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Ectopic expression of a single homeotic gene, the *Petunia* gene *green petal*, is sufficient to convert sepals to petaloid organs

Ursula Halfter[1,2,3], Nazeem Ali[1,3], Jörg Stockhaus, Ling Ren and Nam-Hai Chua

Laboratory of Plant Molecular Biology, The Rockefeller University, 1230 York Avenue, New York, NY 10021, USA
[1]Present address: Department of Plant Sciences, University of Arizona, Tucson, AZ 85721, USA
[2]Corresponding author
[3]Joint first authors

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Genetic studies in *Arabidopsis* and *Antirrhinum* showed that petal determination requires the concomitant expression of two homeotic functions, A and B, whereas the A function alone determines sepal identity. The B function is represented by at least two genes. The *Petunia* homeotic gene *green petal* (*gp*) is essential for petal determination as demonstrated by a *Petunia gp* mutant that has sepals instead of petals. We have used ectopic expression of the *gp* gene as a tool to study flower development in *Petunia*. CaMV 35S-*gp* expression leads to homeotic conversion of sepals into petaloid organs when expressed early in development. This demonstrates that a single homeotic gene is sufficient to induce homeotic conversion of sepals to petals, suggesting that other petal determining genes are regulated in part by ectopically expressed *gp*. Indeed, two other MADS-box-containing genes, *pmads* 2 and *fbp* 1, which show homology to the *Antirrhinum* B function gene *globosa*, are activated in the converted petal tissue. Furthermore, our data provide evidence for autoregulation of *gp* expression in the petaloid tissue and uncover the role of *gp* in fusion of petal tissues.

Key words: autoregulation/ectopic expression/flower development/homeotic conversion/*Petunia*

Introduction

Most dicotyledonous plants produce flowers that are composed of four whorls of organ types: sepals, petals, stamens and carpel, named from the outermost to the innermost whorl. Genetic studies on homeotic flower mutants of *Arabidopsis thaliana* and *Antirrhinum majus* (snapdragon) have led to the proposal of a model for floral development (Bowman *et al.*, 1991; Coen and Meyerowitz, 1991). Although *Arabidopsis* and *Antirrhinum* are only distantly related, similar functions determining flower development have been identified in these two species. Three functions, A, B and C, have been proposed, each acting in two neighbouring whorls (A in whorls 1 and 2, B in 2 and 3, C in 3 and 4), which define floral organ identity by their sole or combinatorial action. Petal identity, for example, requires the concomitant expression of the A and B functions. The B function itself requires the expression of at least two genes, *apetala* 3 and *pistillata* in *Arabidopsis*, and *deficiens* and *globosa* in *Antirrhinum*. Loss of function mutations in any of these homeotic genes result in loss of petal identity. If any of the B function genes is mutated, petals are transformed into sepals or sepaloid petals. In the case of an *Arabidopsis* A function mutation petals are converted into stamens or staminoid carpels. Although no *Antirrhinum* A function mutants have been identified yet, a similar function has been proposed for *Antirrhinum* (Bradley *et al.*, 1993). Concomitant expression of the B and C functions determines stamen identity and if any gene representing the B function is mutated, stamens are transformed into carpels or carpelloid stamens and in the case of a C function mutation into petals, as the A and C functions are antagonistic to one another.

Some of these homeotic genes have been isolated, e.g. *deficiens* (*def*A, Sommer *et al.*, 1990), *globosa* (*glo*, Tröbner *et al.*, 1992) and *plena* (*ple*, Bradley *et al.*, 1993) from *Antirrhinum* and *agamous* (*ag*, Yanofsky *et al.*, 1990) and *apetala* 3 (*ap*3, Jack *et al.*, 1992) from *Arabidopsis*, enabling molecular studies. These genes encode proteins that resemble transcription factors in that they have a region with high sequence homology to known transcription factors MCM1 (yeast, Dubois *et al.*, 1987; Herskowitz, 1989) and SRF (human, Norman *et al.*, 1988). This conserved domain, designated the MADS-box (MCM1, AG, DEF A and SRF), constitutes part of the putative DNA binding domain (Schwarz-Sommer *et al.*, 1990). Furthermore, *in situ* expression analysis demonstrated that accumulation of RNA for *def*A (Schwarz-Sommer *et al.*, 1992), *glo* (Tröbner *et al.*, 1992), *ple* (Bradley *et al.*, 1993), *ap*3 (Jack *et al.*, 1992) and *ag* (Yanofsky *et al.*, 1990; Drews *et al.*, 1991) precedes the initiation of the organ primordia whose fate they specify, and that the expression of these genes is maintained throughout organ development. Recently, ectopic expression was used as a tool to confirm further the validity of the model: *Arabidopsis* plants overexpressing *ag* mirrored the phenotype of *apetala* 2 mutants (Mizukami and Ma, 1992) and ectopic expression of *ple*, the *Antirrhinum* homologue of *ag*, has been shown to be the cause of the *ovulata* phenotype (Bradley *et al.*, 1993). As the A and C functions act antagonistically towards each other, the resultant altered floral organ identity obtained in both cases are as predicted by the model. Moreover, even when the *Brassica napus ag* gene is overexpressed in the distantly related species tobacco, the observed phenotype is as predicted by the model (Mandel *et al.*, 1992a) indicating its general validity for most dicotyledonous plants.

