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Flower Development in Petunia

Alexander R. van der Krol[1] and Nam-Hai Chua[2]

Laboratory of Plant Molecular Biology, The Rockefeller University, 1230 York Avenue, New York, New York 10021

INTRODUCTION

Recent genetic studies on both Arabidopsis and Antirrhinum have led to a common genetic model (reviewed in Coen and Meyerowitz, 1991; see also Coen and Carpenter, 1993, this issue). This model for flower pattern formation proposes three gene functions, *a*, *b*, and *c*, that are active in the floral meristem in partly overlapping whorls (*a* and *b* or *b* and *c*) or complementing whorls (*a* and *c*). The *a* function is active in whorls 1 and 2, *b* is active in whorls 2 and 3, and *c* is active in whorls 3 and 4. 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* determines petal differentiation; in the third whorl, *b* and *c* specify stamens; and in the central fourth whorl, *c* alone determines carpel formation. This model serves as a useful framework for the more detailed analysis of molecular interactions between the different homeotic genes in Arabidopsis and Antirrhinum. Why then study flower development in petunia or other species?

First, the development of many wild-type flowers or putative homeotic mutant flowers cannot be easily reconciled with the model because in these flowers a homeotic conversion is limited to one whorl only (e.g., homeotic mutants in petunia, tulip, and lily). Only by expanding our floral developmental studies to such floral mutants will we be able to determine which part of the model has general validity and which part is species specific.

Second, petunia has the advantage that it is susceptible to Agrobacterium-mediated transformation, which allows the evaluation of gene function by transgenic technology. Moreover, the large flowers of petunia render it convenient for the isolation and biochemical characterization of homeotic proteins as they occur in the plant. Similar arguments can be made for tomato, and, indeed, putative homeotic genes have now been cloned from both plant species (Pnueli et al., 1991; Angenent et al., 1992; Kush et al., 1993; Tsuchimoto et al., 1993). In this review, we will focus mainly on homeotic genes from petunia and their roles in floral development.

[1] Current address: Department of Plant Physiology, Agricultural University Wageningen, Arboretumlaan 4, 6703 BD, Wageningen, The Netherlands.

[2] To whom correspondence should be addressed.

THE HISTORY OF THE CULTIVAR *PETUNIA HYBRIDA*

Since Jusseau established the genus *Petunia* in 1803, ~30 species have been described, which have their geographic origins in South America (Sink, 1984). For many decades, the attractive large flowers of *Petunia hybrida* have rendered this plant the focus of genetic and biochemical studies on floral pigmentation. Because the early taxonomy of the different petunia species was not consistent, the exact origin of the cultivated *P. hybrida* is not clear. It is believed that *P. hybrida* originated from a cross between a member of the white flowering *P. axillaris*/*P. parodii* group and a member of the colored flowering *P. violaceae*/*P. inflata*/*P. integrifolia*/*P. parviflora* group. The results from chemical and protein taxonomic analysis of ancestor and hybrid petunia lines are not completely consistent; nevertheless, they indicate that *P. hybrida* was derived from *P. axillaris* and *P. inflata* (Sink, 1984). Restriction fragment length polymorphism analysis using the chalcone isomerase A (CHI-A) gene as a probe shows that the hybrid line V30 and ancestor lines *P. axillaris* and *P. parodii* have CHI-A restriction fragments of similar size, whereas the CHI-A restriction fragment of the hybrid line W30 is similar in size to those of *P. inflata* and *P. violaceae* (van Tunen, 1990). These results indicate that the putative ancestor lines have contributed differently to each of the cultivated hybrid lines and that caution should be exercised in comparing gene expression among different hybrid lines.

WILD-TYPE AND MUTANT FLOWERS

During vegetative growth, petunia produces leaves with an alternate setting that upon flowering changes to an opposite leaf setting. The floral meristem is formed by two-thirds of the inflorescence meristem; the continuous growth of the remaining part of the meristem pushes the developing floral bud into its lateral position. Like those of Arabidopsis and Antirrhinum, the mature organs of the petunia flower are organized in four concentric whorls. There are five sepals, five petals, five stamens, and a bilobed carpel in the inner whorl, as shown in Figures 1 and 2A. The sepals as well as the petals are fused at their bases to form a calyx and corolla tube, respectively, and the basal parts of the stamen filaments are fused to the corolla tube (Turlier and Alabouvette, 1989).

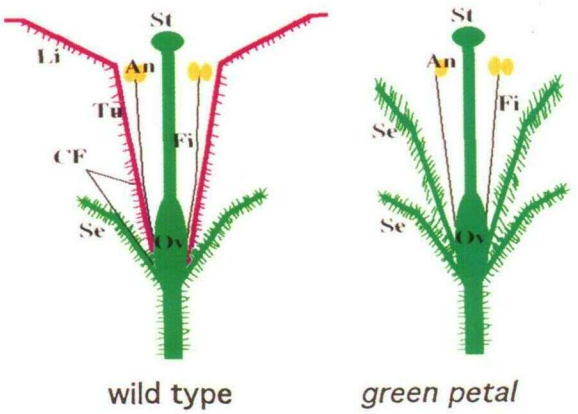

Figure 1. Petunia Flower Structure.

Schematic diagrams of a longitudinal section of a petunia wild-type flower (left) and a *green petal* (*gp*) flower (right). Li, limb; An, anther; St, stigma; Tu, corolla tube; Fi, stamen filament; Se, sepal; Ov, ovary; CF, congenital fusion.

