Functional analysis of petunia floral homeotic MADS box gene *pMADS1*

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The petunia mutant *green petal* (*gp*, line PLV) shows a homeotic effect in one floral whorl, that is, the conversion of petal to sepal. We demonstrate that this mutant contains a chromosomal deletion, including the petunia MADS box gene *pMADS1*. Second whorl petal development in this null mutant can be restored with a CaMV 35S-*pMADS1* transgene, demonstrating the essential role of *pMADS1* in this process. Because *gp* (PLV) shows only a minor effect on stamen development, the homeotic effects of *pMADS1* are different from those of B-type genes in *Antirrhinum* and *Arabidopsis*. Two other MADS box genes, *pMADS2* and *fbp1* (Angenent et al. 1992), require *pMADS1* to maintain expression in the second whorl. However, in the absence of *pMADS1* these two genes continue to be expressed in the third whorl. The functions assigned to *pMADS1* are further supported by experiments in which we phenocopy *gp* by cosuppression of *pMADS1* gene expression. The flowers, obtained through cosuppression and phenotype restoration, display different degrees of sepal to petal conversion. Analysis of these flowers indicate that *pMADS1* controls growth under the zone of petal and stamen initiation, which causes the corolla tube and stamen filaments to emerge as a congenitally fused structure.

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The flower is the reproductive structure of angiosperms and consists of sepal, petal, stamen, and carpel, which are usually organized in four concentric whorls. To generate this arrangement, the cells of the organ primordia in each of the whorls must be spatially defined to execute the correct developmental program. Recent genetic studies on floral development in both *Arabidopsis* and *Antirrhinum* have led to a model explaining the roles of floral homeotic genes in determining floral organ identity (for review, see Coen and Meyerowitz 1991). The model proposes three gene functions, A, B and C, that are active in partly overlapping fields (A and B or B and C) or complementing fields (A and C) within the floral meristem. Gene function A is active in whorls one and two, gene function B is active in whorls two and three, and C is active in whorls three and four. When only A is active, as in the first whorl, the organs will develop into sepals. In the second whorl the combination of A and B functions determines petal formation. In the third whorl, B and C produce stamens, and in the central fourth whorl, C alone leads to carpel formation. The model was derived from the observation that a single floral homeotic mutation produces an effect in two adjacent whorls and it successfully predicts the phenotypes of double and triple mutants of gene functions A, B, and C in *Arabidopsis*

(Bowman et al. 1991a). The recent cloning and expression studies of some of the B-function genes, like the *Antirrhinum* genes *Deficiens-A* (*DEFA*) (Sommer et al. 1990) and *Globosa* (*GLO*) (Trobner et al. 1992) and the *Arabidopsis* gene *AP3* (Jack et al. 1992), have shown that these are expressed in the second and third whorls as predicted by the genetic model. Similarly, investigation of the expression profile of the C-type gene *Agamous* (*AG*) from *Arabidopsis* confirmed that it is expressed only in the inner two whorls (Yanofsky et al. 1990). Sequence analysis of the B- and C-type-encoded proteins shows that they contain a putative DNA-binding domain, the MADS box (MCM1, AG, DEFA, and SRF), which is similar to the DNA-binding domain of the yeast transcription factor MCM1 (Dubois et al. 1987; Christ and Tye 1991) and the human transcription factor SRF (Norman et al. 1988). This indicates that *DEFA*, *GLO*, *AP3*, and *AG* are likely to be plant transcription factors that regulate the genes that determine floral organ identity.

Although the analysis of *Antirrhinum* and *Arabidopsis* flower development resulted in a common genetic model, it is not known to what extent this model can be applied to other plant species. For example, in the case of the *Petunia hybrida*, the mutant *green petal* (*gp*), the second whorl organs are sepaloid instead of petals, whereas the first, third, and fourth whorl organs are mostly unaffected in their structure. Thus, analysis of petunia floral development should give additional insight into the principles that govern floral organ identity.

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The isolation of some of the *Antirrhinum* and *Arabidopsis* floral homeotic genes has made it possible to initiate floral development studies in other plant species, and floral-specific MADS box genes have now been isolated from tomato (Pnueli et al. 1991), Brassica (Mandel et al. 1993) and petunia (Angenent et al. 1992; Kush et al. 1993). Here, we describe the wild-type petunia flower and the flower of the homeotic mutant *gp* (line PLV), and we show that the latter contains a deletion for the petunia MADS box gene *pMADS1*. Moreover, we demonstrate the role of *pMADS1* in second whorl petal and third whorl stamen development, through both phenotypic restoration and cosuppression studies. With these experiments we created a series of flowers with different end points of second whorl petal development, thus enabling us to dissect the differentiation pathway for the conversion of a sepaloid structure into a petal. By analyzing the expression patterns of five MADS box genes from *P. hybrida* during wild-type and mutant flower development, we addressed the question why *gp* (PLV) is mainly affected in one whorl. Our results indicate that the components for floral development are similar in different plant species, but their different interactions may lead to differences in floral organ determination.

Results

The wild-type flower

Flower development in petunia has been described in detail (Prior 1957; Ntarella and Sink 1971; Sink 1984; Turler and Alabouvette 1988). Because the *P. hybrida* line V26 was used for transformations described here, we will give a short description of the essential features of the V26 flower for the purpose of comparison with mutant or transgenic flowers. The mature flower of V26 (Figs. 1 and 2A) has five sepals in the first whorl that are fused at the base to form a calyx tube (Fig. 2B). Regions in the calyx tube contain cells that make less chlorophyll, as judged from the white color of the parenchyma cells. Growth of the corolla tube and of the filaments occurs in part under the zone of interpetalous initiation, resulting in congenital fusion of the filaments to the corolla tube (Figs. 1 and 2C). Figure 2D shows a stained cross section near the base of a 10-mm-long floral bud illustrating the fusion of the filament to the tube. From the point of separation the filaments become a smooth, round structure with flat, elongated epidermal cells that are lightly pigmented near the anther sacs (Fig. 2E).

The lower and upper epidermal cell layer of the sepal appear similar morphologically, consisting of jigsaw-shaped epidermal cells, stomata, and trichomes (Fig. 2F). The inner and outer epidermal cell layer of the corolla tube are comprised of flat, elongated cells that may be pigmented (Fig. 2G), and trichomes are only present on the outside of the corolla tube (see Fig. 2C). On the limb, trichomes are only found on the lower epidermis and are mainly associated with the main vascular bundles. At the upper epidermal cell layer of the limb, the cells are round, cone-shaped, and pigmented with anthocyanins

Figure 1. Schematic diagrams of the *Petunia hybrida* wild-type flower (V26). (Left) A longitudinal section illustrating the fusion of the stamen filaments to the corolla tube and the faces on which trichomes can be detected. CF (congenital fusion) indicates the region where the stamen filament is fused to the corolla tube. (Se) Sepal; (Li) corolla limb; (Tu) corolla tube; (An) anther; (St) style and stigma; (Ov) ovary. (Right) Floral diagram. (Se) Sepal; (Pe) petal; (St) stamen; (Ca) carpel.