Like *def*A and *ap*3, the *Petunia* gene *green petal* (*gp*, *pmads*1; Kush *et al.*, 1993) is expressed in petals and stamens. Furthermore, comparison of the amino acid sequences of the *Petunia* GP and the *Antirrhinum* DEF A proteins shows that the MADS-box region is highly conserved and that there is overall protein homology (Kush *et al.*, 1993). The protein homology and the analogous patterns of *gp* and *def*A expression could suggest a homologous function for *gp* in *Petunia* flower development,

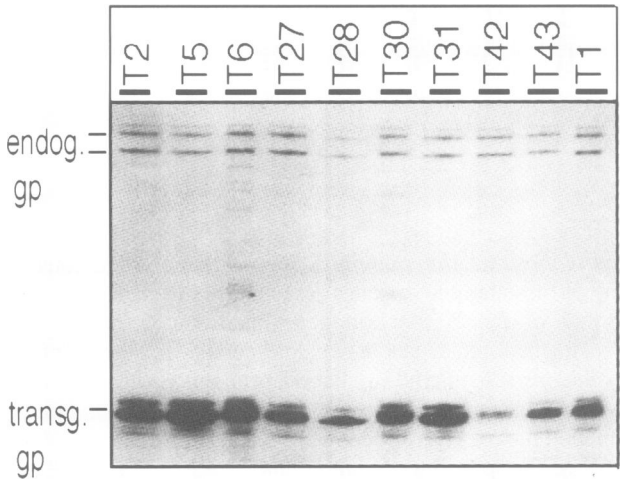

Fig. 1. Analysis of *gp* expression in different *Petunia* lines harbouring the 35S-*gp* transgene. The transgene lacks the *gp* leader sequence thus allowing separate detection of the endogenous and transgenic *gp* mRNAs by primer extension analysis. Shown is an autoradiograph of primer extension analysis of *gp* expression performed on total RNA isolated from young flower buds (1−1.5 cm in length) from different transgenic *Petunia* lines. The signals that correspond to the endogenous *gp* RNA and the transgenic RNA are shown. The expression of the 35S-*gp* transgene varies from strong (line T5) to weak (line T42) among these transgenic lines.

namely that *gp* is essential for petal and stamen determination. In this case a mutation in *gp* would lead to homeotic conversion of both petals to sepals and of stamens to carpels. However, the *Petunia* mutant *gp*(line PLV), which suffers a deletion of the *gp* gene (van der Krol *et al.*, 1993), displays only conversion of petals to sepals but continues to develop stamens (Wiering *et al.*, 1979). It is, however, possible that the stamen determining function of *gp* might be supplanted by another MADS-box gene in the mutant and consequently this particular function of *gp* might be masked in this loss of function background. We have used ectopic expression of the *gp* gene as a tool to obtain further insights into *Petunia* flower development.

Here we report the analysis of *Petunia* ectopically expressing the floral gene *gp*. The altered phenotype of the transgenic flowers, which display a homeotic conversion of sepals into petaloid organs, shows that ectopic expression of a single B function gene, when expressed early in development, is sufficient to determine petal identity in the first whorl. Moreover, our results suggest that genes essential for petal determination are regulated by ectopically expressed *gp*. Furthermore, we provide evidence that *gp* autoregulates its expression in the converted petal tissue.

Results

Ectopic gp expression leads to a homeotic conversion of sepals to petaloid organs

The *gp* coding region was put under control of the cauliflower mosaic virus (CaMV) promoter and the resulting construct used to transform *Petunia hybrida* line V26. Forty independent plants were generated which express the transgene at different levels (Figure 1) and display altered floral phenotypes ranging from weak to severe.

Figure 2A shows a wild type *Petunia* flower which consists of five green sepals occupying the first whorl and, alternate to them, in the second whorl, five coloured petals are positioned which are connately fused in their lower region, creating a tube, while they expand free in the upper region, forming a limb. The generative organs are in the inner two whorls, five stamens in the third whorl and two fused carpels in the fourth whorl. By contrast, transgenic *Petunia* plants ectopically expressing *gp* exhibit altered perianth organs in the outer two whorls, whereas the generative and non-floral organs appear normal. Lines expressing low levels of the 35S-*gp* transgene display only a weak alteration in phenotype (Figure 2B) whereas the most severely altered phenotypes were displayed by two lines expressing the *gp* transgene at high levels. The first whorl organ of a flower ectopically expressing *gp* is petaloid as a result of a conversion of sepal into petal tissue. In the case of a weak and moderate phenotype the lower parts of the sepals are transformed into tissue that resembles the second whorl petal tube (Figure 2C). In plants exhibiting a severe phenotype the first whorl is occupied by five petaloid organs which, in the lower part, form a petal tube like structure and additionally on top have tissue comparable to the petal limb (Figure 2D and E). The development of this part of the organ, reconstituting the petal limb, involves lateral growth of the organ. As in the petal limb, there are on the adaxial surface of this part of the converted organ cone-shaped cells with a high anthocyanin content. In the case of the most severely affected phenotype, however, the petaloid organs are less fused than the wild type petals. The conversion is not fully complete as green tips of sepals are seen on top of the converted petal tissue in the first whorl. The conversion of sepals to petaloid organs in the severely affected phenotypes (Figure 2E) demonstrates that almost the entire first whorl organs can develop as petals.

