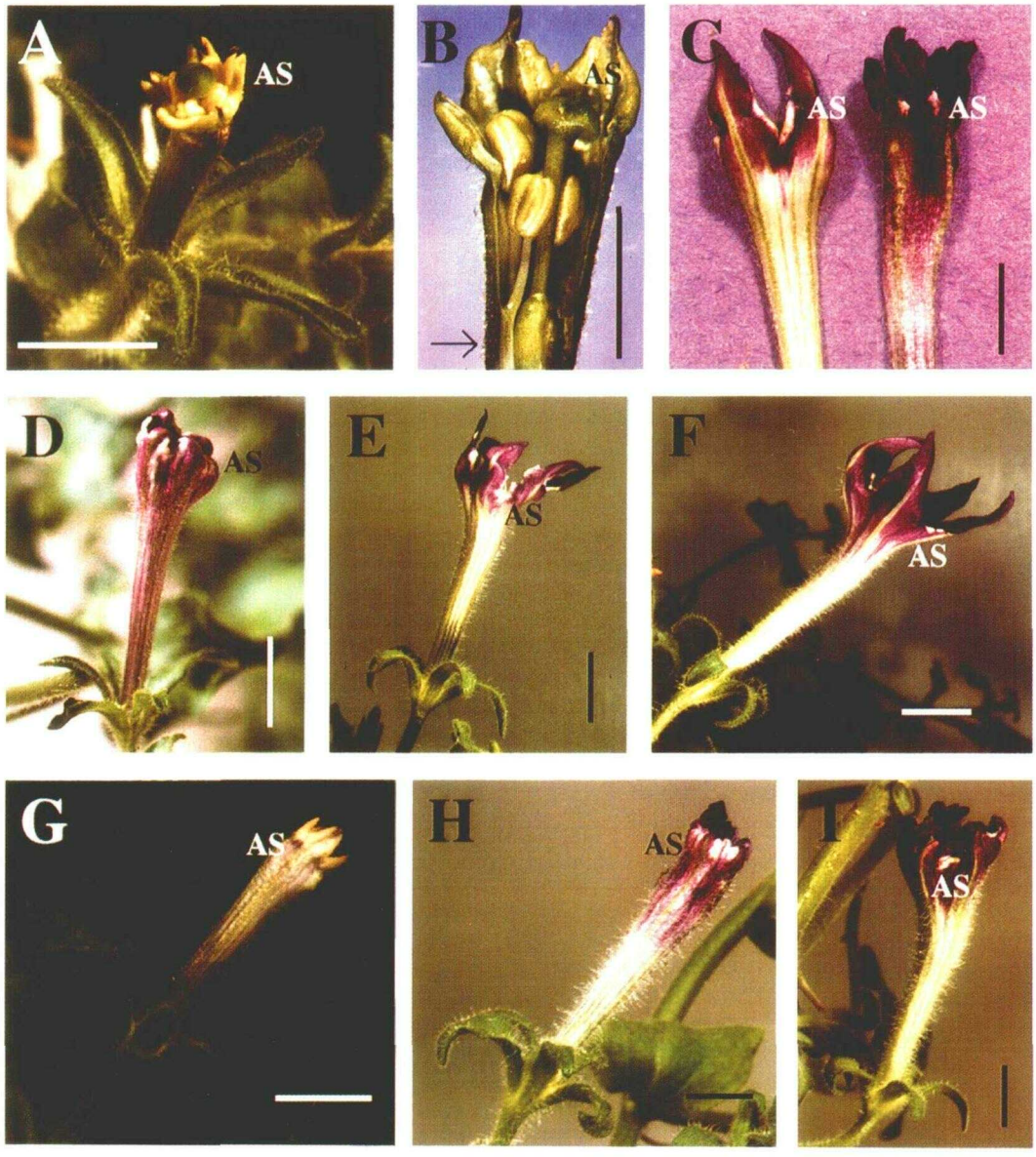


Figure 5. Phenotypic Analyses of pMADS3 Transgenic Petunia Flowers.