(Fig. 2H). In contrast, the epidermal cells at the lower side of the limb vary from jigsaw-shaped to round (Fig. 2I,J) and may have the characteristic cone shape of the upper epidermal cells. Near the main vein the lower epidermal cells are not always pigmented (Fig. 2J).

The *gp* (PLV) flower

The petunia *gp* mutant is characterized by a homeotic conversion of the second whorl petal into sepal. The *gp* phenotype was obtained in plants by a spontaneous mutation (line M64), by ethylmethane sulfonate (EMS) treatment (line R100), and by gamma radiation mutagenesis (line PLV). All of these mutations are recessive. Here, we describe the flowers of the *gp* line PLV (de Vlaming et al. 1984). Sections of young flower buds (up to 3 mm long, measured from the base to the tip of the first whorl sepals) of V26 and *gp* (PLV) are almost indistinguishable morphologically (Fig. 3, cf. A with B). In both V26 and *gp* (PLV), trichomes begin to develop on the abaxial face of the second whorl organs when the flower buds are ~2 mm long. When the *gp* (PLV) floral buds are ~4 mm long, the formation of trichomes can be detected at the adaxial face of the second whorl organs, whereas no trichomes are detected on the adaxial face of the V26 second whorl petal at this or any later stage of flower development. Figure 3, C and D, shows a cross section through a 15-mm-long flower bud of V26 and of *gp* (PLV), respectively. The parenchyma cells of the second whorl organ in *gp* (PLV) are not as large as those of the first whorl sepal but smaller than those of the petal, and the cell wall staining is more like that of sepal than petal (Fig. 3E,F).

Figure 4A shows the mature flower of *gp* (PLV). The

Figure 2. The petunia V26 flower. (A) A V26 flower. (B) Two sepals from the first whorl. (Note that the sepals are fused at the base, which contains less chlorophyll.) (C) A longitudinal-cut flower bud with two sepals removed. (Note the stamen filaments (Fi) that are fused near the base to the corolla tube (Tu) and the trichomes at the outer face of the corolla tube.) (D) A stained cross section of a V26 flower bud, near the base, showing the fusion of the filaments to the corolla tube. (E) Close-up of a stamen filament near the anther. (F) An upper epidermal peel from the first whorl sepal tissue. (G) An epidermal peel from the adaxial face of the second whorl tube tissue. (H) An upper epidermal peel from the second whorl petal limb. (I) A lower epidermal peel from the second whorl petal limb, between the main veins. (J) A lower epidermal peel from the second whorl petal limb, near the main vein. (Tu) Tube; (Se) sepal; (An) anther; (Fi) filament; (St) style; (Ov) ovary. Vertical bar, 1 cm; thick horizontal bar, 1 mm; thin horizontal bar, 0.1 mm.

first whorl sepals are fused at their base, as in V26, and near the base, show only a very slight reduction in chlorophyll pigmentation as compared with the V26 sepals (cf. Fig. 4B with Fig. 1B). The upper and lower epidermal cell layers of the *gp* (PLV) first whorl sepal are similar to those of the V26 sepal (not shown). The *gp* (PLV) second whorl sepals are slightly thinner than the first whorl sepal tissue and show no marked reduction of chlorophyll synthesis near their base (Fig. 4C). Figure 4, D and E, shows an epidermal peel from the abaxial and adaxial face of the second whorl sepal, respectively. The epidermal cells on both faces resemble those of the V26 sepal (jigsaw-shaped cells, stomata, and trichomes). Thus, the second whorl organs of *gp* (PLV) are sepals by virtue of their green pigmentation, cell size and shape, and the presence of trichomes and stomata on both faces.

Although stamen development in the *gp* (PLV) mutant is similar to that in wild-type petunia and leads to the formation of anther sacs that produce viable pollen, some developmental differences are apparent. In contrast to V26, the stamen filaments of *gp* (PLV) are not fused to the second whorl (Fig. 4F; see also cross section in Fig.

3D). Sepaloid structures, however, often emerge from the third whorl stamens (Fig. 4G), or additional sepaloid third whorl organs are initiated between the stamen filaments (Fig. 4H,I). Upon maturation of the *gp* (PLV) flower (after anthesis has occurred), these green sepaloid structures develop regions with petaloid characteristics: cone-shaped epidermal cells with anthocyanins (Fig. 4H). Also, frequently, petaloid epidermal cells and/or trichomes are detected on the stamen filaments (Fig. 4J), which clearly differ from the long, elongated epidermal cells found on wild-type stamen filaments (Fig. 2E). The occurrence of the extra sepaloid structures and sepal/petaloid stamens in the third whorl may vary throughout the plant life cycle and may be influenced by growth conditions.

Four *P. hybrida* MADS box genes

In this paper we describe the expression pattern in V26 and *gp* (PLV) flowers of four petunia MADS box genes that were isolated in our laboratory (Kush et al. 1993; S. Tsuchimoto, A. van der Krol, and N.-H. Chua, in prep.)

Figure 3. Sections of V26 and *gp* (PLV) flowers. (A) A longitudinal section of a V26 inflorescence. The numbers indicate the whorl. (L) Leaf. (B) A longitudinal section of a *gp* (PLV) inflorescence. The numbers indicate the whorl. (C) A transverse section of a V26 flower. (D) A transverse section of a *gp* (PLV) flower. (Note that the stamen filaments are not fused to the second whorl sepal tube (cf. Fig. 2D).) (E) Close-up of a transverse section of a V26 flower, showing sepal and petal tissue. (F) Close-up of transverse section of a *gp* (PLV) flower, showing first and second whorl sepals. Numbers refer to the floral whorl. (L) Leaf; (Se) sepal; (Pe) petal; (St) style; (Ov) ovary; (An) anther; (Fi) filament. Vertical bar, 1 cm; thick horizontal bar, 1 mm; thin horizontal bar, 0.1 mm.

and *fbp1*, a petunia MADS box gene isolated by Angenent et al. (1992). *pMADS1* has a 693-bp open reading frame encoding a protein that shows 93% identity to the *Antirrhinum DEFA* MADS box region and 77% identity outside of the MADS box region (Sommer et al. 1990). *pMADS2* is 972 bp long and encodes a protein of 213 amino acids. This gene shares 87% identity with the *Antirrhinum GLO* gene MADS box region and 60% amino acid homology outside the MADS box region (Trobner et al. 1992). *fbp1* also shares homology to *GLO* (87% identity within the MADS box and 66% identity outside of the MADS box). Both *fbp1* and *pMADS2* genes are present in the hybrid lines W115 (used to isolate *pMADS1-4*) and R27 (used to isolate *fbp1*), as well as in the different presumed ancestor lines of petunia (see Materials and methods; Southern analysis not shown). The two other petunia MADS box genes that were sequenced, *pMADS3* and *pMADS4*, show homology to the *Arabidopsis AGAMOUS* gene (Yanofsky et al. 1990) and

the *AGL6* gene (Ma et al. 1991), respectively. Sequence analysis of these genes will be presented elsewhere (S. Tsuchimoto, A. van der Krol, and N.-H. Chua, in prep.).