Evidence for petal tissue in the first whorl was obtained by morphological and molecular data. *Petunia* petals can be distinguished from sepals by the different distribution of trichomes in the two tissues: sepals have trichomes on both the adaxial and abaxial surfaces, while petals have trichomes only on the abaxial side, especially in the region of the tube. Furthermore, petal specific, cone-shaped cells form the adaxial epidermis in the region of the limb. These cause a velvet-like shimmer and harbour the flower pigment, anthocyanin. These features hold true for the first whorl tissue, confirming the petal nature of the converted tissue. On the other hand, sepal characteristic features such as adaxial trichomes, stomata and chlorophyll content are suppressed in this tissue.

A further effect of *gp* overexpression was found in the second whorl petals where additional petal tissue has been formed in an inside-out orientation (Figure 2C, open arrow). To trace the origin and development of this appendage, cross-sections of young transgenic flower buds were examined. Comparison of Figure 2F and Gi−iii shows that the developing petals failed to fuse early in development but instead continued to grow laterally, producing additional inverted petal tissue. Delayed fusion occurred at the original petal margin, nevertheless, resulting in an appendage that became partially detached. Transgenic plants expressing the *gp* transgene at a low level show only minor effects in the sepals and display additional petal tissue in the second whorl (Figure 2B), albeit less than in the severely affected phenotypes. In very weakly affected phenotypes these appendages are comparatively small and peel off (not shown). Such additional petal structures are not observed in wild type V26 *Petunia* flowers grown under normal