During the last few decades, novel pigmentation mutants of petunia have been obtained by a number of mutagenic treatments (ethyl methanesulfonate, γ-ray, or x-ray treatment or activation of petunia transposons; for a listing, see de Vlaming et al., 1984). By contrast, only a limited number of floral homeotic mutants were obtained in these experiments. The mutants that show a homeotic conversion of one or more of the floral organs are *green petal* (*gp*; Figure 2B); *blind* (*bl*; Figure 2C and Vallade et al., 1987); *phoenix* (*px*); *Double* (*Do1*, Figure 2D; *do2*, Natarella and Sink, 1971); and *ph3* (Turlier et al., 1990). Other petunia mutants do not show a homeotic conversion of floral organs but are specifically affected in floral development. Examples are *choripetal* (*ch2*), *crumbled* (*cr*), *Trumpet* (*Tu*), and *Undulated* (*Un*). Table 1 lists the floral structural mutants, together with a short description of the mutant phenotypes.

Most floral homeotic mutants of Arabidopsis and Antirrhinum show an effect on organ differentiation in two consecutive whorls. By contrast, the petunia mutants *gp* and *bl* were previously reported as homeotic mutants that are affected in one whorl only (Vallade et al., 1987; van Tunen and Angenent, 1991; Angenent et al., 1992); thus, both mutants were thought to be at variance with the proposed genetic model for flower patterning in Arabidopsis and Antirrhinum. In the *gp* mutant, the second whorl is sepaloid instead of petaloid, and in *bl*, the petal limb is converted to an antheroid structure. Recently, however, more careful characterization of these mutants has revealed that they do show some small homeotic effects in another floral whorl. In *gp*, petaloid cells are found on the stamen filaments, a finding that still cannot be easily reconciled with the proposed genetic model. In *bl*, stigmatoid cells are formed on the tip of the sepal (Tsuchimoto et al., 1993); therefore, this mutant resembles the Arabidopsis *apetala2* (*ap2*) mutant and can be accommodated within the framework of the genetic model.

In *ph3*, a female-sterile petunia mutant, antheroid structures are detected within the central carpel. The *ph3* mutation also changes corolla pigmentation by altering the pH (Turlier et al., 1990).

It could be that these mutants are mostly affected in one whorl because they are weak alleles of homeotic genes. For instance, different mutant alleles of the homeotic *b* gene *DEFICIENS-A* (*DEFA*) in Antirrhinum have different effects on second and third whorl development (Schwarz-Sommer et al., 1990). However, we have shown that the *gp* phenotype in petunia line PLV is caused by a chromosomal deletion (van der Krol et al., 1993), making this a homeotic null mutant that affects mainly one whorl. Thus, functional analyses of different petunia floral homeotic genes should provide additional insights into the principles that govern floral organ identity.

PETUNIA MADS BOX GENES

The recent cloning and characterization of some *b* and *c* type genes from both Arabidopsis and Antirrhinum have shown that these genes encode putative transcription factors (*DEFA*, Schwarz-Sommer et al., 1990; *GLOBOSA* [*GLO*], Trobner et al., 1992; *AGAMOUS* [*AG*], Yanofsky et al., 1990). These factors share a similar structural motif, the MADS box, which is a putative DNA binding domain. The homeotic MADS box genes are a subset of a larger MADS box gene family, some of whose members are expressed in all tissues, whereas others display a tissue-specific or organ-specific expression profile. Because of the important roles played by MADS box genes

Table 1. Petunia Floral Structure Mutants[a]

Mutant	Phenotype
Homeotic	
green petal (*gp*)	Petals with shape and color as sepals; petaloid cells on the stamen filaments
blind (*bl*)	Antheroid structures develop in place of the petal limb; stigmatoid cells on the tip of the sepals
Double (*Do1*)	Flower with increased number of petals and stamens within the second whorl
ph3	Female sterile; antheroid structures develop within the carpel; corolla has a bluish hue
phoenix (*px*)	Twist in the corolla tube, sometimes not terminally differentiated because new flower can emerge from the receptacle
Nonhomeotic	
choripetalous (*ch2*)	Flower limb with strong incisions between the petals
crumpled (*cr*)	Flower limb is crumpled
Trumpet (*Tu*)	Flower is trumpet shaped
Undulated (*Un*)	Flower limb and calyx undulated; leaves yellowish green

[a] Adapted from de Vlaming et al. (1984).