(A) A young (2.5-cm-long) DMP12 flower with a severe phenotype.
(B) Inside of a young (2-cm-long) DMP5 flower bud with a severe phenotype. Arrow points to the fusion of the filament to the tube.
(C) Mature petals of DMP5 (left) and DMP12 (right) flowers.
(D) to **(F)** Mature DMP5 flowers with a severe **(D)**, intermediate **(E)**, and weak **(F)** phenotype.
(G) to **(I)** Mature DMP12 flowers with a severe **(G)**, intermediate **(H)**, and weak **(I)** phenotype.
AS, antheroid structure. Bars = 1 cm.

extra carpelloid structures along the edge of the sepals in transgenic plants; these structures were similar to those seen at the apices of the *bl* sepals. These results support the notion that ectopic expression of *pMADS3* is involved in the homeotic conversions in the *bl* mutant and indicate that *pMADS3* is a homeotic gene involved at least in the development of anther, stigma, and style. We also observed morphological changes similar to the *bl* mutant in leaves of transgenic line DMP5. This

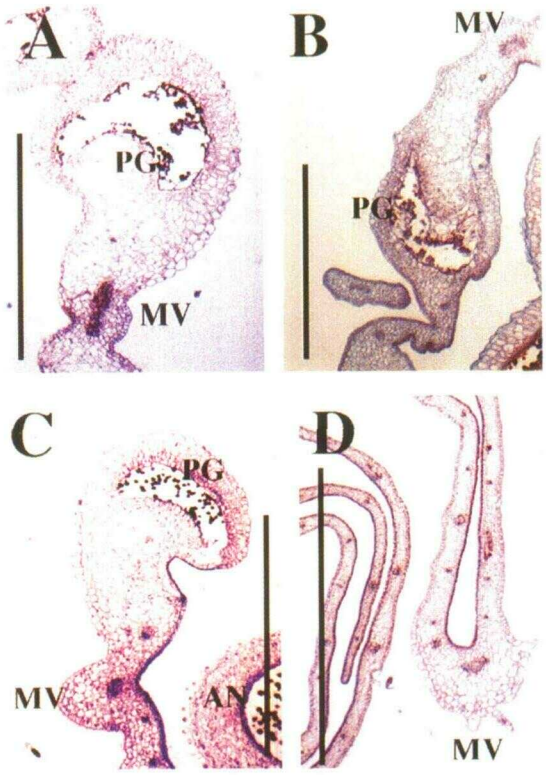


Figure 6. Transverse Petal Sections of Young Floral Buds.

Transverse sections of the upper part of petals that show antheroid structures. The wild type is shown in **(D)** for comparison.
(A) DMP5.
(B) DMP12.
(C) *bl* mutant.
(D) Wild type.
PG, pollen grain; MV, main vein; AN, anther. Bars = 1 mm.

suggests that the expression of *pMADS3* in leaves may lead to the abnormal leaf development, but the significance of this expression remains to be clarified.

Ectopic Expression of *pMADS3* in Tobacco Plants

We generated transgenic tobacco plants carrying the same chimeric modified 35S-*pMADS3* gene that was used to transform petunia plants, which are described above, to see the effect of the petunia *pMADS3* gene in a different species. Figure 8A shows the flower of wild-type tobacco that is similar to the wild-type petunia flower (Figure 1), consisting of five sepals, five petals, five stamens, and a bilobed carpel. The bases of the petals are fused with one another to form a tube, and stamen filaments are fused to the lower part of the latter. In contrast to petunia, tobacco sepals are fused to a greater extent.

