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Divergence of Function and Regulation of Class B Floral Organ Identity Genes

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Regulatory mechanisms controlling basic aspects of floral morphogenesis seem to be highly conserved among plant species. The class B organ identity genes, which are required to establish the identity of organs in the second (petals) and third (stamens) floral whorls, are a good example of such conservation. This work compares the function of two similar class B genes in the same genetic background. The *DEFICIENS* (*DEF*) gene from Antirrhinum, including its promoter, was transformed into Arabidopsis and compared in function and expression with the Arabidopsis class B genes *APETALA3* (*AP3*) and *PISTILLATA* (*PI*). The *DEF* gene was expressed in the second, third, and fourth whorls, as was *PI*. Functionally, *DEF* could replace *AP3* in making petals and stamens. The *DEF* gene's *AP3*-like function and *PI*-like expression caused transformation of fourth-whorl carpels to stamens. Like *AP3*, all aspects of *DEF* function in Arabidopsis required a functional PI protein. Surprisingly, DEF could not replace the AP3 protein in properly maintaining *AP3* transcripts (autoregulation). Our data allow us to revise the current model for class B autoregulation and propose a hypothesis for the evolution of class B gene expression in dicotyledonous plants.

INTRODUCTION

Antirrhinum and Arabidopsis are distantly related dicotyledonous species (occupying different subclasses of Dicotyledoneae) with similar but distinct floral morphologies. The analysis of floral homeotic mutants in both species has permitted the identification and characterization of many regulatory genes that direct floral morphogenesis. Surprisingly, on the basis of recessive mutant phenotypes, amino acid sequence identity, and patterns of transcript distribution, the set of floral regulatory genes identified in Arabidopsis is almost identical to the set identified in Antirrhinum. Thus, the regulatory mechanisms controlling basic aspects of floral morphogenesis seem to be highly conserved (reviewed in Davies and Schwarz-Sommer, 1994; Ma, 1994; Weigel and Meyerowitz, 1994; Haughn et al., 1995).

Among the best-studied floral regulatory genes are the class B organ identity genes, which are required in part to establish the identity of organs in the second (petal) and third (stamen) floral whorls. Two class B genes are known for both Antirrhinum (*DEFICIENS* [*DEF*] and *GLOBOSA* [*GLO*]) and Arabidopsis (*APETALA3* [*AP3*] and *PISTILLATA* [*PI*]). Loss-of-function mutations in any one of the four genes result in similar homeotic transformations: petals to sepals and stamens to carpels (Bowman et al., 1989, 1991; Hill and Lord, 1989; Schwarz-Sommer et al., 1990, 1992; Sommer et al., 1990; Tröbner et al., 1992). All four genes have been cloned, and their nucleotide sequences were determined (Sommer et al., 1990; Jack et al., 1992; Tröbner et al., 1992; Goto and Meyerowitz, 1994; Okamoto et al., 1994; Irish and Yamamoto, 1995). Each of the genes contains a MADS box DNA binding domain (Schwarz-Sommer et al., 1990) and a K-box (potential dimerization domain; Ma et al., 1991). On the basis of the deduced amino acid sequence, AP3 is closely related to DEF (61.2% sequence identity) and PI is related to GLO (58.4% sequence identity). In contrast, the promoters of the *AP3* and *DEF* genes are significantly divergent.

The expression of the Arabidopsis and Antirrhinum class B transcripts and protein products has been studied extensively (Schwarz-Sommer et al., 1990, 1992; Jack et al., 1992, 1994; Tröbner et al., 1992; Goto and Meyerowitz, 1994; Zachgo et al., 1995; Krizek and Meyerowitz, 1996a). For all four class B genes, transcripts are initially detected in young flower primordia at the time of sepal initiation (stage 3; stages as defined by Smyth et al., 1990; Zachgo et al., 1995), but there are differences in their spatial distribution. One of the two class B genes from each organism is transcribed in the