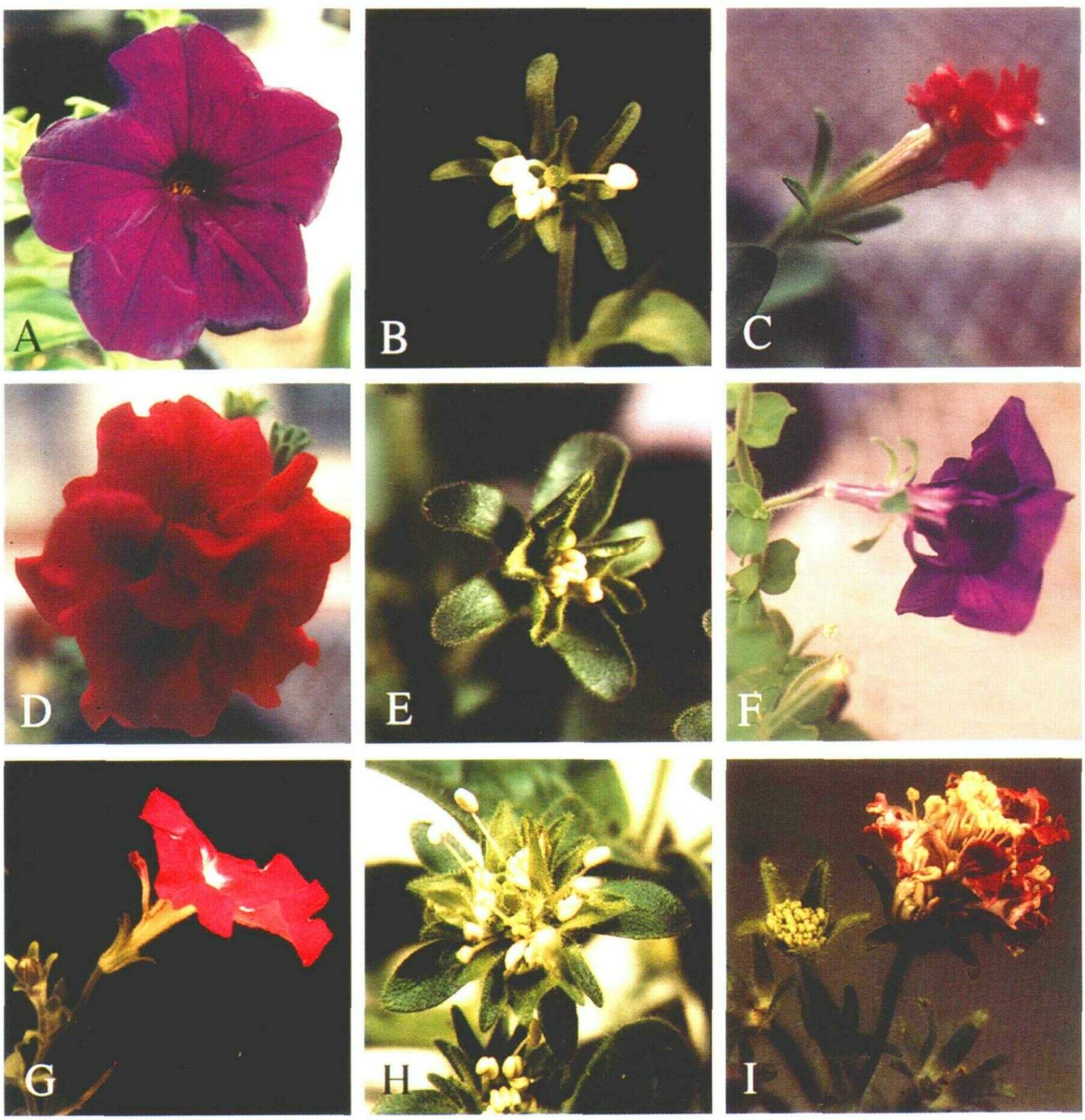

Figure 2. Petunia Floral Structure Mutants.

(A) A V26 (wild-type) flower.
(B) A *green petal* (*gp*, PLV) flower.
(C) A *blind* (*bl*) flower. Note the antheroid structure on top of the corolla tube.
(D) A *Double* (*Do1*) flower. Note the extra petals in the inner whorls.
(E) A V26 flower in which *pMADS1* gene expression is cosuppressed by a 35S-*pMADS1* transgene.
(F) A V26 flower in which the *pMADS1* expression level is elevated by a 35S-*pMADS1* transgene. Note the partial conversion of the base of the first whorl sepals into petal tissue, and the extra petaloid organs on the corolla tube.
(G) A *gp* flower in which petal differentiation has been restored by a 35S-*pMADS1* transgene. Note that the high expression phenotype of *pMADS1* is also observed (see **[F]**).
(H) A flower from a double mutant carrying the *Do1* and *gp* mutations. Note the extra sepals, sepaloid stamens, and normal stamens in the inner two whorls.
(I) A flower from a double mutant carrying the *Do1* and the *bl* mutations. Note the *bl* phenotype of the second whorl petal. The extra stamens in the inner two whorls show reduced petaloid characteristics compared to the *Do1* single mutant (see **[D]**).

in floral development, both Angenent et al. (1992) and our laboratory (Kush et al., 1993; Tsuchimoto et al., 1993) have screened petunia cDNA libraries for such genes. These efforts have resulted in the isolation and characterization of *floral binding protein 1* (*fbp1*) and *fbp2* (Angenent et al., 1992), *pMADS1* and *pMADS2* (Kush et al., 1993), and *pMADS3* and *pMADS4* (Tsuchimoto et al., 1993), whose expression patterns are diagrammed in Table 2.

The predominant expression of *fbp1*, *pMADS1*, and *pMADS2* in the second and third whorls suggests that they are *b* type genes likely to be involved in the determination of organ identity in these two whorls. Indeed, pMADS1 shows homology to Antirrhinum DEFA (93% within and 77% outside the MADS box region) and has now been identified as the *gp* gene product (van der Krol et al., 1993). Both pMADS2 and FBP1 show homology to the protein encoded by another *b* gene, GLO (87% within the MADS box region for both genes, 66% outside the MADS box for FBP1 and 60% for pMADS2). pMADS3 shows homology to the product of the Arabidopsis *c* gene *AG* and is expressed in the third and fourth whorls, similar to *AG* (Tsuchimoto et al., 1993). pMADS4 shows homology to Arabidopsis AGL6, an AG-like protein (Ma et al., 1991). The *fbp2* gene product is 93% identical to the tomato MADS box protein TM5 (Pnueli et al., 1991) and 58% identical to Arabidopsis AGL2 (Ma et al., 1991). This gene is expressed in the second, third, and fourth whorls of wild-type petunia flowers.