As illustrated in Figures 8B and 8C, three of 12 transgenic tobacco plants showed completely fused sepals with stigmatoid structures on the apices. Figures 8E and 8F compare the stigmatoid structures and the wild-type stigma. Tobacco line DMT4 showed the most severe phenotype (Figure 8B); the upper parts of sepals just below the stigmatoid structure were fused together, assuming a stylelike shape. These structures impeded the growth of the second, third, and fourth whorl floral organs, causing them to be morphologically distorted and to senesce prematurely. The growth of the interior organs,

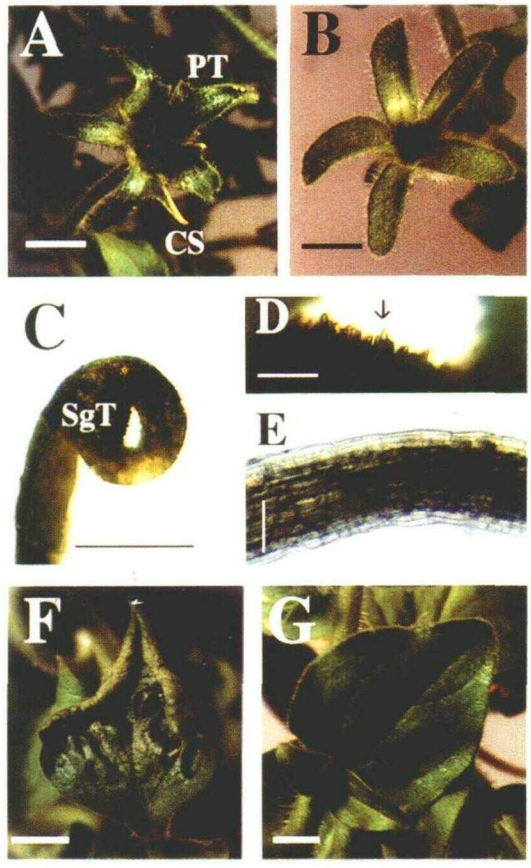


Figure 7. Phenotypic Analyses of Sepals and Leaves of Transgenic Petunia Overexpressing pMADS3.

(A) Rolled-up DMP5 sepals with curly filamentous structures.
(B) Wild-type (V26) sepals.
(C) A curly filamentous structure with stigmatoid tissue on the apex.
(D) A higher magnification of stigmatoid tissue in **(C)** showing the stigmatic papillae (arrow).
(E) A curly filamentous structure showing stylelike epidermal cells.
(F) Curled-up DMP5 leaf.
(G) Wild-type (V26) leaf.
PT, pale-colored tissue; CS, curly structure; SgT, stigmatoid tissue. Thick bars = 1 cm **(A)**, **(B)**, **(F)**, and **(G)**; thin black bar = 1 mm **(C)**; thin white bars = 0.1 mm **(D)** and **(E)**.