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second-, third-, and fourth-whorl primordial cells (*DEF* and *PI*), whereas transcripts of the second class B gene are limited mainly to the second- and third-whorl primordial cells (*GLO* and *AP3*). The Arabidopsis and Antirrhinum class B genes expressed in fourth-whorl primordial cells (*DEF* and *PI*) are not considered to be orthologous genes on the basis of amino acid sequence similarity. At later stages, class B gene transcripts are maintained at a high level only in the second- and third-whorl primordial cells of developing floral shoots, with transcripts persisting in developing petals and stamens until the completion of flower development. However, low levels of transcript of some of the class B genes have been detected in first-whorl (*DEF* and *AP3*) and fourth-whorl (*DEF*) organs of flowers in later stages of development. Class B proteins become detectable in the second- and third-whorl primordial cells at stage 4, when sepal primordia have become separated from the floral apex and start to overgrow it (Jack et al., 1994; Zachgo et al., 1995). Class B proteins have not been found in the first- or fourth-whorl organ primordia.

The complexity of the expression pattern of class B genes suggests that they are highly regulated. Indeed, several genes are known to be required for this process (Schultz et al., 1991; Bowman et al., 1992; Weigel and Meyerowitz, 1993; Simon et al., 1994; Ingram et al., 1995; Levin and Meyerowitz, 1995; Sakai et al., 1995; Wilkinson and Haughn, 1995). Functional class B proteins are themselves required to maintain class B gene expression in developing second- and third-whorl organs (Schwarz-Sommer et al., 1992; Tröbner et al., 1992; Goto and Meyerowitz, 1994; Jack et al., 1994).

Genetic, molecular, and biochemical studies with class B genes from both species have helped us to understand some aspects of how the class B organ identity genes function. Early in floral development (at the time of sepal initiation), *DEF* and *GLO* genes in Antirrhinum and *AP3* and *PI* genes in Arabidopsis are transcribed in overlapping domains (Sommer et al., 1990; Jack et al., 1992; Schwarz-Sommer et al., 1992; Tröbner et al., 1992; Goto and Meyerowitz, 1994; Krizek and Meyerowitz, 1996a). In the region of overlap (second- and third-whorl primordial cells), the two different class B polypeptides specifically interact to form a heterodimeric protein complex (Schwarz-Sommer et al., 1992; Tröbner et al., 1992; Goto and Meyerowitz, 1994; Davies et al., 1996a; Riechmann et al., 1996; S.E. Kohalmi and W.L. Crosby, unpublished results). Formation of the heterodimer protein stabilizes the class B polypeptides (Jack et al., 1994; Zachgo et al., 1995; Krizek and Meyerowitz, 1996a), allows entry into the nucleus (in Arabidopsis; McGonigle et al., 1996), and permits binding to MADS domain target sequences (CarG motifs; Schwarz-Sommer et al., 1992; Tröbner et al., 1992; Riechmann et al., 1996). Targets of the heterodimeric class B protein might include the promoters of the class B genes themselves (autoregulation) because maintenance of class B transcripts depends on the availability of a functional class B protein (Schwarz-Sommer et al., 1992; Tröbner et al., 1992; Goto and Meyerowitz, 1994; Jack et al., 1994; Zachgo et al., 1995; Krizek and Meyerowitz, 1996a). The requirement of both proteins in a cell for both stability and autoregulation could explain why expression in the first or fourth whorls of just one of the pair is transient.

Despite the similarities between Arabidopsis and Antirrhinum class B floral regulatory genes, a closer look at the putative class B orthologous genes *AP3* and *DEF* shows that they differ in many ways. First, mutations in *DEF* eliminate fourth-whorl organs; yet, in Arabidopsis class B mutants, fourth-whorl organs still develop (Jack et al., 1992; Schwarz-Sommer et al., 1992). Second, temperature shift assays with temperature-sensitive mutants show a difference in the time they are required for normal stamen development (Bowman et al., 1989; Zachgo et al., 1995). Third, initial transcript expression is different: *AP3* is expressed in the base of the sepals, whereas *DEF* is initially expressed in the fourth whorl and is slightly expressed in sepals later in development (Jack et al., 1992, 1994; Schwarz-Sommer et al., 1992). Finally, although *DEF* expression depends on a functional GLO protein, transcriptional regulation of *AP3* in second-whorl organs seems to be independent of *PI* function (Schwarz-Sommer et al., 1992; Goto and Meyerowitz, 1994).