Table 2. Expression Patterns of Petunia MADS Box Genes in Mature Wild-Type and *green petal* Flowers

Gene	*Whorl 1*	*Whorl 2*	*Whorl 3*	*Whorl 4*
Wild-type flower				
pMADS1	+[a]	+ + +	+ +	–
pMADS2	–	+ + +	+ +	–
fbp1	–	+ + +	+ +	–
pMADS3	–	–	+ +	+ + +
pMADS4	+ + +	+ + +	+	+ + +
fbp2	–	+ + +	+ +	+ + +
green petal flower				
pMADS1	–	–	–	–
pMADS2	–	–	+ + +	–
fbp1	–	–	+ + +	–
pMADS3	–	–	+ +	+ + +
pMADS4	+ + +	+ + +	+	+ + +

[a] –, no detectable expression; +, low expression; + +, intermediate expression; + + +, high expression.

MADS BOX GENE EXPRESSION AND ACTIVITY

To understand the relationship between gene expression and organ differentiation, it is important to separate the initial state of gene expression from gene expression at later developmental stages. Some homeotic MADS box genes that are expressed in the floral primordia may only be upregulated at late stages of floral development under the appropriate feedback conditions. Feedback may be autonomous or provided by or in conjunction with other putative transcription factors. Studies in Antirrhinum have provided evidence for an "induced" and "maintained" state of MADS box gene expression (Sommer et al., 1990; Trobner et al., 1992). *GLO* expression is initiated normally but fails to be maintained in the absence of *DEFA* and vice versa. Moreover, the upstream promoter region of both *DEFA* and *GLO* contains potential binding sites for the *DEFA/GLO* heterodimer (Trobner et al., 1992; see also Coen and Carpenter, 1993, this issue).

pMADS2 and *fbp1* are expressed at an early stage of floral development in the second and third whorls of both wild-type and *gp* mutant flowers (Angenent et al., 1992; van der Krol et al., 1993). In the mature *gp* flower, however, these genes are no longer expressed in the second whorl (van der Krol et al., 1993), indicating that in this whorl the *gp* gene product pMADS1 is necessary for the maintenance and/or upregulation of expression of these genes. In the third whorl of the *gp* (PLV) flower, both *pMADS2* and *fbp1* are expressed at an early as well as a late stage of development. This is in contrast to the situation in the Antirrhinum *DEFA* mutant, in which *GLO* expresssion is initiated but not maintained. The result obtained with the *gp* mutant is consistent with the fact that its third whorl development is mostly unaffected, whereas in the *DEFA* mutant a homeotic conversion from stamen to carpel occurs in the third whorl. Thus, depending on the plant species, similar homeotic genes may interact in different ways, leading to differences in maintenance of gene expression and, consequently, different homeotic conversion consequences.

Besides transcription, post-transcriptional and/or post-translational control mechanisms may determine the activity of MADS box genes. In this regard, Angenent et al. (1992) have claimed that although *fbp1* mRNA is present in both the second and the third whorls of petunia flowers, FBP1 protein could be detected only in nuclear protein fractions prepared from the second whorl. Unfortunately, in these experiments no positive control for stamen nuclear protein preparations was shown. Moreover, because the antibodies were raised against the entire FBP1 protein, which includes the conserved MADS box region, it is not clear that the signal obtained with the second whorl nuclear protein fraction is indeed specific for FBP1. Therefore, the conclusion of a translational control of the *fbp1* gene in the third whorl may be premature. Several groups have pointed out the strong conservation of a potential phosphorylation site within the MADS box, suggesting that phosphorylation may be important for MADS box protein function by controlling subcellular localization (i. e., nuclear import), DNA binding, and/or protein–protein interactions.

FUNCTIONS OF MADS BOX GENES

pMADS1 Is Essential for Second Whorl Petal Development but Redundant for Stamen Development

Among all the petunia MADS box genes, the function of the *gp* gene product, pMADS1, during floral development has been studied most extensively (van der Krol et al., 1993). Petal differentiation in the second whorl in the *gp* (PLV) mutant can be restored by a *pMADS1* transgene in which the cauliflower mosaic virus 35S promoter is used to drive *pMADS1* expression, demonstrating that pMADS1 is essential for this function. pMADS1 has a more subtle function in the third whorl, where it suppresses the formation of petaloid cells on the stamen filaments and the formation of extra sepaloid organs. Moreover, expression of *pMADS1* under the control of the 35S promoter also causes the stamen filaments to be partially fused to the petal tube as in the wild-type petunia flower.

Additional evidence for pMADS1 functions was obtained from cosuppression experiments. Introduction of a 35S-*pMADS1* transgene into wild-type petunia resulted, in some cases, in the suppression of both endogenous and transgenic *pMADS1* expression (cosuppression; see Napoli et al., 1990 and van der Krol et al., 1990). The cosuppression resulted in a homeotic conversion of petals into sepals and caused stamen development similar to that in the *pMADS1* null mutant *gp* (PLV) (Figure 2E). The cosuppression phenotype correlated with reduced *pMADS2* and *fbp1* expression in the second whorl, again demonstrating that *pMADS1* controls *pMADS2* and *fbp1* expression in the second whorl. The cosuppression was specific for *pMADS1*, because most transgenic plants showed a flower phenotype varying from wild type to a phenocopy of the *gp* flower. In some plants, however, second and third whorl development was more severely affected than in *gp* mutants, resulting in an absence of second whorl organs and in petaloid stamens. This indicates that genes other than *pMADS1* may also be suppressed by the 35S-*pMADS1* transgene, presumably through homology in the conserved MADS box region.