MADS box gene expression

We analyzed the expression pattern of the five petunia MADS box genes and found that *pMADS1*, *pMADS2*, and *pMADS3* are expressed in flowers but not in vegetative organs, whereas *pMADS4* is expressed in leaves as well as in flowers (S. Tsuchimoto, unpubl.). The expression pattern of *fbp1* in petunia has been reported previously by Angenent et al. (1992). Initial experiments showed that the steady-state mRNA levels in the different floral organs vary throughout development. We therefore show expression levels at early and late stages of development (for a description of stages, see Materials and methods). These initial studies also showed that the steady-state mRNA levels of some *pMADS* box genes

Figure 4. The flower of *gp* (PLV). (A) A mature *gp* (PLV) flower. (B) Two sepals from the first whorl. (Note that the sepals are fused near the base.) (C) Two sepals from the second whorl. (Note that the sepals are fused for half of their length.) (D) An upper epidermal peel from the second whorl sepal. (E) A lower epidermal peel from the second whorl sepal. (F) A longitudinal section of a mature *gp* (PLV) flower. (Note that the stamen filaments are not fused to the second whorl sepals.) (G) A sepaloid stamen often found in *gp* (PLV) flowers. (H) A *gp* (PLV) flower at a late stage of development. (Note that the extra third whorl sepaloid organs can develop regions with petaloid characteristics (arrow).) (I) Close-up of *gp* (PLV) flower with the first and second whorl sepals removed. (Note the stamen filaments (not fused to the second whorl organs) and a sepaloid sixth organ, initiated between the stamen (arrow).) (J) Close-up of a *gp* (PLV) stamen filament, showing petaloid cells and trichomes. Vertical bar, 1 cm; thick horizontal bar, 1 mm; thin horizontal bar, 0.1 mm. For abbreviations, see legend to Fig. 2.

may vary at similar stages of floral development among different lines, presumably reflecting the different rates at which floral organs mature in different genetic backgrounds. Two processes that are specifically associated with petal development are suppression of chlorophyll synthesis and enhancement of anthocyanin pigmentation in the petals. To monitor these events we also analyzed the expression of the chlorophyll a/b binding protein (*CAB*) gene and the chalcone synthase (*CHS*) gene. For easy comparison, we grouped, for each of the MADS box genes, the expression profile in V26 (Fig. 5A–G, top panels) and *gp* (PLV) (Fig. 5A–G, middle panels) floral tissue.

pMADS1 In V26 this gene is mainly expressed in the second and third whorls (Fig. 5A, top panel). No expression could be detected in *gp* (PLV), either at an early or late stage of flower development (Fig. 5A, middle panel).

pMADS2 Figure 5B (top panel) shows that the expression of this gene in V26 is mainly in the second and third whorls. In the second whorl tissue of *gp* (PLV), this gene is expressed at a very low level and only at young stages of flower development (Fig. 5B, middle panel), whereas its expression in the third whorl (stamens) is increased in both young and mature *gp* (PLV) floral buds compared with that in V26.

fbp1 In V26 the *fbp1* gene, like *pMADS2*, is expressed in the second and third whorls (Fig. 5C). In *gp* (PLV), *fbp1* expression is only detected in the second whorl at a very low level in early stages of development, whereas no expression is detected at the late stage of second whorl development. However, in the third whorl of *gp* (PLV), *fbp1* expression is elevated both in young and mature flower buds compared with that in V26 (Fig. 5C, middle panel).

pMADS3 Figure 5D shows that this gene is expressed in the third and fourth whorls of V26 (Fig. 5D, top panel) as well as in *gp* (PLV) (Fig. 5D, middle panel). In the fourth whorl of *gp* (PLV) the mRNA level is slightly higher than in the wild-type V26.

pMADS4 The expression of this gene is mainly detected in the first, second, and fourth whorls of V26 flowers (Fig. 5E, top panel). Expression in the mature second whorl is lower and in the mature fourth whorl is higher in *gp* (PLV), compared with V26 (Fig. 5E, cf. top and middle panels).

CHS *CHS* expression is detected in all four whorls of the V26 flower, but the expression is elevated in the mature petal tissue. In the mutant *gp* (PLV) the *CHS* gene is also expressed in all four whorls (Fig. 5F, middle

Figure 5. Expression of MADS box genes and *CAB* in floral organs of V26, *gp* (PLV), and cosuppression plants. Total RNA was isolated from young and mature flower buds of V26 (top), PLV (middle), and SD15c (bottom). Filters containing 7 μ g of RNA per lane were hybridized to specific probes derived from the genes indicated: (A) *pMADS1*; (B) *pMADS2*; (C) *fbp1*; (D) *pMADS3*; (E) *pMADS4*; (F) *CHS*; (G) *CAB*. The panels within one box were derived from one filter; therefore, the strength of the hybridization signal can be compared directly within a box. Lanes 1, 2, 3, and 4 represent RNA isolated from whorls 1, 2, 3, and 4, respectively. (H) Total RNA was isolated from young and mature flowers of transgenic line SD15d. Lane 2/3 represents RNA isolated from the combined second and third whorl tissues. Lanes 1 and 4 represent RNAs isolated from whorls 1 and 4, respectively.

panel); however, its up-regulation in the mature second whorl is no longer detected. In contrast, in the third whorl of the *gp* (PLV) flowers, the *CHS* expression level is higher compared with that of mature stamens of V26.