There is still little direct evidence concerning the functional equivalence of putative orthologous floral regulatory genes in general and for *DEF* and *AP3* in particular. We have introduced the *DEF* gene into Arabidopsis and compared its function with that of *AP3*. Our data show that *DEF* can complement the strong *ap3-3* mutation. Because complementation depends on the appropriate spatial and temporal expression of the *DEF* gene, the interaction of the DEF protein with PI, and the activation of the appropriate target genes, our data strongly support the hypothesis that *DEF* is orthologous to *AP3*. This work also allows us to identify clear differences between the two genes, providing new insights on class B function, maintenance, and evolution.

RESULTS

Transgenic Arabidopsis Plants Carrying the *DEF* Gene Show a Homeotic Floral Phenotype

Our primary objective was to examine the effect of introducing the genomic Antirrhinum *DEF* gene (including its own promoter) into Arabidopsis. For this purpose, we cloned a 7-kb EcoRI genomic fragment from Antirrhinum containing the *DEF* gene (Schwarz-Sommer et al., 1992) into a binary transformation vector (see Methods) with selective markers for both kanamycin resistance and glucuronidase (GUS) activity. This construct was transformed into wild-type Arabidopsis (T_1) by using Agrobacterium-mediated transformation. Three independent kanamycin-resistant T_2 transformants were examined. Kanamycin resistance and GUS activity segregated among the T_3 progeny at a frequency suggesting that each line carried three (TDF1) or one (TDF2 and TDF3) loci

with a T-DNA. DNA gel blot analyses indicated that the transformants contained an intact *DEF* gene (data not shown) with a copy number of one (TDF3), two (TDF2), or approximately six (TDF1).

Two of the three lines containing *DEF*, TDF1 and TDF2, had a floral phenotype distinct from the wild type that segregated with the kanamycin resistance marker. The phenotype, designated Tdef, was a homeotic transformation of the fourth-whorl carpels into stamens. Whereas the first emerging flowers were phenotypically similar to wild-type flowers, each successive flower displayed a stronger homeotic transformation than did the previous one. Figure 1 shows wild-type flowers and flowers with a typical Tdef phenotype from plant line TDF1. The earlier flowers were either indistinguishable from the wild type or had distinctly curved pistils (Figure 1B). Later flowers had gynoecia consisting of both stamen and carpel tissue fused together that resulted in split and distorted gynoecia (stamen–carpel organs; Figures 1C, 2J, and 2K). There was considerable variation in the ratio of stamen to carpel tissue in such flowers. Finally, for some of the latest emerging flowers in the inflorescence, the gynoecium was replaced by a variable number of stamens and stamen–carpel organs (Figure 1D), with the maximum number of extra stamens being four. Although all three floral phenotypes were seen in TDF1 and TDF2 plants, both the number of mutant flowers and the degree to which each flower was affected (expressivity) was higher in TDF1 than in TDF2.

To determine whether the severity of the phenotype was due to loci number, the TDF1 plant was outcrossed to the wild type to obtain lines segregating for only one locus. All single-loci lines showed a Tdef phenotype, proving that one locus of the *DEF* gene is enough to cause fourth-whorl transformations. One of these TDF1 derivative lines (TDF1-1), carrying two loci, was used in subsequent crosses. Significantly, the Tdef floral phenotype is similar to that of Arabidopsis floral morphogenesis mutant 10 (Flo10, also known as Superman) mutants or transgenic plants in which the *AP3* gene is under the control of the cauliflower mosaic virus 35S promoter (Schultz et al., 1991; Bowman et al., 1992; Jack et al., 1994). In both of these latter mutants, the stamen tissue in the fourth whorl has been correlated with and attributed to expression of *AP3* and *PI* in cells of the fourth whorl. One major difference between the Tdef and Flo10 phenotypes is that as one moves along the inflorescence in an acropetal direction, the Tdef floral phenotype becomes more severe whereas the Flo10 phenotype becomes less severe (Bowman et al., 1992). Unlike Flo10 mutants (Gaiser et al., 1995), the Tdef floral phenotype did not include any changes in ovule development or structure.