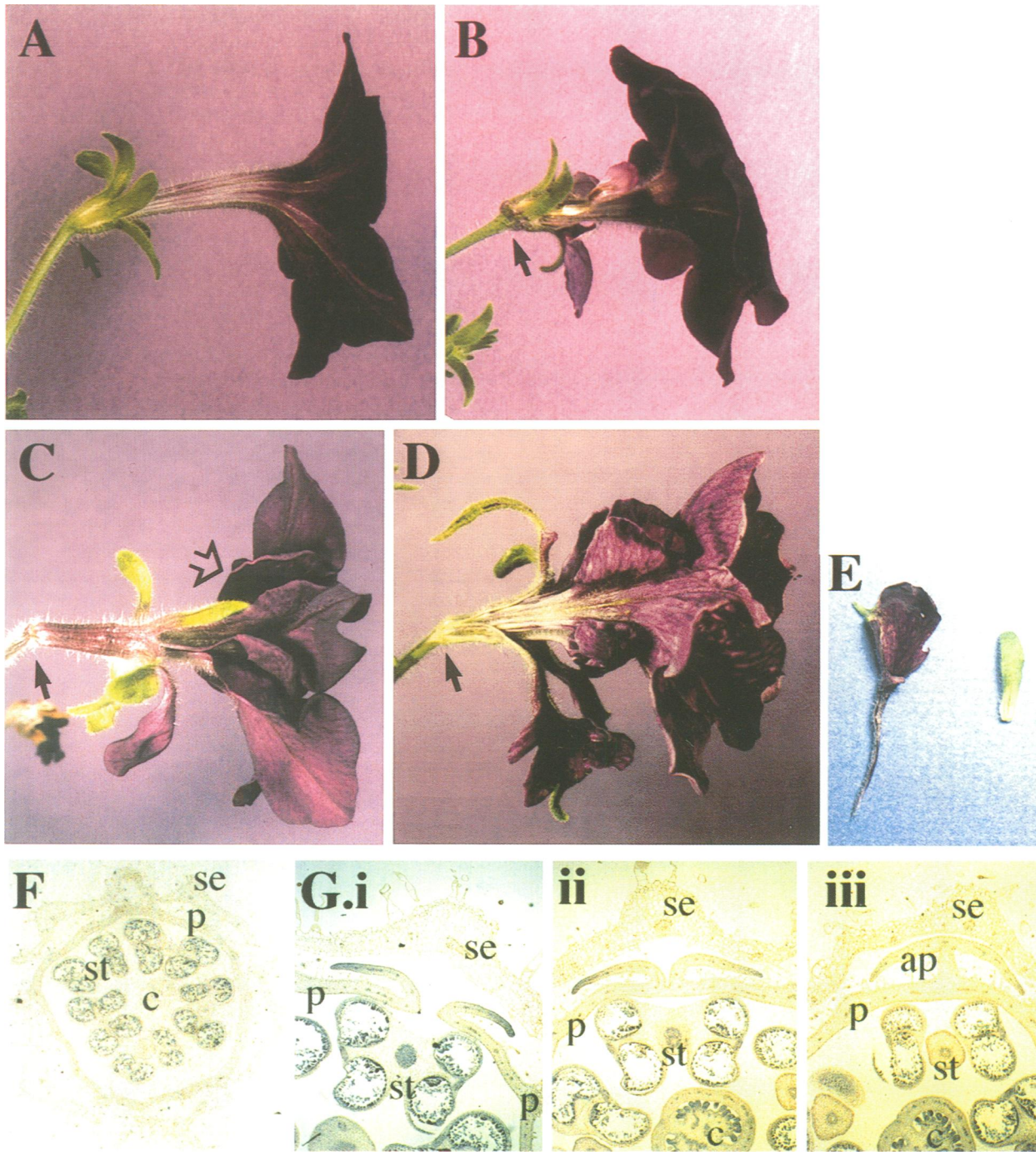

Fig. 2. Phenotype of *Petunia* flowers. (**A**) The flower of wild type line V26 consists of four organ types occupying four whorls: five green sepals (first whorl), five coloured petals (second whorl) which are fused in their lower region, forming a tube, and expand in their upper region, forming limbs, five stamens (third whorl) and carpel (fourth whorl). (**B–D**) *Petunia* flowers transgenic for 35S-*gp*. The flowers display different degrees of conversion of sepals into petaloid organs in the first whorl. (B) A transgenic flower which shows a weakly affected phenotype (line T43 which expresses a low level of the 35S-*gp* transgene, cf. Figure 1). (C) In a flower with an intermediate phenotype (line T2) a petal tube-like structure is formed in the first whorl which resembles the second whorl petal tube. The second whorl petals form additional tissue with an inside-out orientation (open arrow in panel C; see also panels Gi–iii). (D) Flower of line T5 which shows a severely affected phenotype and the highest level of transgenic *gp* expression (cf. Figure 1). Note the formation of petaloid organs that in addition have tissue which resembles the second whorl petal limb; however, sepal tips can still be seen. (Black arrows in panels A–D point to the base of the flowers for orientation.) (**E**) Comparison of first whorl organs: wild type sepal (right) and transgenic petaloid organ (left). (**F**) Cross-section of a young wild type flower bud, displaying four concentric whorls: sepals (se), petals (p), stamens (st) and carpel (c). In the second whorl the petals have fused forming a tube. (**G**) A series of cross-sections of T5 flower buds to elucidate the origin of the additional petal tissue: (i) neighbouring petals failed to fuse connately but continued to grow laterally, producing additional tissue that became inverted; (ii) fusion occurred at the original position; (iii) the petal appendage expanded and became detached from the petal limb.

conditions. In contrast to the first whorl transformation of sepals into petaloid organs, there was no conversion of carpel into stamens in the fourth whorl of the transgenic flowers.

The above described phenotypes of petaloid first whorl organs and additional petal tissue are inherited by the progeny of the transgenic plants.

Early gp expression is needed for homeotic conversion of sepals

The following detailed analysis of *gp* expression was carried out with line T5 which shows a high level of transgenic *gp* expression and a severely affected phenotype. RNA was isolated from almost mature flowers which had been dissected into different whorl organs. In particular, the petaloid organ of the first whorl was dissected into sepal and petal tissue. High levels of RNA from the *gp* transgene were detected in all tissues analysed: leaves, converted petal tissue, petals, stamens and carpel and in particular in the non-converted parts of the sepals (Figure 3), confirming that the 35S promoter directs transcription in the different floral organs as well as in non-floral tissue of mature plants.

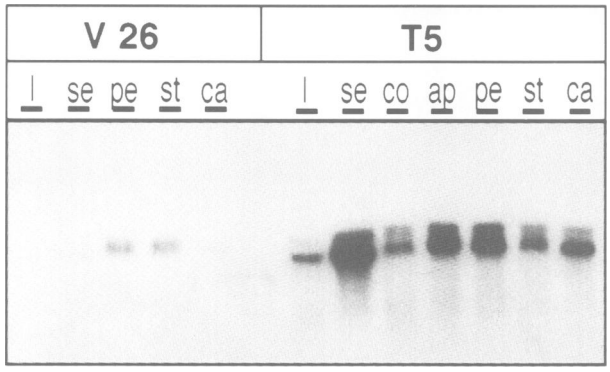

Fig. 3. Northern blot analysis of *gp* expression in wild type and transgenic flowers. RNAs were isolated from leaves (l), and from flowers dissected (before opening) into sepals (se), petals (pe), additional petal tissue (ap), stamens (st) and carpel (ca). The transgenic petaloid organs were separated into sepal tips (se) and converted petal tissue (co). In wild type flowers (V26, left panel) *gp* is expressed in petals and stamens, whereas in line T5 (right panel) transgenic *gp* transcript is detected in all tissues. In T5 RNA samples, the major and minor signals presumably represent *gp* transcripts with different lengths of poly(A) tail.