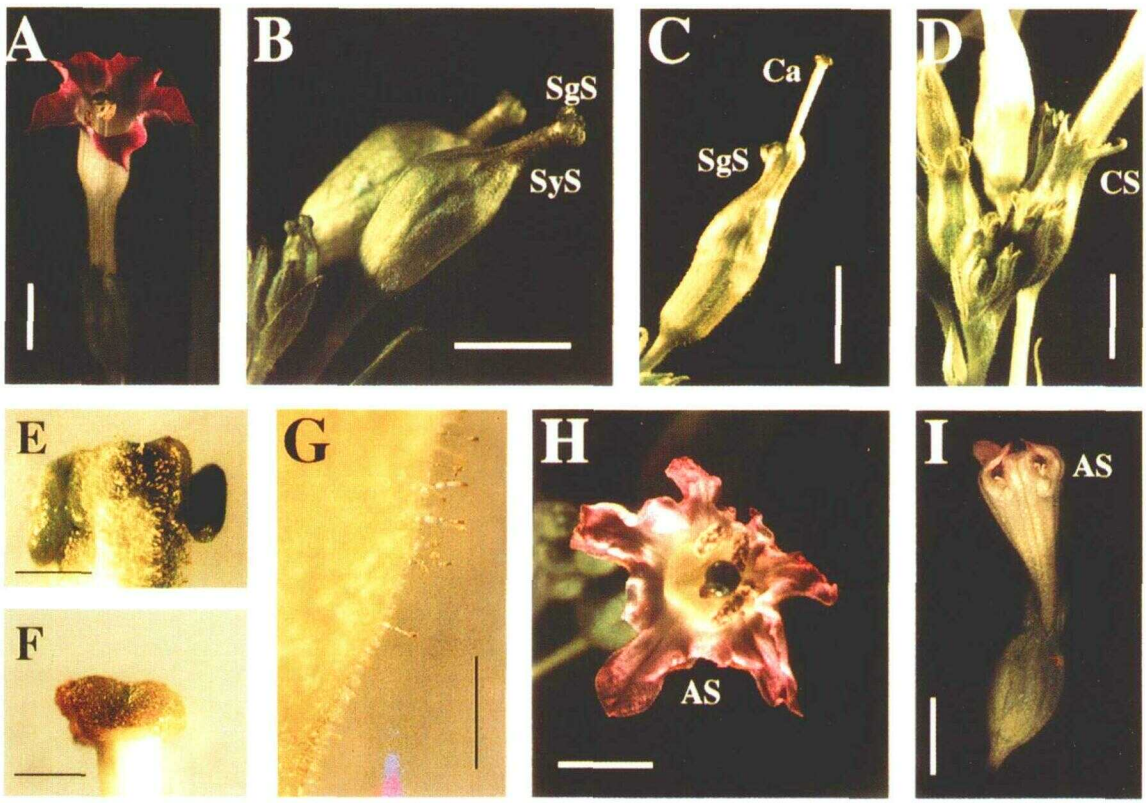


Figure 8. Phenotypic Analyses of Mature Flowers of Transgenic Tobacco Overexpressing *pMADS3*.

(A) Wild-type (SR1) flower.
(B) Two DMT4 flowers showing only carpelloid sepals covering the interior organs.
(C) A DMT1 flower showing carpelloid sepals and carpel.
(D) DMT1 flowers with a weak phenotype showing curly structures arising from sepals.
(E) Stigmatoid tissue on the apices of DMT4 sepals.
(F) Stigmatic tissue of wild-type carpel.
(G) Lower part of DMT4 sepals with trichomes.
(H) A DMT5 flower, top view.
(I) A DMT4 flower that developed after the encasing sepals were cut open.
SgS, stigmatoid structure; SyS, stylelike structure; Ca, carpel; CS, curly structure; AS, antheroid structure. Thick white bars = 1 cm **(A)** to **(D)**, **(H)** and **(I)**; thin black bars = 1 mm **(E)** to **(G)**.

however, could be restored by cutting open the fused sepals of young floral buds. DMT1 showed a weaker floral phenotype (Figure 8C). Its stigmatoid structures were sometimes altered to curly filamentous structures that are similar to those found in the transgenic petunia plant DMP5 (Figure 8D). This suggests that the curly structures of petunia are malformed stigmatoid structures. The other transgenic tobacco plant (line DMT5) with stigmatoid structures showed the weakest phenotype among the three transgenic lines (data not shown). The lower parts of the fused sepals in all transgenic lines did not show any carpelloid features, such as orange colored tissue or placental tissue, and they were covered with trichomes (Figure 8G). Six transgenic plants, including DMT1, DMT4, and DMT5, had antheroid structures along the edges of the limbs, but no morphological alterations were detected in the tube (Figures 8H and 8I). All of the transgenic tobacco lines were fertile, in contrast to the transgenic petunia plants that were male sterile.

Figures 9A, 9B, 9D, and 9E show transverse sections of the fused sepals of line DMT4. The stigmatoid structures on the apices were chimeric organs composed of the sepaloid, stigmatoid, and stylelike tissue (Figures 9A and 9B). The wild-type stigma and style are shown in Figure 9C for comparison. In the stylelike structure (Figure 9D), the fused sepals formed a ring-shaped organ with the stigmatoid tissue fused on the inside surface like the wild-type style (Figure 9C). The lower

part of the sepals was devoid of the carpelloid tissue (Figure 9E). Figure 9F shows the antheroid structure of the transgenic tobacco (DMT1), which contained pollen grains, like those of the transgenic petunia. The petal tissue was also observed in the middle of the limb.