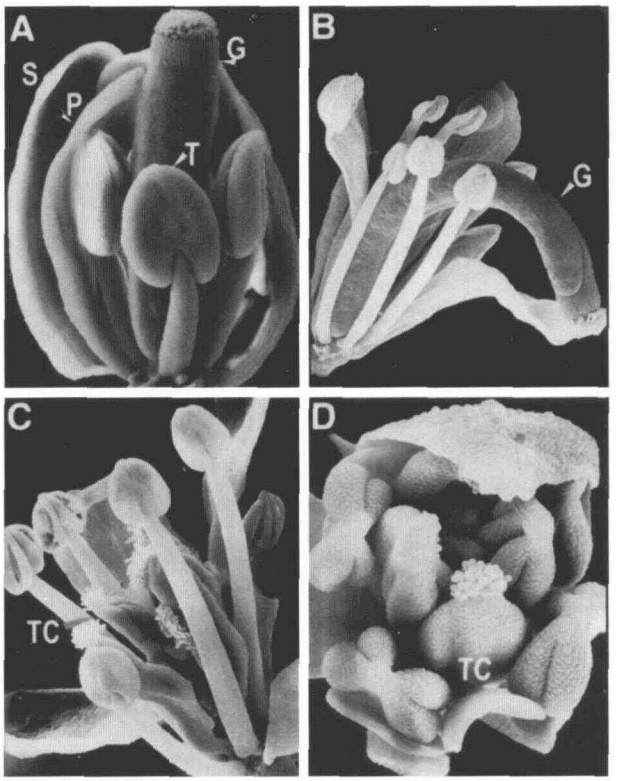

Figure 1. Scanning Electron Microscopy of Flowers from Transgenic Arabidopsis Plants Expressing the *DEF* Gene.

Some perianth organs were removed to show more clearly the reproductive organs.

(A) Wild-type flower with first-whorl sepals (S), second-whorl petals (P), third-whorl stamens (T), and a fourth-whorl gynoecium (G). Magnification is ×34.

(B) A flower from the TDF1 line showing a weak Tdef phenotype. Notice the distinctly curved gynoecium (G). Magnification is ×18.

(C) A flower from the TDF1 line with fourth-whorl stamen–carpel organs (TC). Notice the stigmatic papillae on the fourth-whorl stamen-like organs. Magnification is ×26.

(D) A flower from the TDF1 line in which the gynoecium is replaced by stamens and stamen–carpel organs (TC). Magnification is ×62.

Expression of Class B Organ Identity Genes in Transgenic Arabidopsis Tdef Plants

Transcripts of the class B organ identity genes of both Arabidopsis and Antirrhinum are present at high levels in cells of the second- and third-whorl primordia and developing petals and stamens of wild-type plants (Jack et al., 1992, 1994; Schwarz-Sommer et al., 1992; Tröbner et al., 1992; Goto and Meyerowitz, 1994). In addition, *DEF* and *PI* transcripts are present in fourth-whorl primordial cells in young floral primordia until just before the emergence of petal and stamen primordia. In Flo10 mutants or transgenic Arabidopsis plants in which the *AP3* gene is under the control of the cauliflower mosaic virus 35S promoter, the class B genes are also expressed in cells of the developing fourth whorl (Bowman et al., 1992; Goto and Meyerowitz, 1994; Jack et al., 1994).