As described above, the conversion of sepals into petals was not complete. This was surprising, because the level of *gp* RNA was highest in the green tips of the sepals when compared with all other tissues analysed (Figure 3, lane T5, se), yet no conversion was observed. An explanation for this may be found in the expression profile of the CaMV 35S promoter. Although referred to as constitutive, this promoter contains regulatory upstream promoter elements that confer cell-type specific expression (Benfey and Chua, 1990). We have analysed the expression patterns of the 35S promoter during development of *Petunia* sepals. 35S promoter activity was not detectable in very young sepals (Figure 4A–C), indicating that this promoter is not active during the initiation of sepals. Later in development expression can be detected in the lower part of young sepals (Figure 4D, left se). At a slightly later developmental stage (Figure 4D, right se) 35S-directed expression was first seen in the tip of the sepal and then spread downwards, accompanied by a strong reduction in expression at the base of the sepal (Figure 3, compare 'co' and 'se' lanes and Figure 4D). This expression pattern of the 35S promoter seems to be independent of the position of the insertion into the *Petunia* genome as it is seen with transgenic plants which express 35S-*gp* (monitored by *in situ* hybridization) and 35S-GUS (shown by GUS staining). The initial absence and the subsequent turning on of 35S promoter activity in the developing sepals result in the observed mosaic first whorl organ. From this observation we expect that the first whorl would be completely converted to a petal if the transgene were expressed early enough in development, presumably at the time of whorl initiation. In the other organs, petals, stamens and carpel, *gp* transcripts were detected already at very early stages of development (Figure 4A).

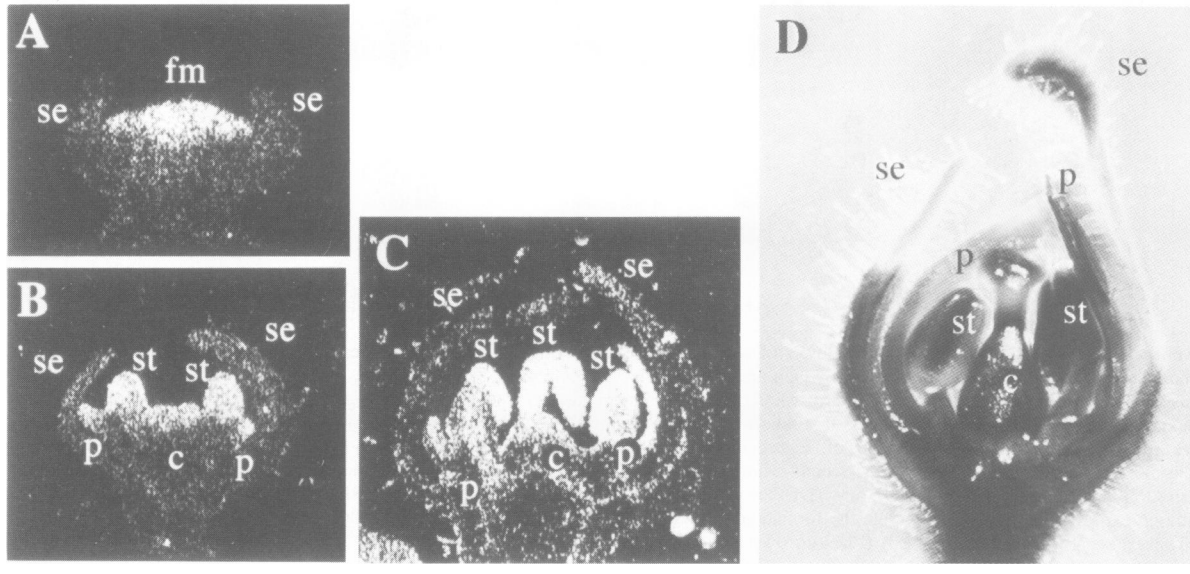

Fig. 4. (**A–C**) Expression of *gp* in young T5 flower buds. Longitudinal sections during development: (A) at an early stage, sepal primordia and floral meristem (fm) are seen. (B) shows young sepals, petal- and stamen- and carpel primordia; (C) shows a later stage of development. The expression pattern was determined by *in situ* hybridization to longitudinal sections using a ^{35}S-radiolabelled *gp* specific antisense RNA probe. Very young sepals do not display *gp* expression conferred by the 35S promoter whereas *gp* expression in the fourth whorl is detectable already at the initiation of the carpel. (**D**) 35S promoter expression pattern in a young flower bud using β-glucuronidase (GUS) as a reporter gene. The pattern of 35S promoter activity monitored by GUS staining changes with the development of the sepals. The sepal to the left (se) shows strong expression in the lower part but no expression in the upper part, whereas the sepal to the right, which is slightly further developed, displays expression in the tip which is maintained in the mature sepal at a high level (see also Figure 3, lane T5 se).