These results showed that the ectopic expression of the petunia *pMADS3* gene in tobacco can lead to a homeotic conversion of corolla limbs into antheroid structures with pollen grains and the formation of stigmatoid and stylelike structures on the apices of fused sepals. The formation of carpelloid structures in the first whorl and antheroid structures in the second whorl indicates that petunia *pMADS3* functions in tobacco in the specification of stamens and carpels, as occurs in petunia.

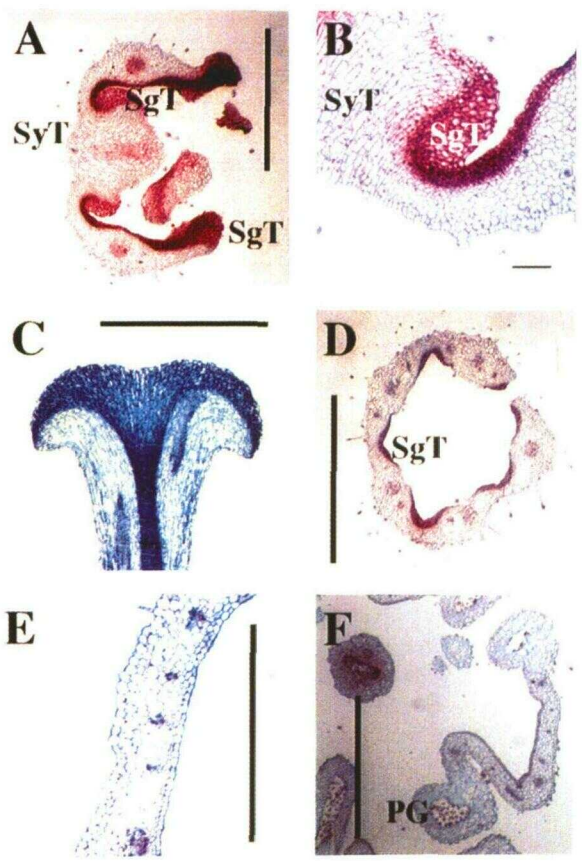


Figure 9. Tissue Sections of Tobacco Wild-Type (SR1) and Transgenic Flowers.

All tissues were collected from young (2-cm-long) flower buds.
(A) A transverse section of stigmatoid structures on the apices of DMT4 sepals.
(B) Higher magnification of **(A)**.
(C) A longitudinal section of wild-type carpel.
(D) A transverse section of stylelike structure on DMT4 sepals.
(E) A transverse section of the lower part of DMT4 sepals.
(F) A transverse section of DMT1 petal.
SgT, stigmatoid tissue; SyT, stylelike tissue; PG, pollen grain. Thick bars = 1 mm; thin bar = 0.1 mm.

DISCUSSION

pMADS3 Is Involved in Stamen and Carpel Development

In this work we have isolated the *pMADS3* cDNA by cross-hybridization to an *Arabidopsis AG* cDNA fragment containing the MADS-box region. Therefore, it is not surprising that the amino acid sequence of the MADS-box region is identical between the two proteins. Based on three lines of evidence, we concluded that, like *AG* or Antirrhinum *PLE*, *pMADS3* is also involved in the determination of stamens and carpels: (1) *pMADS3* is expressed exclusively in stamens and carpels of wild-type flowers (Figure 4A; Yanofsky et al., 1990; Bradley et al., 1993); (2) in the *bl* mutant, the homeotic conversions of corolla limbs to antheroid structures and small parts of sepals into carpelloid tissue are correlated with the ectopic expression of *pMADS3* in the first and second whorls (Figure 4C); and (3) overexpression of a modified 35S-*pMADS3* transgene in wild-type petunia flowers results in phenocopies of *bl*. Similar results have been recently reported by Mizukami and Ma (1992). They showed that the ectopic expression of a CaMV 35S-*AG* transgene in Arabidopsis can lead to altered whorl identity similar to that seen with the *ap2* mutant (Bowman et al., 1989, 1991). Although the petunia *GP* gene does not seem to fit to the ABC floral development model (see Introduction), functions of the *pMADS3* gene are similar to those of the C function gene as described in the model in terms of determination of floral organs in the third and fourth whorls. However, there are also some minor differences between phenotypes caused by the ectopic expression of *pMADS3* and of the C function genes of *Arabidopsis* (Bowman et al., 1991; Mizukami and Ma, 1992), Antirrhinum (Carpenter and Coen, 1990; Bradley et al., 1993), and Brassica (Mandel et al., 1992).