Upregulation of *pMADS2* and *fbp1* in the Second Whorl Correlates with Petal Development

To date there are no genetic data on the function of *pMADS2* and *fbp1* in petunia flower development. Introduction of a 35S-*pMADS2* transgene into wild-type petunia does not result in any visible phenotypic changes, either through ectopic expression or cosuppression (A. Brunelle and A. R. van der Krol, unpublished data). Interestingly, both genes share extensive homology to the Antirrhinum *b* gene *GLO* and have a similar (i.e., second and third whorl) expression pattern. We have established that the presence of two such genes is not due to the hybrid character of *P. hybrida* because a copy of each gene is present in different hybrid lines as well as in the petunia ancestor lines (van der Krol et al., 1993). It is not known whether there are multiple *GLO*-like genes in the Antirrhinum genome. If both *pMADS2* and *fbp1* indeed function similarly to *GLO*, the redundancy in gene function would preclude the isolation of *pMADS2* or *fbp1* mutant plants. Similar redundancy in other gene functions may explain in part the low number of petunia floral homeotic mutants recovered to date.

RNA gel blot analysis of *gp* plants in which petal growth has been restored by a 35S-*pMADS1* transgene showed that *pMADS1* is necessary to maintain and upregulate *pMADS2* and *fbp1* expression in the second whorl. This indicates that *pMADS2* and *fbp1* are downstream targets of *pMADS1* and, therefore, that they may be involved in petal differentiation. Another correlation between *pMADS2* and *fbp1* expression and petal cell differentiation can be found in the third whorl of *gp* (PLV) flowers or *pMADS1* cosuppression plants. In these plants, expression of *fbp1* and *pMADS2* is upregulated in the mature third whorl compared to wild-type mature stamens (see Table 2). Also, in these plants, petaloid cells are found on the stamen filaments (van der Krol et al., 1993). In situ hybridization experiments, however, are needed to ascertain whether the cells that assume petaloid characteristics do indeed express high levels of *pMADS2* and *fbp1.*

Expression of *pMADS2* and *fbp1* in the Third Whorl Correlates with Stamen Development

In Antirrhinum, expression of *GLO* in the third whorl is initiated but not maintained in the absence of *DEFA*, thus correlating expression of both *DEFA* and *GLO* with stamen development. By contrast, in the third whorl of the petunia *gp* (PLV) flower the putative *GLO* homologs *pMADS2* and *fbp1* maintain their expression in the absence of *pMADS1* (van der Krol et al., 1993). Also, in contrast to *defA* mutants, the petunia *pMADS1* mutant (*gp*) does not show any homeotic conversion of stamens to carpels. From these observations, it can be inferred that both *pMADS2* and *fbp1* are involved in third whorl stamen development.

pMADS3 Expression Correlates with Stamen and Carpel Development

The expression pattern (third and fourth whorls) of *pMADS3* and the sequence homology of its protein product to the Arabidopsis *c* type gene product AG suggest a similar function for this gene in stamen and carpel differentiation as has been described for *AG* (Yanofsky et al., 1990). No petunia *pMADS3* mutant has yet been identified, and efforts to induce suppression of the *pMADS3* gene expression in transgenic plants have not been successful. However, ectopic expression of a 35S-*pMADS3* transgene in wild-type petunia can phenocopy the *bl* mutant. Indeed, in the *bl* mutant the spatial expression of *pMADS3* is deregulated, and ectopic expression

is observed throughout the inflorescence (Tsuchimoto et al., 1993). The ectopic expression of *pMADS3* in the first whorl correlates with the presence of stigmatoid cells on the sepals (Tsuchimoto et al., 1993), and the ectopic expression in the second whorl correlates with the replacement of the petal limb by antheroid structures. Taken together, these results indicate that the *bl* mutation is equivalent to the *ap2* mutation in Arabidopsis.

pMADS4 and *fbp2* Functions Are Unknown

The *pMADS4* gene is expressed in leaf tissue and in all floral whorls, albeit at very low levels in the immature third whorl. This implies that *pMADS4* has a more general function in organ development than the other MADS box genes. The *fbp2* gene is expressed in the second, third, and fourth floral whorls (Angenent et al., 1992), and this expression pattern matches that of the tomato gene *TM5* (Pnueli et al., 1991). The function of FBP2 and TM5 is not known.

ECTOPIC EXPRESSION OF *pMADS1* PARTIALLY CONVERTS SEPALS TO PETALS

Overexpression of a 35S-*pMADS1* transgene in wild-type petunia results in a partial conversion of sepal to petal, indicating that in part of the sepal, pMADS1 is the limiting factor for petal differentiation (U. Halfter, N. Ali, L. Ren, A. Kush, and N.-H. Chua, unpublished results). The top part of the sepal is not converted to petal. Because no effect in leaves is observed from ectopic expression of *pMADS1*, it is reasonable to conclude that only "preconditioned cells" can differentiate into petal cells. It could be that such preconditioned cells are located only near the base of the sepal.

One precondition that could possibly make a cell responsive to *pMADS1* expression is an already existing low-level expression of "petal" genes. In this case, the *pMADS1* transgene elevates the expression of these genes to a level that allows phenotypic expression. Indeed, cells near the base of wild-type sepals show some degree of petal differentiation (lack of chlorophyll, small parenchyma cells). Also, *pMADS1* is expressed at a very low level in wild-type sepals (van der Krol et al., 1993). In *gp* plants transformed with a 35S-*pMADS1* transgene, petal differentiation is restored when expression of the transgene is high, but no restoration is seen when transgene expression is low (van der Krol et al., 1993). This shows that low levels of *pMADS1* expression, like that observed in wild-type sepals, may not be sufficient for full expression of the petal phenotype.