gp activates its own expression

A quantitative analysis of *gp* expression levels using primer extension analysis on total RNA isolated from almost mature flowers allowed us to determine the expression levels of endogenous and transgenic *gp* RNA. The results are consistent with those obtained from the Northern analysis. The above-mentioned high levels of transgenic *gp* transcript in all transgenic floral organs are confirmed (Figure 5A). Wild type and T5 flowers express endogenous *gp* in petals and stamens. In a prolonged exposure very weak signals for endogenous *gp* transcript can be detected in sepals and carpel as well (Figure 5A). This analysis also shows endogenous *gp* expression in petal tissue of the first whorl (Figure 5A, 'con' lane) at a level comparable to that found in petals (second whorl). We conclude that in the converted tissue ectopically expressed GP leads to the up-regulation of endogenous *gp* expression from the low levels detectable in sepals to the level found in the petals of the second whorl. These results demonstrate that GP can activate its own expression. Because no up-regulation of the endogenous *gp* expression was seen in non-petal tissue, this process likely involves tissue specificity provided by other factors.

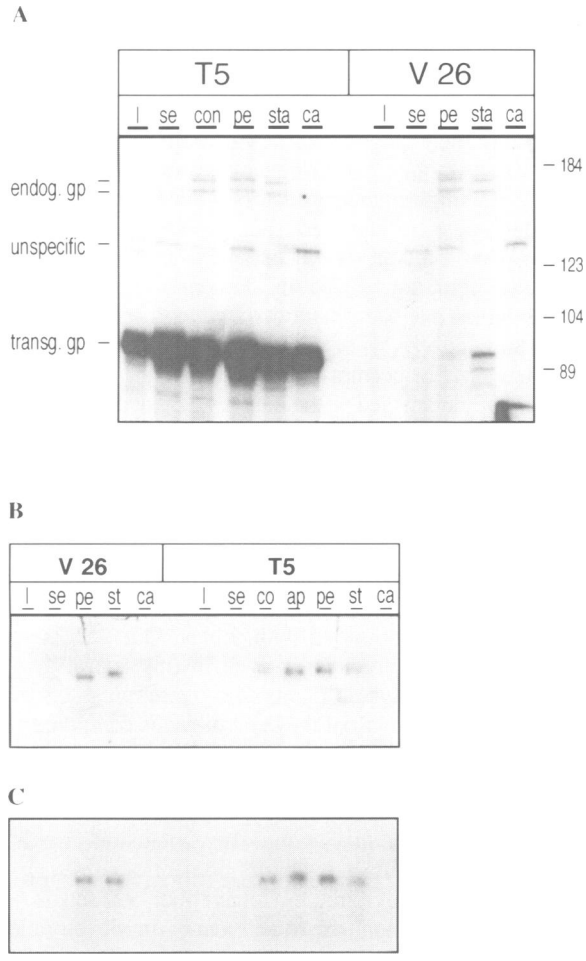

Fig. 5. (**A**) Detection of *gp* transcripts in wild type and line T5. Autoradiograph of primer extension analysis of *gp* transcript of RNA isolated from V26 and line T5. RNA samples were as in Figure 3. Endogenous *gp* expression in petals and stamens and transgenic *gp* pattern were as in Figure 3, but additionally, the first whorl converted petal tissue ('con' lane) shows a signal for the endogenous *gp* transcript. (Signals in the V26 'sta' lane which are of a similar size to the transgenic *gp* in T5 are nonspecific as they do not comigrate with the transgenic *gp* signals when the gels were run for a longer period.) A longer exposure shows very weak signals for the endogenous *gp* RNA in sepals and carpel in V26 as well as in sepal tissue and carpel in line T5. (**B**) Activation of *pmads2* by ectopic *gp* expression. Autoradiograph of a Northern blot filter hybridized to a *pmads2* gene specific probe using RNA samples as in Figure 3. A 3′ fragment of the *pmads2* cDNA (Kush *et al.*, 1993) excluding the MADS-box was used as a probe. *pmads2* RNA was detected in petals and stamens and also in the first whorl petal tissue (co). (**C**) Activation of *fbp1* by 35S-*gp*. Northern blot analysis probed for *fbp1* (Angenent *et al.*, 1992) expression with a gene specific probe. RNAs like in (B). *fbp1* RNA was detectable in petals and stamens and as well in the first whorl petal tissue (co).

Ectopic gp expression activates fbp1 and pmads2

Two other *Petunia* MADS-box genes, *fbp1* and *pmads2*, have been isolated which are expressed in petals and stamens (Angenent *et al.*, 1992; Kush *et al.*, 1993). Both show sequence homology to *glo*, the other B function gene in *Antirrhinum*. In the plants transgenic for 35S-*gp*, expression of *pmads2* and *fbp1* was also detected in the converted petal tissue of the first whorl (Figure 5B and C, 'co' lane) indicating their activation by the ectopically expressed *gp*.