CAB The *CAB* gene expression is high in the first two whorls of both V26 (Fig. 5G, top panel) and *gp* (PLV) (Fig. 5G, middle panel). In the mature wild-type flower the *CAB* gene expression level diminishes in petals and carpels and the mRNA is not detected in mature stamens.

pMADS1 is deleted from the genome of *gp* (PLV)

The lack of any *pMADS1* expression in *gp* (PLV) prompted us to analyze its genomic DNA for the state of the *pMADS1* gene. Figure 6 shows the hybridization pro-

file of a *pMADS1* probe to genomic DNA of three wild-type hybrid lines (V26, V30, and W115) and a segregating population of *gp* mutant and wild-type plants (4 of 20 plants analyzed are shown). We found that DNA isolated from *gp* plants did not hybridize to the *pMADS1* probe, demonstrating that this gene is deleted from the genome. Therefore, *gp* (PLV) is a null mutant for *pMADS1*. This is not surprising because the mutation in *gp* (PLV) was induced by gamma radiation treatment, which is known to cause chromosomal deletions. Using the *pMADS1* probe and under low stringency hybridization conditions, we did not detect any additional band that is present in the wild type and absent from the *gp* mutant, indicating that no other *pMADS1*-related gene is deleted from the *gp* genome. The other four petunia MADS box genes are present and intact in the mutant genome, as confirmed by Southern and Northern blot analyses (see above). The *gp* (PLV) phenotype is a phenotypic marker for chromosome IV of *P. hybrida*, thus placing the *pMADS1* gene on chromosome IV (de Vlaming et al. 1984).

pMADS1 restores petal development in *gp* (PLV)

To prove that *pMADS1* is an essential gene for petunia petal development, it was necessary to show that the *gp* phenotype can be restored to the wild-type phenotype by the *pMADS1* gene function. Because of regeneration problems associated with *gp* (PLV), we performed a cross between this mutant and V26. A plant (GP/*gp*) from the progeny was used for leaf disc transformation to introduce a 35S-*pMADS1* gene (J84; see Materials and methods). One of the resulting transgenic plants, carrying three independent inserts of J84 and showing an overexpression phenotype (U. Halfter, N. Ali, L. Ren, A. Kush, and N.-H. Chua, in prep.; Fig. 7A), was backcrossed to *gp/gp* (PLV) plants. The progeny plants were analyzed for the presence of the wild-type *pMADS1* gene and the 35S-

Figure 6. Southern blot analysis of wild-type and *gp* (PLV) genomic DNA. Genomic DNAs were digested with *HindIII*, size fractionated on an agarose gel, and blotted onto a GeneScreen Plus filter. The blot was hybridized to a full-length *pMADS1* cDNA (see Materials and methods), and after hybridization it was washed under high-stringent conditions. The three *pMADS1* gene fragments are indicated by arrows. (Lane 1) V26; (lane 2) V30; (lane 3) W115; (lanes 4–7) a segregating population of wild-type and *gp* (PLV) plants; (lanes 4,6) wild-type plants; (lane 5,7) *gp* (PLV) plants.

pMADS1 transgene by Southern blot hybridization and for their floral phenotype. Among the 33 progeny plants analyzed, three plants were identified that neither contained a wild-type *pMADS1* gene nor a 35S-*pMADS1* transgene in their genome, and these plants had a *gp* phenotype. Ten other plants did not contain any wild-type *pMADS1* gene but had one or more copies of the 35S-*pMADS1* transgene. Four of these still exhibit the *gp* phenotype, suggesting a lack of phenotypic restoration (M1a). One plant showed small red sectors on a sepaloid second whorl (M1b; Fig. 7B), indicating a partial restoration by the 35S-*pMADS1* transgene. At a later stage this same plant showed a very weak restoration (Fig. 7C,D; before and after anthesis, respectively). Figure 7, G and H, shows that the late differentiation into petal tissue in the second whorl tissue of M1b resulted in the presence of trichomes on the upper epidermal layer of the petal limb. Three plants showed nearly complete (e.g., M1c Fig. 7E) and two showed complete (e.g., M1d Fig. 7F) petal development in the second whorl. In these plants the maturation rate of the petal was clearly slower compared with that of V26 (Fig. 7J,K). The late restoration resulted in trichomes being present on the adaxial face of the petal tube in M1c [cf. inner face of wild-type tube (Fig. 7L) with that of the M1c tube (Fig. 7M)]. Figure 7I shows that the cells constituting the second whorl sepal structure in the *gp* flowers can still be recognized in flowers of plants that showed partial restoration (M1c). In plants that showed no or weak restoration of the *pMADS1* gene function in the second whorl, the ectopic expression of the 35S-*pMADS1* gene in the third whorl did not suppress the development of extra organs. However, these extra organs in the third whorl and structures on the stamen, which in *gp* (PLV) showed only partial petal characteristics, then developed into full petaloid tissue (Fig. 7N). Unlike the filaments of the *gp* (PLV) flowers, the base of the filaments of the M1c and M1d flowers were fused to the second whorl tissue, as in V26.

Figure 8A shows a Northern blot analysis of RNA isolated from mature flowers of M1a plants and M1c plus M1d plants. In both sets of plants, the *pMADS1* transgene shows high expression in the first two whorls and low expression in the inner two whorls. The expression

of the transgene in M1a is very low (expression in the third and fourth whorls is only visible after prolonged exposure), which correlates with the lack of restoration in petal development in these transgenic plants. In the M1c and M1d plants, the restoration of petal development correlated with a high expression level of the 35S-*pMADS1* transgene, as well as an up-regulation of *pMADS2* and *fbp1* expression in the second whorl, indicating that the expression of these two genes in this whorl is controlled by *pMADS1*. Overexpression in petunia *gp* (PLV) of a similar *pMADS2* gene construct (see Materials and methods) did not result in any restoration of petal development nor did it affect expression of *fbp1* (Fig. 8B).

Phenocopy of *gp* (PLV) by cosuppression of *pMADS1*

Supporting evidence for the function of *pMADS1* in flower development was obtained from V26 transgenic plants in which introduction of a m35S-*pMADS1* chimeric gene resulted in the cosuppression of *pMADS1* expression, in some cases leading to a complete phenocopy of *gp*. The cosuppression of *pMADS1* was manifested in a gradation of phenotypes ranging from a decrease in petal pigmentation (5 of 20 transgenic plants, SD15; see Fig. 9A), reduced petal growth (1 of 20, SD6; see Fig. 9B), and reduced growth and differentiation (1 of 20, SD12; see Fig. 9C) to a complete lack of petal differentiation, resulting in sepaloid structures in the second whorl (1 of 20, SD3; see Fig. 9D). A partial petal differentiation of the second floral whorl in SD3 plants could occur with the aging of the plant (mainly after anthesis), resulting in slightly pigmented, sepaloid second whorl structures (SD3; Fig. 9E). In all of the transgenic plants mentioned above, cosuppression resulted in the formation of third whorl stamens with filaments that were not fused to the second whorl (e.g., see SD12; Fig. 9F). Also, in the case of a strong cosuppression phenotype, the third whorl organ number was often altered by the appearance of small additional sepaloid structures between the stamen filaments (arrow; Fig. 9F). The partial petal differentiation in SD3, SD6, and SD12 in the second whorl differed by cell layer and could occur in sharply defined sectors (SD3; Fig. 9G). Figure 9, H–J, shows the different