In the second whorl, the high ectopic expression of *pMADS1* gives rise to the formation of extra petal tissue on the corolla tube (U. Halfter, N. Ali, L. Ren, A. Kush, and N.-H. Chua, unpublished results; see Figure 2F). Similar, but smaller, extra petal structures are also occasionally observed on the petal tube of nontransgenic petunias (A. R. van der Krol, unpublished observations). Extra "petals" are also occasionally observed in *gp* plants in which a 35S-*pMADS1* transgene fully restores petal development (Figure 2G).

pMADS1 INFLUENCES THE COORDINATION OF PETAL GROWTH AND FUSION COMPETENCE

Analyses of petunia plants that overexpress the homeotic gene *pMADS1* and those in which the *pMADS1* expression level is reduced by cosuppression have provided insight into the different processes that make a petal. The results show that *pMADS1* plays a role in the coordination of growth and fusion of the five petals as well as in the fusion of the stamen filaments to the corolla tube. Contrary to the situation in Arabidopsis, both the first and second whorl organs of petunia are fused at their bases to form tubelike structures. In petunia, both the calyx and the corolla tube are thought to be phylogenetically (also called congenitally) fused structures. Congenital fusion can be viewed as an extreme case of postgenital fusion; the fusion event occurs simultaneous with the development of the structures rather than after distinct structures form and can be recognized (reviewed by Verbeke, 1992; see also Gasser and Robinson-Beers, 1993, this issue).

Overexpression of *pMADS1* converts the fusion of the five petals in the corolla tube from a congenital to a postgenital event by changing the coordination of growth and fusion competence of the cells. The delayed fusion results in extra petal tissue emerging from the corolla tube (U. Halfter, N. Ali, L. Ren, A. Kush, and N.-H. Chua, unpublished results). *pMADS1* does not control the fusion competence itself, because in the *pMADS1* null mutant (*gp*, line PLV) congenital fusion still occurs among the first whorl sepals and among the second whorl sepals. It could be that a high level of *pMADS1* expression at an early stage of development stimulates extra lateral cell divisions, which occur before cell fusion competence is activated. Alternatively, *pMADS1* could delay the onset of the fusion program. If this is the case, it is necessary to assume that the fusion event is itself normally a signal for the inhibition of further lateral cell divisions.

pMADS1 also affects the fusion of the stamen filaments to the corolla tube. In wild-type petunia, the corolla tube and stamen filament grow both above and below the zones of petal and stamen initiation. It is growth under the zones of initiation that causes the basal part of the stamen filaments to emerge fused to the corolla tube (congenital fusion; Turlier and Alabouvette, 1988). In the absence of *pMADS1*, however, growth occurs only above the petal and stamen initiation zones, resulting in separate stamen filaments and corolla tube (see Figure 1). Unfused stamens are thus observed in *gp* flowers, in *pMADS1* cosuppression plants, and in *gp* plants that show only

partial phenotypic restoration with a 35S-*pMADS1* transgene (van der Krol et al., 1993).

PETUNIA PETAL DEVELOPMENT

Cosuppression and restoration experiments with *pMADS1* generated a series of plants whose mature flowers show different degrees of sepal-to-petal conversion in the second whorl (van der Krol et al., 1993). Analysis of these flowers reveals that at the cellular level there is no sharp division between "sepal" and "petal" differentiation and indicates that the morphological characteristics of a cell only reflect the differentiation pathway that has been most active during the life of the cell. Morphological characteristics thus do not identify the differentiation pathway that is presently operative in that cell. Petal differentiation can be activated late during organ development and still result in fully differentiated petal tissue when the cells undergo extra cell divisions because in the daughter cells, the petal differentiation pathway is active from the very beginning of the cell's life.

At the cellular level, petal differentiation can be recognized by the appearance of two cell types, epidermal and parenchyma. Mature sepals contain epidermal cells that are flat, unpigmented, and jigsaw shaped, and there are numerous stomata and multicellular trichomes on both the adaxial and abaxial faces. In the mature sepal parenchyma, the cells are big, round, and contain chlorophyll. By contrast, the mature petal epidermal cell is small (approximately one-third the diameter of a sepal epidermal cell), cone shaped, and strongly pigmented with anthocyanins. In addition, petal differentiation prevents the formation of trichomes and stomata on the epidermis. The mature petal parenchyma cell is small and round and is deficient in chlorophyll. In second whorl development, the extent of petal characteristics apparently depends on the duration and level of *pMADS1* expression throughout the life of a cell. In partially restored *gp* plants or *pMADS1* cosuppression plants, a petal epidermis is often seen adjacent to a green parenchyma cell layer. However, the reverse situation, i.e., a white parenchyma cell layer covered by a sepal-like epidermis, is never observed. This may indicate that differentiation of the epidermis precedes that of the internal cell layers.

Within a layer, petal differentiation is cell autonomous because sectors of fully differentiated petal epidermal cells are sometimes seen next to sepal-type epidermal cells in partially cosuppressed or restored flowers (van der Krol et al., 1993). When petal differentiation is active from an early stage of development, as in most parts of the wild-type petal, formation of sepaloid structures (e.g., trichomes and stomata) is suppressed. However, there are parts of the wild-type petal where petal differentiation lags, resulting in more sepaloid characteristics in these localized regions. For example, on the abaxial face of the wild-type petal, the parenchyma cells near the main veins remain green, and the epidermis has trichomes and jigsaw-shaped cells, some of which are pigmented with anthocyanins (van der Krol et al., 1993).