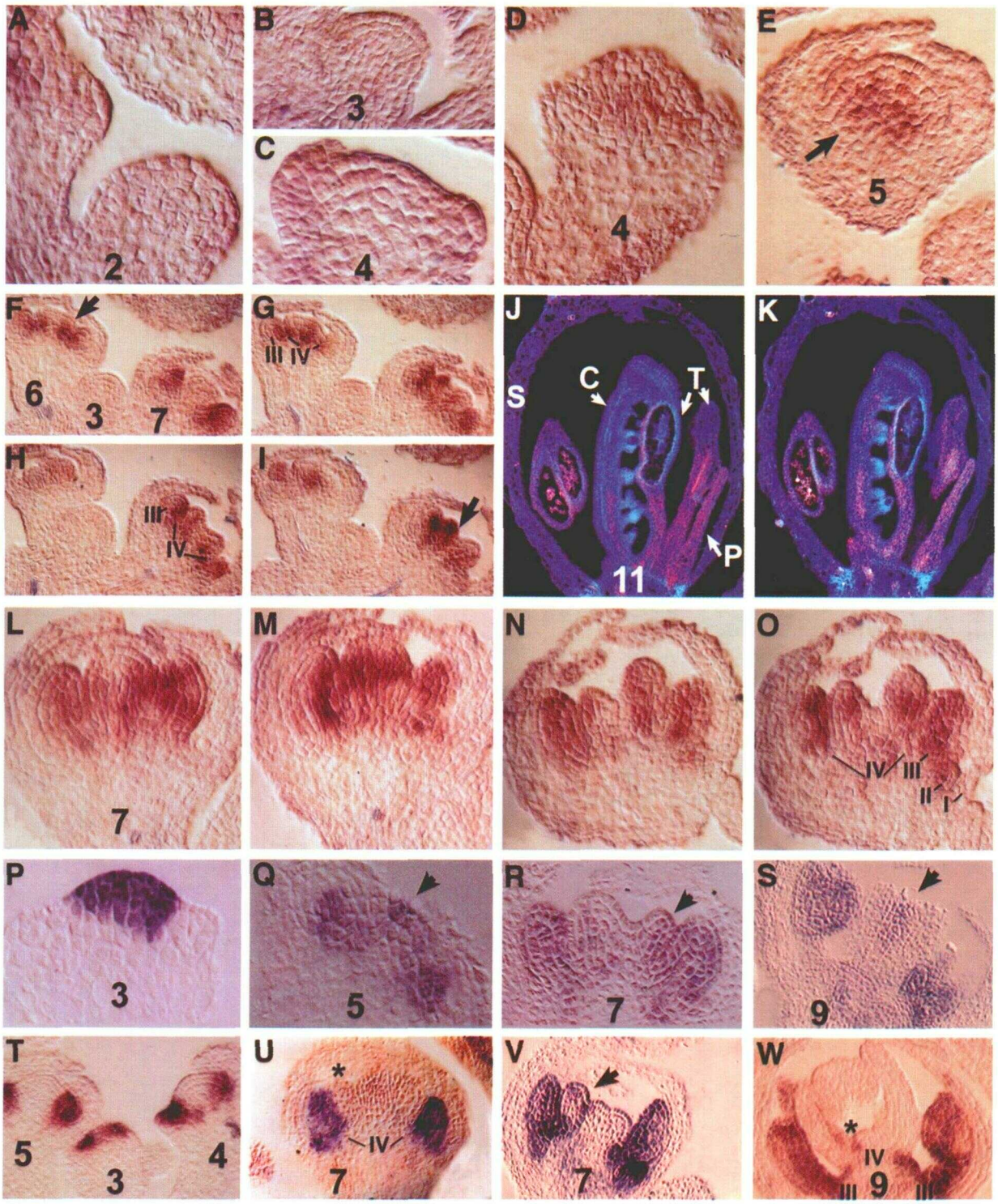

Figure 2. Distribution of Class B mRNAs in Inflorescences and Flowers of Transgenic TDF Plants.