In wild-type petunia flowers, *pMADS3* is expressed in anthers, filaments, the upper part of carpels (the stigma and style), and ovary. Although the expression profile suggests that *pMADS3* is involved in the development of all parts of the stamens and carpels, it is not clear whether it is involved in the development of the filaments and the ovary because the expression of *pMADS3* in the first and second whorls of the *bl* mutant and transgenic plants did not lead to the formation of these organs, in contrast to Arabidopsis *AG* or Antirrhinum *PLE*. There are three possible explanations for these phenotypes: (1) the *pMADS3* gene is not involved in the development of these organs; (2) *pMADS3* alone is not sufficient for the development of the filaments or the ovary; and (3) the level of *pMADS3* expression is not high enough. In the case of the first two explanations, there might be another homeotic gene that is necessary for the development of whole stamens and carpels.

Petunia *pMADS3* appears to have a similar function in tobacco because ectopic expression of *pMADS3* in the latter leads to the conversion of corolla limbs to antheroid structures, as well as the fusion of sepals to form stylelike structures with stigmatoid tissue on the apex (Figure 8). Mandel et al. (1992) have recently reported that the expression of Brassica *BAG1*, a presumptive *AG* homolog, in tobacco can convert sepals and petals into carpelloid and staminoid structures, respectively. Although both petunia *pMADS3* and Brassica *BAG1* can carry out C function in tobacco, there are some differences in the phenotypes of the transgenic tobacco plants: (1) *pMADS3* can promote the production of pollen grains in the antheroid structures, but *BAG1* cannot; (2) *BAG1*, but not *pMADS3*, can convert the tube into filaments and the lower sepals into ovary; and (3) *pMADS3* transgenic plants are fertile, whereas *BAG1* transgenic plants are male sterile. One possibility is that tobacco might have two genes, one homologous to *pMADS3* and the other to *BAG1*, and the two genes perform slightly different functions, as we assumed in petunia. On the other hand, the phenotypic differences between the two groups of transgenic plants could simply be due to the use of different promoters, i.e., modified CaMV-35S *versus* CaMV-35S.

The *BL* Gene Product Is a Negative Regulator of *pMADS3*

Although it has been supposed that the *bl* mutant is a single-whorl floral homeotic mutant affected only in the second whorl (de Vlaming et al., 1984; Angenent et al., 1992), our detailed analysis showed that the mutation also leads to the homeotic conversion in the first whorl. Because the loss of its function results in the expanded expression of *pMADS3* in sepals, petals, and leaves, it is reasonable to conclude that the *BL* gene product is a negative regulator of *pMADS3*, and, consequently, the *BL* gene product itself must be expressed in these organs. Although it has been recently reported that the *Ovulata* mutant of Antirrhinum, a semidominant mutant that has similar phenotypes as *bl*, turned out to be a gain-of-function mutant of the *PLE* gene (Bradley et al., 1993), *bl* may not be a mutation in the *pMADS3* gene because it is a recessive mutation (de Vlaming et al., 1984). We note that the profile of *BL* as a negative regulator of *pMADS3* is similar to that of the Arabidopsis *AP2* gene as a negative regulator of *AG* (Drews et al., 1991). Therefore, it is likely that *BL* is a homolog of *AP2*, an A function gene of Arabidopsis. A weak allele of the *ap2* mutant has stigmatoid tissue on the apices of sepals like the *bl* mutant (Bowman et al., 1989). This suggests that *bl* might be a weak allele. On the other hand, because the corolla tube remains intact even when the limbs are completely converted to antheroid structures in *bl*, the *BL* gene product could be a multidomain protein with each domain being responsible for a particular function; alternatively, there might be an additional homeotic gene that is responsible for the development of the tube. This issue could be resolved by the isolation and characterization of additional alleles, particularly a null allele, in the *BL* gene.