In many of the transgenic plants with *pMADS1* cosuppression or petal restoration, petal differentiation is delayed compared to that in wild-type plants. The extended sepaloid growth in the second whorl of these plants results in an extended sepaloid phenotype, which is reflected by a different trichome distribution over both the abaxial and adaxial face of the petals. Trichome distribution is a marker frequently used for species identification, and our results indicate that this type of difference between species may originate from a heterochronic shift in a single developmental program.

THE *Do1* MUTATION DOES NOT INTERACT WITH *gp* OR *bl*

All the commercially available "double" petunia plants carry the dominant *Do1* mutation. There is also a recessive petunia double mutant, *do2*, which produces multiple flower parts, but this mutant has not been fully characterized (de Vlaming et al., 1984). The dominant mutation *Do1* causes the appearance of many additional organ primordia in the region of the third and fourth whorls. These develop into petals, petaloid stamens, or normal stamens, depending on their proximity to the center of the flower (Natarella and Sink, 1971; Figure 2D). The phenotype of the *Do1* mutation resembles that of the *superman* (*sup*) mutation in Arabidopsis, which causes the production of extra stamens at the expense of the central carpels. Because *SUP* only controls the spatial expression of the *b*-type genes *AP3* and *PISTILLATA*, in the absence of these two genes the effect of the *sup* mutation is not manifested (Bowman et al., 1992). By contrast, analyses of *Do1/gp* and *Do1/bl* double mutants show that these genes act independently of each other (A. R. van der Krol, unpublished results). The *Do1/gp* double mutant has sepaloid first and second whorls, with many sepaloid and normal stamens making up the center of the flower (Figure 2H). The second whorl of the *Do1/bl* double mutant has antheroid structures replacing the limb, similar to that of the single *bl* mutant. In the central portion of the flower, there are numerous stamens that are less petaloid in character than those of the *Do1* single mutant (Figure 2I). Further analysis of the mutants will be necessary to see how the different phenotypes relate to the expression of the different MADS box genes.

PHYSIOLOGY AND FLOWER DEVELOPMENT

Floral development can vary extensively on one plant depending on its age and growth conditions. These physiological and environmental factors affect organ differentiation specifically but not growth itself, indicating that environmental and physiological conditions can alter the function of homeotic genes.

Examples of environmental effects on floral development are the variation in extra third whorl organs and sepaloid structures on stamens in *gp* flowers and the extent of petal limb structures in *bl* flowers. In addition, the effect of the *Do1* mutation on carpel development is sensitive to growth conditions: under poor growth conditions (e.g., old plants) the number of extra stamens is severely reduced, allowing a carpel to develop in the center of the flower. A similar variability in floral development has also been reported for Arabidopsis (Bowman et al., 1992) and Antirrhinum (Schwarz-Sommer et al., 1990) floral mutants. Some of the physiological effects might reflect the activity of modifying enzymes that influence the activity of the homeotic gene products. Such changes could in turn influence homeotic gene transcription by feedback mechanisms.

Cytoplasmic factors can also affect the action of nuclear floral homeotic genes. Several mutant tobacco lines show a homeotic conversion of the stamen to petaloid structures, resulting in male-sterile plants (Kofer et al., 1991). In these lines, the effect on stamen development was correlated with a rearrangement in the mitochondrial DNA. An even more extreme example of a cytoplasmic mutation affecting the action of floral homeotic genes can be found in carrot. Kitagawa et al. (1993) have analyzed the morphological effects on flower development of a homeotic male-sterile cytoplasmic mutation in combination with two different nuclear genotypes. The early stages of flower development are not affected in either nuclear/cytoplasmic combination. At later stages, however, the third whorl organs of one sterile line (W33A) differentiate into petaloid organs instead of stamens. In the other sterile line (W259A), both the second whorl petals and the third whorl stamens are replaced by sepaloid or bractlike organs. Because both fertile wild-type lines bear normal flowers, the different sterile phenotypes likely result from the interactions of distinct nuclear genotypes with one sterility-inducing cytoplasm, presumably culminating in different actions of the nuclear floral homeotic genes (Kitagawa et al., 1993). Taken together, these data suggest that action of wild-type floral homeotic genes is influenced by a number of factors, some of which may be non-nuclear.

FUTURE CHALLENGES

Many gaps in our knowledge still need to be filled before we can understand the mechanisms that control flower development in petunia. In this respect, analysis of floral mutants will continue to play an important role. Because the number of petunia floral mutants isolated thus far is quite small, it is likely that previous mutagenesis efforts have not saturated the genome. Therefore, attempts should be made to isolate additional floral mutants after chemical or transposon mutagenesis.

To understand how the petunia pMADS proteins interact with one another during floral differentiation, a thorough biochemical characterization of these factors is required. We need to determine the DNA binding capacity of all potential homodimers and heterodimers, identify their target sites, and demonstrate that these factors indeed function as transcription factors. The identification of potential target binding sites of MADS box proteins should help identify putative downstream target genes. Also, an inventory of potential DNA binding sites in the promoters of MADS box genes may provide insight into the putative feedback mechanisms that control the expression of these genes. Finally, we need to examine the factor(s) that may affect the action of the MADS box genes, e.g., the effects of protein modifications such as phosphorylation on dimerization and/or DNA binding properties. Modifier activities could potentially play a role in the feedback mechanisms that establish defined zones of expression of the MADS box genes and could be involved in changes in cellular expression patterns during floral organ maturation.

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REFERENCES

Angenent, G.C., Buscher, M., Franken, J., Mol, J.N.M., and van Tunen, A.J. (1992). Differential expression of two MADS box genes in wild-type and mutant petunia flowers. Plant Cell **4,** 983–993.

Bowman, J.L., Sakai, J.H., Jack, T., Weigel, D., Mayer, U., and Meyerowitz, E.M. (1992). SUPERMAN, a regulator of floral homeotic genes in Arabidopsis. Development **114,** 599–615.