Sections in **(J)** and **(K)** were hybridized with ^{35}S-labeled antisense *DEF* RNA. The dark-field exposure, used to detect the silver grains, is superimposed on a UV fluorescence image to visualize the underlying tissue stained with calcofluor white. All other sections were hybridized with digoxigenin-labeled antisense RNA probes and photographed under differential interference contrast optics, with the transcript signal visible as dark brown or blue. All sections, except for the one shown in **(V)** (TDF2), are from TDF1 plants. All sections are longitudinal.

In situ hybridization was used to determine the pattern of *DEF*, *AP3*, and *PI* transcripts in TDF1 and TDF2 plants (Figure 2). The *DEF* transcript was not detected in wild-type Arabidopsis flowers (control), indicating that under the hybridization conditions used, the *DEF* probe was specific for the *DEF* transcript (data not shown). In early stages of development of Tdef flowers (stages 2 to 4), no *DEF* transcript was detected above background levels (Figures 2A to 2D). The *DEF* transcript was first clearly detected in early stage 5 flowers in the center of flower primordia (Figure 2E). Later in flower development, after the initiation of petal and stamen primordia, the *DEF* probe detected transcripts in the developing second and third whorls of Tdef flowers of all stages (Figures 2F to 2O). The signal within these primordia seemed less uniform than that normally found for *AP3* and *PI* in wild-type flowers (Figures 2L to 2O). In addition, and consistent with the abnormal fourth-whorl phenotype of Tdef flowers, *DEF* was expressed in sectors of variable size in fourth-whorl primordia (Figures 2F to 2O). In mature flowers, such fourth-whorl expression was observed only in flowers with curved (small sectors) or staminoid (large sectors) fourth-whorl organs. In the latter type, the larger sectors containing *DEF* transcript always encompassed those parts of the gynoecium that were obviously staminoid (Figures 2J and 2K).

The *PI* (Figures 2P to 2S) pattern of expression in second- and third-whorl organs of Tdef flowers was similar to that of the wild type. In wild-type flowers, expression in fourth-whorl primordial cells is transient, whereas in Tdef plants, *PI* transcript was maintained in sectors of the developing fourth-whorl organs of most Tdef flowers (Figures 2Q to 2S). The *AP3* (Figures 2T to 2W) pattern of expression in second- and third-whorl organs of Tdef flowers was also similar to that of wild-type plants. Unlike *PI* and *DEF*, in most Tdef flowers, *AP3* transcripts were not detected in any fourth-whorl organs, even when they were clearly abnormal (Figure 2U). In a few Tdef flowers (~10% of flowers with abnormal fourth-whorl organs), *AP3* transcripts were detected in sectors of fourth-whorl staminoid organs (Figures 2V and 2W), with the level of signal being usually lower than that found in the same tissues of second- and third-whorl organs.

The Tdef Phenotype Is Dependent on *PI* and Is Masked by *flo10*

The results discussed above suggest that *DEF* is expressed and can influence organ identity in Arabidopsis. To determine which genes are required for the Tdef phenotype, the TDF1-1 plant was crossed to Pi and Flo10 mutant backgrounds. *pi-1* is a recessive nonsense allele that results in a relatively strong phenotype (Bowman et al., 1989; Goto and Meyerowitz, 1994). Pi-1 flowers have sepaloid second-whorl organs and third-whorl organs that are freestanding filamentous structures or carpelloid organs fused to the fourth-whorl carpels. F_2 progeny homozygous for the *pi-1* allele and carrying the *DEF* transgene were identified genetically, and their floral phenotype was characterized. These Pi-1/Tdef plants had only Pi-1 flowers, suggesting that *DEF* cannot complement a *pi* mutation and that the Tdef fourth-whorl phenotype requires *PI* function. Indeed, TDF1-1 plants heterozygous for the *pi-1* allele had primarily wild-type flowers, with only the latest flowers showing a weak Tdef fourth-whorl phenotype, suggesting that this phenotype is sensitive to the level of PI protein in the plant.