METHODS

Plant Material and Transformations

Petunia and tobacco plants were grown under standard greenhouse conditions. The *pMADS3* cDNA was isolated from *Petunia hybrida* line W115. *P. hybrida blind* (*bl*) mutant line Sb1 is a spontaneous mutant and was kindly provided by E. Farcy (INRA, Dijon, France). *P. violecea* (line S10) was kindly provided by R. Koes (Free University, Amsterdam, The Netherlands) and was used in a cross with the *bl* mutant line Sb1. *P. hybrida* line V26 and *Nicotiana tabacum* cv SR1 were also used. Plant transformations were performed, as described by Horsch et al. (1985), using leaf discs from petunia V26 or tobacco SR1.

DNA Cloning Strategies

The *pMADS3* cDNAs were isolated from a petunia cDNA library prepared from floral bud RNA using a 720-bp polymerase chain reaction probe containing the Arabidopsis *AGAMOUS* (*AG*) coding region (from the MADS box to the C terminus). The modified cauliflower mosaic virus (CaMV) 35S-*pMADS3* construct was made by cloning the *pMADS3* cDNA fragment into a vector derived from pMON721 that contained a modified CaMV 35S promoter and a poly(A) addition signal (van der Krol et al., 1993).

RNA Gel Blot Analysis

Floral buds longer than 1 cm were dissected and used for total RNA isolation by the RNaid procedure (BIO 101, La Jolla, CA). Equal amounts of total RNA (20, 15, or 10 μg) were fractionated on 1.2% agarose gels containing 1 M formaldehyde. Gels were blotted onto GeneScreen plus membranes (Du Pont) according to the manufacturer's instructions and hybridized to a random primer-labeled DNA probe in 20% formamide, 5 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate), 1% SDS, 5 × Denhardt's (1 × Denhardt's solution is 0.02% Ficoll, 0.02% PVP, 0.02% BSA), and 10 μg/mL of salmon sperm DNA at 42°C. Blots were washed in 2 × SSC, 1% SDS at 65°C. cDNA fragments without the MADS-box region of the *pMADS3* gene (a 0.7-kb BglII-SalI fragment) and the *GREEN PETAL* (*GP*) gene (a 0.7-kb EcoRI fragment) were labeled and used as gene-specific probes. A cDNA fragment encoding the *Nicotiana plumbaginifolia* β ATPase (Boutry and Chua, 1985) was used as a positive control.

Phenotypic Analysis and Imaging

Flower close-ups were photographed under a stereomicroscope (model SMA-U; Nikon Inc., Melville, NY). Microscopic sections were prepared and stained as described by Natarella and Sink (1971) and photographed in bright field under the same microscope. All images were processed by the Adobe Photoshop program (Adobe Systems Inc., Mountain View, CA) and assembled in an Aldus Pagemaker (Aldus Co., Seattle, WA).

ACKNOWLEDGMENTS

We thank Shan-Ping Wang and Chun-Hai Dong for tissue sections; Hong Rong for plant transformation; Alex Gasch for technical advice; and Alan Brunelle, Anil Kush, and Ulla Halfter for helpful discussions. E. Farcy (INRA, Dijon, France) and Ronald Koes (Free University, Amsterdam, The Netherlands) kindly provided the plant material used in this work. S.T. was supported by Kanebo Ltd. This work was supported in part by a grant from Monsanto Co.

Received May 17, 1993; accepted June 21, 1993.

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