Figure 7. Phenotypic analyses of flowers of *pMADS1* restoration plants. Leaf tissue from hybrid GP/*gp* was used for transformation with the 35S-*pMADS1* gene construct J84 (see Materials and methods), and one transgenic line (M1) that showed an overexpression phenotype (see U. Halfter, N. Ali, L. Ren, A. Kush, and N.-H. Chua, in prep.) was back-crossed with *gp* (PLV). Genomic DNAs of progeny plants (M1a–z) were analyzed for the presence of wild-type *pMADS1* and the 35S-*pMADS1* transgene. Flowers of plants that did not carry the wild-type *pMADS1* gene but contained one or more copies of the 35S-*pMADS1* transgene (M1a–d) were analyzed (B–F). (A) The transformed GP/*gp* plant that showed an overexpression phenotype (M1). This plant was crossed with *gp* (PLV), and M1a–d are some of the progeny plants. (B) Close-up of a M1b flower, showing a *gp* phenotype but with small sectors of petal tissue in the second whorl. (C, D) A flower on M1b at a late stage of plant development, before and after anthesis. (Note the petal tissue that has developed after anthesis.) (E) M1c, heterozygous for the 35S-*pMADS1* transgene (Southern analysis not shown). (F) M1d, homozygous for the same 35S-*pMADS1* transgene insert (Southern analysis not shown). (G) Second whorl of M1b, showing the partial petal development. (H) Close-up of second whorl of M1b. (Note the trichomes on the adaxial face of the petal.) (I) A corolla of M1c. (Note that the sepal structure can still be recognized through the corolla tissue, indicating that most of the corolla limb is derived from the second whorl (default) sepal.) (J) Flower buds of V26. (K) Flower buds of M1c. (L) Inside of a V26 second whorl corolla tube. (Note the absence of trichomes on the adaxial face.) (M) Inside of a M1c second whorl corolla tube. (Note the presence of trichomes on the adaxial face.) (N) A petaloid stamen of M1c. Vertical bar, 1 cm; thick horizontal bar, 1 mm; thin horizontal bar, 0.1 mm.

Figure 7. (See facing page for legend.)

Figure 8. Northern blot analysis of *gp* transgenic plants expressing *pMADS1* or *pMADS2*. Total RNA was isolated from mature flower buds of *gp* (PLV) transgenic plants expressing the 35S-*pMADS1* transgene J84 (A) or the 35S-*pMADS2* transgene VIP186 (B). Equal amounts (7 μ g) of RNA were analyzed on identical Northern blots using gene-specific probes. Lanes 1, 2, 3, and 4 represent RNA isolated from whorls 1, 2, 3, and 4, respectively. The signal for *pMADS2* and *fbp1* in B can be compared directly with the signal in Fig. 5, B and C, respectively (same hybridization). (A) In M1(a) the expression of the 35S-*pMADS1* transgene is very low and can only be detected in the third and fourth whorls upon prolonged exposure (not shown). In these transgenic plants the expression of *pMADS2* and *fbp1* is primarily in the mature third whorl. In M1(c+d) the average expression of the 35S-*pMADS1* transgene is higher in all four whorls. In these plants the expression of *pMADS2* and *fbp1* is up-regulated in the second whorl, compared with nontransformed *gp* plants (cf. Fig. 5, B and C). (B) Transgenic *gp* plants that express the 35S-*pMADS2* transgene (M2). Tissue from five independent transgenic flowers was combined for Northern analysis. The average expression of *pMADS2* in the first, second, and fourth whorls is low (presumably from the transgene) and has no effect on the expression of *fbp1* (cf. Fig. 5C).

end stages of petal development that can be detected in these second whorl floral organs. In the epidermal peels the petaloid cells could be seen next to nonpigmented sepaloid cells, indicating that petal differentiation is cell autonomous (Fig. 9I,J). On the plants with mild to strong cosuppression phenotype, the filaments of the stamen showed regions with petaloid cells, pigmented with anthocyanins, and occasionally a trichome (Fig. 9K).

We attributed the changed phenotype of the transgenic flowers to a cosuppression of the *pMADS1* gene because the *pMADS1* mRNA steady-state level was substantially reduced in these transgenic lines (see below). The cosuppression phenotype was stably inherited to the next generation for lines SD12 and SD3. The progeny from the selfed transgenic line SD15 showed a segregating population of plants among which petal development varied from wild-type (SD15a), medium petal development (SD15b) to sepaloid petals (SD15c). This was the result of the segregation of three independent inserts of the m35S-*pMADS1* transgene (Southern blot analysis not shown). Of 20 progeny plants, 3 showed a floral phenotype that was not consistent with a suppression of just the *pMADS1* gene function. In these flowers the second whorl petals often did not develop or were fused to the third whorl stamens to form petaloid stamen (e.g., SD15d; see Fig. 9L,M).

RNA analysis of two cosuppression plants

To demonstrate that the phenotype in the transgenic lines carrying the m35S-*pMADS1* transgene was the result of cosuppression of *pMADS1* expression, we analyzed the steady-state mRNA levels of the different *pMADS* genes during floral development in SD15c, a transgenic line that showed a *gp* phenotype (Fig. 5A–G, bottom panels), and in SD15d, a transgenic line that showed a limited second whorl development and petaloid stamens (Fig. 5H). Because there was no clear separation between second and third whorl in SD15d, we combined tissue of these two whorls for RNA analysis. In SD15c the *pMADS1* mRNA steady-state levels were much reduced both in the second as well as in the third whorl, indicating a cosuppression of the *pMADS1* trans- and endogenous gene (Fig. 5A, cf. lanes 2 and 3 of top and bottom panels). The transgenic line SD15d had a much reduced *pMADS1* steady-state mRNA level in young floral buds, but the *pMADS1* gene was not suppressed in later stages of floral development (Fig. 5G, top panel). The lack of a significant high level of *pMADS1* mRNA steady-state level in SD15c indicates that the change in flower phenotype in these plants was caused by a cosuppression of the *pMADS1* trans- and endogenous gene.

To see whether the cosuppression effect was specific

Figure 9. Phenotypic analysis of *pMADS1* cosuppression plants. Wild-type petunia plants (V26) were transformed with a 35S-*pMADS1* chimeric gene construct (VIP162). Transgenic plant SD15 was selfed, and flowers of progeny plants (SD15a–d), showing different degrees of cosuppression, were analyzed. (A) SD15a. Although the effect on second whorl petal development is mild, the third whorl stamen filaments are not fused to the petal tube of this flower. (B) SD12. The tube and petal tissue of this flower are not fully developed. (C) SD15b. The second whorl tube is much reduced, and in the limb, sectors of petal or petaloid tissue have developed. (D) SD15c. The flowers from this line are an almost complete phenocopy of *gp* (PLV) flowers. (E) SD15c. Same flower as D, but 1 week later. After anthesis the second whorl can develop some petaloid characteristics. (F) Inside of a SD15b flower. Part of the first and second whorl organs were removed to show that the stamen filaments are not fused to the second whorl organs and that extra, third whorl sepaloid organs develop in this flower (arrow). (G) Second whorl sepaloid structure of SD3, showing sectors of petal tissue. (H) Close-up of second whorl sepaloid structure of SD3, showing sectors of green cells, white cells, and cells pigmented with anthocyanins. (I, J) An epidermal peel from SD3 second whorl tissue (see H), showing small, fully pigmented petal cells next to nonpigmented and slightly pigmented jigsaw-shaped epidermal cells. (K) Close-up of a stamen filament of a SD3 flower, showing petaloid cells and trichomes. (L) SD15d. Most of the second whorl organs in these flowers do not develop or are fused to the stamens. (M) SD15d. All of the organs of the second/third whorl that are produced in one flower. Vertical bar, 1 cm; thick horizontal bar, 1 mm; thin horizontal bar, 0.1 mm.