Discussion

A single homeotic gene, gp, is sufficient for petal determination in the first whorl

Genetic studies on floral mutants in *Arabidopsis* and *Antirrhinum* have suggested that three functions, A, B and C, determine floral organ identity by their sole or combinatorial action. Expression of the A function alone, for example, results in sepal identity whereas the combination of the A and B functions determines petal identity. In the two plant species studied so far the B function requires at least two genes. Here, we show that ectopic expression of the *Petunia* MADS-box gene *gp* results in conversion of sepals into petaloid organs. This homeotic conversion provides evidence that ectopic expression of a single B function gene, the *gp* gene, is sufficient for petal determination in a whorl that normally assumes sepal identity. That the ectopic expression of a single homeotic gene in *Petunia* is sufficient to cause this conversion is surprising, since the petal determining B function in both *Antirrhinum* and *Arabidopsis* requires the expression of at least two genes. The observed conversion strongly suggests either that other petal determining gene(s) must be regulated in part by *gp* and ectopic expression of *gp* activates these genes as well or that petal determination in *Petunia* requires only one gene. Two other *Petunia* MADS-box genes, *pmads2* and *fbp1*, which show sequence homology to another *Antirrhinum* B function gene, *glo*, are activated in the converted petal tissue of the first whorl in plants harbouring the 35S-*gp* transgene. This observation might support the first hypothesis, namely that *pmads2* and/or *fbp1* are involved in petal determination in *Petunia*, but further functional analysis of these genes will be necessary to establish their roles in *Petunia* flower development.

Although *gp* is sufficient for the homeotic conversion of sepals into petaloid organs in the first whorl, the fourth whorl is not affected as no staminoid organs develop there, which might have been expected as conversion of the first whorl occurs. Even though the *Petunia* mutant *gp*(PLV), which lacks the *gp* gene, develops stamens, the possibility cannot be excluded that the stamen determining role of *gp* has been supplanted by another MADS-box gene in the mutant

background. Our results using ectopic *gp* expression show that *gp* can determine petal development in the first whorl but cannot determine stamens in the fourth whorl, confirming that the *gp* gene is essential only for petal development and plays at most a minor role in stamen development. Analysis of floral development in other plant species should show whether the organization of the B function in *Petunia* is exceptional among flowering plants.

Early gp expression is essential for petal determination in the first whorl

The mosaic nature of the petaloid organ of the first whorl provides clues as to the timing of *gp* expression for tissue determination, because the delayed expression of 35S-*gp* leads to the formation of sepal tissue on top of the converted organ. Although in these sepaloid cells *gp* transcript was detected at very high levels later in development, no conversion into cells displaying petal features occurred. The lack of *gp* expression at early stages of organ development therefore results in sepal identity, indicating that in the first whorl there is only a certain developmental 'time window' during which petal determination is possible.

Late differentiation of a sector of petal tissue in second whorl sepaloid organs has been reported recently in *gp* plants that express a 35S-*gp* transgene (van der Krol *et al.*, 1993). However, an alternative explanation (as opposed to late differentiation) might be that these sectors result from the mosaic expression of the 35S transgene during early organ determination, so that some cells express the transgene early, giving rise to petaloid sectors, whereas neighbouring cells may not express the transgene, resulting in a mosaic organ with sepaloid sectors next to petaloid sectors. The use of an inducible promoter for *gp* expression or a temperature sensitive *gp* allele should be helpful for investigating whether trans-differentiation of sepal to petal tissue can occur in *Petunia* at late stages of development.

Although *gp* transcript is detected in vegetative as well as in floral organs in the transgenic plant, conversion to petal tissue occurs only in the first floral whorl and is not detected in, for example, leaves. This means that the cells of the first and second whorl are distinguished from non-floral cells by the expression of one or more genes. This may include gene(s) for the A function, which are presumably activated in the first and second whorls during the transition from a vegetative meristem to an inflorescence and floral meristem (Irish and Nelson, 1991; Coen *et al.*, 1990; Mandel *et al.*, 1992b; Weigel *et al.*, 1992; Weigel and Meyerowitz, 1993). This predetermining process would result in the activation of the A function in the primordia of the first and second whorls. The presence or absence of GP in either of these two whorls then acts as the key signal for the tissue to develop into either petals or sepals as proposed by the combinatorial features of the flower model.