Coen, E.S., and Carpenter, R. (1993). The metamorphosis of flowers. Plant Cell **5,** 1175–1181.

Coen, E.S., and Meyerowitz, E.M. (1991). The war of the whorls: Genetic interactions controlling flower development. Nature **353,** 31–37.

de Vlaming, P., Gerats, A.G.M., Wiering, H., and Wijsman, H.J.W. (1984). *Petunia hybrida*: A short description of the action of 91 genes, their origin and their map location. Plant Mol. Biol. Rep. **2,** 21–42.

Gasser, C.S., and Robinson-Beers, K. (1993). Pistil development. Plant Cell **5,** 1231–1239.

Kitagawa, J., Posluszny, U., Gerrath, J.M., and Wolyn, D.J. (1993). Developmental and morphological analyses of homeotic cytoplasmic male sterile and fertile carrot flowers. Sex. Plant Reprod., in press.

Kofer, W., Glimelius, K., and Bonnett, H.T. (1991). Modifications of mitochondrial DNA cause changes in floral development in homeotic-like mutants of tobacco. Plant Cell **3,** 759–769.

Kush, A., Brunelle, A., Shevelle, D., and Chua, N.-H. (1993). The cDNA sequence of two MADS box proteins in *Petunia*. Plant Physiol. **102,** 1051–1052.

Ma, H., Yanofsky, M.F., and Meyerowitz, E.M. (1991). *AGL1-AGL6*, an *Arabidopsis* gene family with similarity to floral homeotic and transcription factor genes. Genes Dev. **5,** 484–495.

Napoli, C., Lemieux, C., and Jorgensen, R. (1990). Introduction of a chimeric chalcone synthase gene into petunia results in reversible cosuppression of homologous genes *in trans*. Plant Cell **2,** 279–289.

Natarella, N.J., and Sink, K.C. (1971). The morphogenesis of double flowering in *Petunia hybrida*. J. Am. Soc. Hort. Sci. **96,** 600–602.

Pnueli, L., Abu-Abeid, M., Zamir, D., Nacken, W., Schwarz-Sommer, Z., and Lifschitz, E. (1991). The MADS box gene family in tomato: Temporal expression during floral development, conserved secondary structures and homology with homeotic genes from *Antirrhinum* and *Arabidopsis*. Plant J. **1,** 255–266.

Schwarz-Sommer, Z., Huijser, P., Nacken, W., Saedler, H., and Sommer, H. (1990). Genetic control of flower development: Homeotic genes in *Antirrhinum majus*. Science **250,** 931–936.

Sink, K.C., ed. (1984). Monographs on Theoretical and Applied Genetics 9: Petunia (Berlin: Springer Verlag).

Sommer, H., Beltrán, J.-P., Huijser, P., Pape, H., Lönnig, W.-E., Saedler, H., and Schwarz-Sommer, Z. (1990). *Deficiens*, a homeotic gene involved in the control of flower morphogenesis in *Antirrhinum majus*: The protein shows homology to transcription factors. EMBO J. **9,** 605–613.

Trobner, W., Ramirez, L., Motte, P. Hue, I., Huijser, P. Lönnig, W.-E., Saedler, H., Sommer, H., and Schwarz-Sommer, Z. (1992). *GLOBOSA*: A homeotic gene which interacts with *DEFICIENS* in the control of *Antirrhinum* floral organogenesis. EMBO J. **11,** 4693–4704.

Tsuchimoto, S., van der Krol, A.R., and Chua, N.-H. (1993). Ectopic expression of *pMADS3* in transgenic petunia phenocopies the petunia *blind* mutant. Plant Cell **5,** 843–853.

Turlier, M.-F., and Alabouvette, J. (1989). La morphogenese florale chez le petunia. II. La fleur normale. Can. J. Bot. **67,** 1985–1997.

Turlier, M.-F., Alabouvette, J., and Doulain-Douvier, D. (1990). La morphogenese florale chez le petunia. III. Deviations dans le programme organogene chez un mutant femelle sterile. Can. J. Bot. **69,** 866–872.

Vallade, J., Maizonnier, D., and Cornu, A. (1987). La morphogenese florale chez le petunia. I. Analyse d'un mutant corolle staminée. Can. J. Bot. **65,** 761–764.

van der Krol, A.R., Mur, L.A., Beld, M., Mol, J.N.M., and Stuitje, A.R. (1990). Flavonoid genes in petunia: Addition of a limited number of gene copies may lead to a suppression of gene expression. Plant Cell **2,** 291–299.

van der Krol, A.R., Brunelle, A., Tsuchimoto, S., and Chua, N.-H. (1993). Functional analysis of the petunia floral homeotic MADS-box gene *pMADS1*. Genes Dev. **7,** 1219–1228.

van Tunen, A.J. (1990). Regulation of Flavonoid Biosynthesis in Petunia. Ph.D. Dissertation (Amsterdam: Vrije Universiteit).

van Tunen, A.J., and Angenent, G.C. (1991). How general are the models describing floral morphogenesis? Flowering Newslett. **12,** 34–37.

Verbeke, J.A. (1992). Fusion events during floral morphogenesis, Annu. Rev. Plant Physiol. Plant Mol. Biol. **43,** 583–598.

Yanofsky, M.F., Ma, H., Bowman, J.L., Drews, G.N., Feldmann, K., and Meyerowitz, E.M. (1990). The protein encoded by the *Arabidopsis* homeotic gene *AGAMOUS* resembles transcription factors. Nature **346,** 35–39.