Figure 9. (See facing page for legend.)

for the *pMADS1* gene, we also analyzed the expression of *pMADS2*, *pMADS3*, *pMADS4*, and *fbp1*. In young sepaloid tissue of the second whorl of SD15c, the *pMADS2* and *fbp1* steady-state mRNA level was very low but similar to that of V26. Upon maturation of this tissue, expression of both genes remained low, in sharp contrast with the increase in *pMADS2* and *fbp1* steady-state mRNA level in mature petals of untransformed V26 flowers (Fig. 5B,C, cf. lane 2 of top and bottom panels). In line SD15d, *pMADS2* and *fbp1* expression in the second/third whorl increased upon maturation (Fig. 5H).

RNA analysis of V26 and *gp* (PLV) and the *pMADS1* restoration plants indicated that both *pMADS2* and *fbp1* are regulated by *pMADS1* (see above). Therefore, the lack of *pMADS2* and *fbp1* expression in the second whorl of SD15c is likely the result of the reduced *pMADS1* gene expression in this whorl, rather than a nonspecific cosuppression effect of the 35S-*pMADS1* transgene. In the mature third floral whorl of *gp* (PLV), the expression of *pMADS2* and *fbp1* is elevated (Fig. 5B,C, middle panels). However, Northern analysis of SD15c showed that the *pMADS2* gene expression is very low in the mature third whorl organs. This indicates that *pMADS2* may also be a target for cosuppression by the *pMADS1* transgene. Also, the phenotype of plant SD15d is stronger than that of *gp* (PLV), a *pMADS1* null mutant, suggesting that genes other than just *pMADS1* are being suppressed at an early stage of flower development in SD15d. The cosuppression effect has little or no influence on *pMADS3* and *pMADS4* expression levels. (Fig. 5D,E, bottom panel; and Fig. 5H).

Analyses of petunia petal development

With the cosuppression of *pMADS1* function and the restoration of *gp* (PLV), we created a series of (mature) flowers with different stages of petal development. These flowers not only help to define the function of *pMADS1* but also to analyze the actual process of petal growth and differentiation in petunia. Figure 10A shows the different end stages of second whorl organ development, starting with the *gp* sepal [Fig. 10A(1)], V26 partial cosuppression [Fig. 10A(2) and (3)], *gp* partial restoration, and ending with wild-type petal. Similar end stages of petal development are shown schematically in Figure 10B. The petal differentiation in the epidermal cell layer suppresses trichome and stomata formation and promotes longitudinal and lateral cell divisions in the tube and the limb. The fully differentiated petaloid epidermal cell is a small, round, and cone-shaped cell with a high level of pigmentation by anthocyanins. The parenchyma cells of the inner cell layers of mature petal tissue are smaller than those in mature sepal tissue and do not show any green pigmentation. Because the (macro) surface area of mature petal tissue is approximately twice that of sepal tissue while the parenchyma cells and epidermal cells of the petal are up to fivefold smaller than those of the sepal, petal development consists of many additional cell divisions besides those that are necessary to make up the (default) sepal structure. The transgenic

line SD15c, which shows a sepaloid second whorl in the mature flower, can still develop petal-like tissue [SD15c before anthesis (Fig. 9D), as well as after anthesis (Fig. 9E)]. The same can be seen in the restoration experiment [petal sector (Fig. 7B) and petal development (Fig. 7C,D) in M1c]. How far a cell can differentiate into a complete petaloid cell depends on when the genes of the petal differentiation pathway are activated. A mature sepaloid cell may not be able to change its shape but can become pigmented with anthocyanins, whereas a young sepaloid cell may change its shape and/or divide to give rise to a fully differentiated petaloid cell.

The growth patterns that transform a sepaloid organ into a petal are illustrated in Figure 10C. The sepal growth (S; Fig. 10C) includes a congenital fusion at the base of the five sepaloid organs, leading to a tube structure. This tube structure corresponds to the fused part of the corolla limb; the corolla tube has no real equivalent in *gp* (see below). When the petal differentiation pathway is activated, S growth is transformed into C growth (Fig. 10C) and extended by additional lateral cell divisions (Fig. 10C, C1), additional longitudinal cell divisions at the base, which make up a part of the corolla tube (Fig. 10C, C2), and additional cell divisions under the base of the sepal and the stamen (Fig. 10C, C3 and F2), which make up the part of the corolla tube with the fused stamen filaments. The C3 and F2 growth are affected most easily by *pMADS1* cosuppression and restored least easily by *pMADS1* expression in *gp* (PLV) plants. Under conditions of partial cosuppression (SD15a) or partial restoration (M1b) of the *pMADS1* function, the tube and filament growth only occurs above the petal and stamen initiation zone (Fig. 10C, C2 and F1), resulting in separate (nonfused) stamen filaments and corolla tube. The differentiation of the sepaloid second whorl organ into petal is mainly responsible for the corolla limb structure (Fig. 10C, C and C1). This is, for instance, illustrated by flowers of M1b (Fig. 7D, G, H), which show petal tissue (C1 growth) at the fringes of the otherwise sepaloid second whorl organs, and in flowers of M1c (Fig. 7I), where the sepaloid structure that forms in *gp* (PLV) flowers can still be seen within the corolla limb that has formed by *pMADS1* restoration. The corolla tube is formed by C2 and C3 growth (Fig. 10C), and equivalent growth is either extremely limited or absent in the sepaloid organs of *gp*.