Autoregulation of gp in petal tissue

In the first whorl petal tissue of the transgenic flower, ectopically expressed GP activates endogenous *gp* expression. Because GP is a MADS-box-containing protein and therefore may be a transcription factor, one simple model for autoregulation is that GP acts directly on its own promoter; however, autoregulation could also be indirect, involving other factors. Autoregulation in *Drosophila* is understood as a mechanism for maintaining the original positional information established by an external signal, ensuring a long-term cellular memory (Bienz and Tremml, 1988; Kuziora and McGinnis, 1988; Jiang *et al.*, 1991). In *Petunia* high levels of *gp* RNA are found throughout petal development. A role for *gp* expression later in development may be indicated by *Petunia* flowers transgenic for antisense *gp* RNA which display faintly coloured or white sectors in the petals because of reduced anthocyanin pigmentation (unpublished observation). Our data suggest that autoregulation may be the molecular basis for maintaining *gp* expression. Autoactivation of the *gp* expression is suggested for the second whorl petal tissue as well, because, in the transgenic plant, the first whorl converted petal tissue has developed in a way similar to the second whorl petal tissue in the wild type plant, namely by providing GP protein in a whorl which would otherwise assume sepal identity. Because this autoregulation is limited to petal tissue (no activation of the endogenous *gp* by ectopically expressed *gp* was found in sepals, carpel or leaves), other positively or negatively acting regulatory factors which determine tissue specificity must be involved. We propose a petal and/or stamen specific factor, as our experiments provide no clue as to autoregulation of *gp* in stamens. In *Antirrhinum*, autoregulation has been demonstrated as a mechanism for maintaining high levels of *def*A and *glo* expression in petals and stamens. Furthermore, genetic analysis of homeotic *Antirrhinum* mutants led to the conclusion that autoregulation of *def*A and *glo* requires an interdependent action of the DEF A and GLO proteins on each other (Schwarz-Sommer *et al.*, 1992; Tröbner *et al.*, 1992).

The phenotype caused by *gp* overexpression shows that too high GP levels may disturb normal petal development, as additional petal tissue is formed and the fusion to a tube is delayed. Petal development is characterized by increased lateral growth as compared with sepals. Our data suggest that GP could be directly involved in this lateral growth process, since transgenic plants that overexpress *gp* show additional lateral growth of the petal tissue as compared with the wild type plants, leading to the additional petal tissue.

Our results demonstrate the usefulness of ectopic expression as a tool for studying the mechanisms governing flower development in *Petunia*. In conclusion, we have shown that in *Petunia* a single homeotic gene, *gp*, is sufficient to induce homeotic conversion of sepals into petaloid organs when expressed early in development. Furthermore, our data show that *gp* is autoregulated and can activate the expression of two other MADS-box genes.

Materials and methods

Transgenic plants

The *gp* cDNA (Kush *et al.*, 1993) was inserted into the *Sma*I site of pMon 530 (Cuozzo *et al.*, 1988), under the control of the CaMV 35S promoter (Benfey and Chua, 1990) and the polyadenylation signal of the *rbcS-E9* gene. Leaf discs of *P.hybrida* line V26 were transformed using *Agrobacterium*-mediated gene transfer (Horsch *et al.*, 1985).

Northern analysis

Total RNA was isolated from almost mature flowers (just before opening) which were dissected into the different organ parts. The tissues were homogenized under liquid nitrogen, transferred to extraction buffer containing 8 M guanidine hydrochloride and extracted with acidic phenol. RNA was ethanol precipitated, dissolved in water and purified from polysaccharide contaminants using the CTAB method (Murray and Thompson, 1980).

Aliquots of 25 μg total RNA were size-fractionated on a 1.5% agarose gel containing 6% formaldehyde and blotted to Hybond membrane

(Amersham). The membrane was hybridized to ^{32}P-radiolabelled gene specific probes of three different *Petunia* MADS-box genes. For *gp* an *Eco*RI−*Sma*I 3′ fragment (0.7 kb) of the *gp* cDNA was used, for *pmads2* a *Cla*I−*Sma*I 3′ fragment of the *pmads2* cDNA was used and for *fbp1* the gene specific fragment was amplified by PCR. Sequences encoding the MADS-box were excluded in all three cases.

Primer extension analysis

Primer extension reactions were carried out using either the U2 oligonucleotide primer, 5′-CTTGATCTGGATCTTTCCACGAGCC-3′ (Figure 1), or the U7 primer, 5′-GGTTTTCTATTCTCTTGATCTGGA-TCTTTCCACGAGCCATAGTG-3′ (Figure 5). The oligonucleotide primer was kinased with [γ-^{32}P]ATP, gel-purified and coprecipitated with aliquots of 30 μg total RNA. Annealing was performed in 80% formamide at 28°C overnight. M-MLV H$^-$ reverse transcriptase (Gibco-BRL) and reaction buffer were used as recommended by the supplier. The extension reaction was performed at 48°C for 2 h and stopped by RNase treatment. The products were size-fractionated on an 8% acrylamide sequencing gel and autoradiographed.

In situ hybridization

Longitudinal sections of paraffin-embedded tissue were prepared and treated for *in situ* hybridization essentially as described by Cox and Goldberg (1988). A 0.7 kb *gp* specific *Eco*RI−*Sma*I 3′ *gp* fragment was cloned into the pBluescript II KS polylinker. An *Eco*RI-linearized template was used for *in vitro* transcription with T7 RNA polymerase (Boehringer) in the presence of [^{35}S]UTP. The RNA was hydrolyzed to 80−100 nt and used for *gp* specific *in situ* hybridization. The sections shown were exposed for 7−10 days.

GUS staining

Young *Petunia* flower buds from plants transgenic for 35S-GUS were stained with X-glucuronide as a substrate using standard techniques (Jefferson *et al.*, 1987).

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