Discussion

The functions of pMADS1 in determining floral organ identity

Here, we provide evidence that the mutant *gp* (PLV) suffers a chromosomal deletion that includes the *pMADS1* locus. Moreover, petal development in *gp* can be restored by a 35S-*pMADS1* transgene, and the *gp* phenotype can be obtained by *pMADS1* cosuppression. These combined results indicate that the *pMADS1* gene can be designated as *GP*. Although *pMADS1* does not control stamen growth in the third whorl, it does have minor effects in this whorl by suppressing formation of petaloid cells and

Figure 10. Different end stages of petal development in petunia. (A) Hand-made tissue sections from mature second whorl organs of the following. (1) *gp* (PLV) sepal. The upper and lower epidermal cell layer are translucent, trichomes are present on both faces, and the inner parenchyma cells are green. (2) *pMADS1* cosuppression (SD15b). The upper and lower epidermal cell layer shows sectors of petaloid cells, pigmented with anthocyanins. (3) *pMADS1* cosuppression (SD12). There is a reduction in trichome number in larger patches of petaloid epidermal cells. The parenchyma cells show only weak green pigmentation. (4) *pMADS1* restoration (M1c). The upper epidermal cell layer is almost completely petaloid, with only a few trichomes on the adaxial face. The inner parenchyma cells are small although still pigmented green. (5) V26 petal. The upper and lower epidermal cell layer consist of small cone-shaped cells, pigmented with anthocyanins. The parenchyma cells are small and white. (B) A schematic presentation of the different end stages of petal development in petunia. The default state of the second whorl organ is sepaloïd, characterized by jigsaw-shaped epidermal cells, trichomes, stomata, and large, green parenchyma cells. Petal differentiation suppresses the formation of trichomes and stomata, whereas the epidermal cells can become pigmented and lose their characteristic jigsaw shape (more round). Full petal differentiation results in small, white parenchyma cells and epidermal cells that are small, cone-shaped, and pigmented purple with anthocyanins. (C) A schematic presentation of the growth stages that transform a sepaloïd organ into a petal. The sepal is formed by growth S. When petal differentiation is activated, this growth is transformed into C and additional growth occurs (C1, C2, and C3), leading to the petal structure. F1 is the growth of the filament that occurs above the zone of stamen initiation. The growth of C3 and F2 occurs under the zone of petal and stamen initiation, resulting in a congenital fusion of stamen filament to the corolla tube.

additional organs. The petal differentiation pathway appears to be dosage dependent, because the degree of restoration is correlated with the expression level (Fig. 8A) and copy number of the 35S-*pMADS1* transgene (Fig.

7E,F). Partial restoration by the 35S-*pMADS1* transgene could occur in defined sectors (Fig. 7B). Such a sector is phenotypically similar to a sector in the *DEFA* (*def-621*) mutant in which somatic reversion has occurred during

second whorl development, resulting in the restoration of *DEFA* expression (Carpenter and Coen 1990). However, the sector in M1b is genetically different because in this tissue the *pMADS1* gene is under control of the "constitutive" CaMV 35S promoter. This result suggests that a weakly expressed *pMADS1* transgene can activate petal differentiation in a cell at a low frequency, and, subsequently, feedback mechanisms might re-enforce this differentiation process. In this connection it should also be noted that a putative MADS box DNA-binding site (CCAAAGATGG) is present in the CaMV 35S promoter. Therefore, expression from this promoter might also be subject to regulation by MADS box genes.

pMADS1 regulates the expression of pMADS2 and fbp1 in the second whorl

Because pMADS box gene expression varies throughout floral development and at similar stages among different *P. hybrida* lines, caution should be exercised in interpreting differences in expression levels among plants with different genetic backgrounds. The effect of the absence of *pMADS1* on *pMADS2* and *fbp1* expression, however, was observed consistently (Figs. 5B,C and 8A). In the second whorl, *pMADS1* up-regulates, whereas in the third whorl it down-regulates *pMADS2* and *fbp1* expression [cf. expression in V26 and *gp* (PLV); (Fig. 5A–C)]. *pMADS1* has also been shown to up-regulate its own and *pMADS2* expression in first whorl tissue of transgenic plants in which the ectopic expression of *pMADS1* resulted in the homeotic conversion of sepals to petals (U. Halfter, N. Ali, L. Ren, A. Kush, and N.-H. Chua, in prep.). The expression of *fbp1* in another *gp* mutant (M68) has been reported previously (Angenent et al. 1992), but the *pMADS1* expression in this line has not been described. Also, in these analyses the effect of floral development on the expression levels of *fbp1* was not considered. The expression of *pMADS3* was largely unaffected by the presence or absence of *pMADS1* gene expression, as was *pMADS4* gene expression.

Our observation that petaloid cells can develop in a *pMADS1* null mutant (Fig. 4 H,J) suggests that *pMADS1* controls petal cell differentiation indirectly. For example, it might regulate the expression of regulatory factors in the second whorl that directly interact with petal-specific genes. These downstream transcription factors apparently do not need the *pMADS1* gene for their expression in cells of the third whorl. We note that the expression profiles of both *pMADS2* and *fbp1* fit that of the above described petal-specific downstream genes.

Cosuppression of pMADS1

The phenomenon of cosuppression was first described by Napoli et al. (1990) and van der Krol et al. (1990) and occurs when a transgene somehow represses its own expression as well as that of other homologous genes, either wild-type genes or other transgenes, present in the plant genome (Napoli et al. 1990). Although cosuppression has now been observed with many different types of genes in transgenic plants, its molecular mechanism re-

mains unknown (for review, see Jorgensen 1990). A cosuppression phenotype was observed with *pMADS1* but not with a similar *pMADS2* gene construct (A.R. van der Krol, unpubl.). In the *pMADS1* cosuppression plants, expression of the transgene was not detected in sepals of line SD15c and SD15d (Fig. 5A, bottom panel, and Fig. 5H, top panel), indicating that in both of these lines the *pMADS1* transgenes are already in a suppressed state. This has also been observed for other cases of cosuppression (e.g., *CHS* and the dihydro-flavonol reductase genes; van der Krol et al. 1990). Most *pMADS1* cosuppression plants showed a phenotype varying from wild type to that of *gp*, indicating that the changes can be ascribed to alterations in *pMADS1* expression level. However, there is some evidence suggesting that the cosuppression may not always be specific for *pMADS1*. The changes in *fbp1* expression in the cosuppression plant SD15c are comparable with the changes in *fbp1* expression in *gp*; however, the changes in *pMADS2* expression are not. It could be that *pMADS2* is also a target for the cosuppression in this transgenic line. Moreover, the SD15d flowers show a phenotype more severe than *gp* flowers, indicating cosuppression of gene functions other than those of *pMADS1*.

Differences between petunia and Antirrhinum and Arabidopsis flower development

The deduced function of *pMADS1* in second whorl organ development agrees with that of *DEFA* and *AP3*. Mutation of one of these genes produces a homeotic conversion of petal into sepal. Both *pMADS2* and *fbp1* show a very low level of expression at early stages of second whorl organ development, and in the absence of *pMADS1* this expression fails to increase to the high level detected in the wild-type mature petal. This expression pattern is similar to that described for *DEFA* and *GLO* in *Antirrhinum*. (Sommer et al. 1990; Trobner et al. 1992) and *AP3* and *Pistillata* (*Pi*) in *Arabidopsis* (Jack et al. 1992). Both set of B-type genes are induced independently at early stages of flower development but show an interdependency for maintenance and increase of expression at later stages of petal development. Sommer et al. (1990) and Trobner et al. (1992) reported that only the DEF/GLO heterodimer can bind to DNA target sites located in the promoter regions of *DEFA* and *GLO*. This result suggests that both genes are involved in up-regulating their own expression (Trobner et al. 1992).

In contrast to *DEFA* and *AP3*, the petunia *pMADS1* is genetically redundant for third whorl stamen development. A minor effect of *pMADS1* in the third whorl is the suppression of petaloid cell formation on the stamen filaments. A similar third whorl function would be masked in *DEFA* and *AP3* mutants because they do not develop stamens. In petunia, stamen development in the absence of *pMADS1* coincides with the up-regulation of *pMADS2* and *fbp1* expression. This is in sharp contrast to the situation in *Antirrhinum* and *Arabidopsis* where, as in the second whorl, both sets of B-type genes show an interdependency for maintenance of expression in the third whorl organs (Jack et al. 1992; Trobner et al. 1992).

Thus, *pMADS2* and *fbp1* proteins can function without the *pMADS1* protein, indicating a different type of interaction between the petunia proteins *pMADS1* and *pMADS2/fbp1* on one hand, and between the *Anthirrium* proteins *DEFA* and *GLO* and the *Arabidopsis* proteins *AP3* and *PI* on the other hand. Future analysis will have to show whether these different interactions between B-type genes in petunia and *Anthirrium* and *Arabidopsis* is reflected in different DNA-binding and dimerization properties of the respective gene products.

Materials and methods

Plant material and transformations

Petunia plants were grown under standard greenhouse conditions. The *pMADS1* and *pMADS2* cDNAs were isolated from *P. hybrida* line W115. The *fbp1* gene was isolated from *P. hybrida* line R27 by Angenent et al. (1992). The petunia *gp* mutant line PLV was obtained by gamma-ray treatment and was kindly provided by Dr. E. Farcy (INRA, Dijon, France). Other hybrid lines used in our experiments were *P. hybrida* line V26 and V30. The (presumed) ancestor petunia lines used were *P. axillaris* (S1 and S2, the different S numbers designate different origins), *P. inflata* (S6 and S14), *P. parviflora* (S4), *P. violacea* (S9 and S10), *P. integrifolia* (S12 and S13), and *P. parodii* (S8). These lines were kindly provided by Dr. R. Koes (Free University, Amsterdam, The Netherlands). Plant transformations were performed as described by Horsch et al. (1985) using leaf discs from V26 (construct VIP162), a hybrid of V26 and PLV (construct J84), or PLV (construct VIP186).

DNA cloning strategies

The overexpression construct J84 was made by cloning *pMADS1* cDNA fragment into a vector containing the CaMV 35S promoter and *rbcsE9* poly(A) addition signal (U. Halfter, N. Ali, L. Ren, A. Kush, and N.-H. Chua, in prep.). The *pMADS1* cosuppression construct VIP162 was made by cloning the cDNA as an *XbaI*–*KpnI* fragment downstream of a modified 35S (m35S) promoter in the vector pBSII (Stratagene) to generate clone VIP160. The modified 35S promoter contains the –90 to +8 fragment of the CaMV 35S promoter (AS10; Benfey and Chua 1990) with four copies of the B3 domain (Benfey and Chua 1990) and four copies of an optimized AS-1-binding site (Katagiri et al. 1989) placed upstream. The m35S promoter has been shown to direct the expression of a β -glucuronidase reporter gene in all cell layers of the petunia petal (A.R. van der Krol, unpubl.). The m35S promoter plus *pMADS1* coding sequence was isolated from VIP160 as a partial *HindIII*–*KpnI* fragment and cloned between the *HindIII*–*KpnI* site of the binary vector VIP26 (van der Krol and Chua 1991) to generate clone VIP162. The *pMADS2* gene construct was made by inserting the *EcoRI* fragment of the *pMADS2* cDNA into a binary vector that contains both the mCaMV 35S promoter and the *rbcsE9* poly(A) addition signal to form VIP186. The following chimeric gene constructs were introduced into the petunia genome:

Southern and Northern analysis

Genomic plant DNA, isolated from ~1 gram of leaf tissue, was digested with restriction endonucleases, size-fractionated on agarose gels, and blotted onto GeneScreen Plus membrane (DuPont). The hybridization and washing conditions were similar as were the Northern blots (see below). Total RNA was isolated from plant tissue using the RNaid isolation procedure (BIO 101). Flower buds were dissected into first, second, third, and fourth whorl tissue. The young flower bud material that was used measured, from the base to the tip of the first whorl sepal, 5–15 mm for V26 and V30, 5–20 mm for W115, and 5–10 mm for PLV. In all lines, at this stage, the second whorl tissue is mainly light green in color and covers the third and fourth whorl organs, and the third whorl stamen filaments have not yet elongated. The mature flower bud material consisted of closed flower buds 5–6 cm long for V26 and V30 (measured from the base to the tip of the second whorl petal), closed flower buds 6–7 cm long for W115, and for PLV, open flowers with stamen filaments fully elongated, but before anthesis. For V30, V26, and W115, the mature floral bud stage coincides with the peak of *CHS* gene expression in the second whorl (Koes et al. 1989).

Equal amounts (7 μ g) of total RNA were fractionated on 1.2% agarose gels containing 6% formaldehyde. Gels were blotted onto GeneScreen Plus (DuPont) according to the manufacturer's instructions and hybridized to random primed labeled DNA (Boehringer Mannheim) in 20% formamide, 5 $\times$ SSC, 1% SDS, 5 $\times$ Denhardt's solution, and 10 μ g/ml of salmon sperm DNA at 42°C. Blots were either washed under nonstringent conditions (0.5 hr, 2 $\times$ SSC, 65°C) or stringent conditions (0.5 hr, 0.2 $\times$ SSC, 65°C). Gene-specific probes (cDNA fragments without the MADS box region) were used for each of the genes. The *fbp1* probe, covering nucleotides 494–760 (Angenent et al. 1992), was generated by PCR amplification using R27 genomic DNA as a template.

Phenotypic analysis and imaging

Flowers and hand-made tissue sections were photographed under a Nikon SMA-U stereomicroscope. Epidermal peels taken from sepal or petal tissue were vacuum infiltrated with water to remove air pockets before photographing. Microscopic sections were made and stained as described by Natarella and Sink (1971) and photographed in bright field either under a Nikon SMZ-U stereo microscope or a Nikon optiphot microscope. All images were processed in a Adobe photoshop and assembled in an Aldus Pagemaker. The Northern images were compressed vertically (20%).

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