A mutation in the *FLO10* gene results in a replacement of the fourth gynoecial whorl by up to 10 stamens, with the average number of extra stamens decreasing in later flowers to 3.1 (Schultz et al., 1991; Bowman et al., 1992). Introducing *DEF* into a *flo10-1* background had no significant effect on the number of fourth-whorl staminoid organs of early and later flowers, compared with Flo10 plants (data not shown). Flo10 is therefore epistatic to the Tdef phenotype, distinguishing this case from experiments in which expressing Arabidopsis class B genes under a constitutive promoter

Figure 2. (continued).

(A) to **(O)** TDF flowers probed with *DEF* antisense RNA probe. **(A)** to **(E)** show TDF1 flowers at early stages of flower development. A clear *DEF* signal is first noticeable in the central dome of a stage 5 flower (**[E]**, arrow). **(F)** to **(I)** are serial sections through stage 3, 6, and 7 flowers. No signal can be detected in the stage 3 flower. *DEF* expression is seen in third-whorl stamen primordia and in fourth-whorl organs. Arrows point to fourth-whorl cells expressing *DEF* transcript. **(J)** and **(K)** show stage 11 flowers. Notice that the fourth whorl consists of both stamen and carpel tissue fused together and that the *DEF* transcript is located mostly in stamen and petal tissue. **(L)** to **(O)** are serial sections through a stage 7 flower. Notice that the primordia of second, third, and fourth whorls do not display uniform expression patterns of *DEF*.

(P) to **(S)** TDF flowers probed with *PI* antisense RNA probe. **(P)** shows a stage 3 flower with *PI* expression in the central dome similar to that of the wild type. **(Q)** to **(S)** show sections through stage 5, 7, and 9 flowers. *PI* is expressed in second- and third-whorl primordia as occurred in the wild type. In addition, ectopic expression in fourth-whorl organs can clearly be seen (arrows).

(T) to **(W)** TDF flowers probed with *AP3* antisense RNA probe. **(T)** and **(U)** show sections through stage 3, 4, 5, and 7 flowers. *AP3* is expressed in second- and third-whorl primordia as occurred in the wild type. Notice in **(U)** that abnormal stamen–carpel primordia (asterisk) in the fourth whorl are devoid of *AP3* signal. **(V)** shows a stage 7 flower in which the *AP3* transcript is found in cells of abnormal fourth-whorl organ primordia (arrow). Unlike this flower, most Tdef flowers showed a pattern similar to that shown in **(U)**. **(W)** shows a stage 9 flower with staminoid fourth-whorl organs. The *AP3* transcript is seen in third-whorl stamens and in a limited number of cells (asterisk) in the fourth whorl.

I, first-whorl organ; II, second-whorl organ; III, third-whorl organ; IV, fourth-whorl organ; C, carpel; P, petal; S, sepal; T, stamen. Numbers refer to stages of floral development. Magnifications are as follows: **(A)** ×417; **(B)** ×282; **(C)** ×467; **(D)** ×350; **(E)** ×310; **(F)** to **(I)** ×150; **(J)** and **(K)** ×81; **(L)** to **(O)** ×262; **(P)** ×367; **(Q)** ×350; **(R)** ×236; **(S)** ×158; **(T)** ×148; **(U)** ×147; **(V)** ×121; **(W)** ×147.

caused an enhancement of the Flo10 phenotype (Krizek and Meyerowitz, 1996a).

The *DEF* Gene Complements *ap3* Mutations

To determine whether *DEF* can functionally replace its putative Arabidopsis homolog *AP3*, crosses were made between TDF1-1 and both Ap3-1 and Ap3-3 plants. *ap3-1* is a temperature-sensitive, recessive allele resulting from a missense mutation in the K-box. The Ap3-1 phenotype is relatively weak, with sepaloid organs in the second whorl and typically freestanding carpelloid stamens in the third whorl when the plant is grown at 22°C (Bowman et al., 1989). *ap3-3* is a recessive allele resulting from a nonsense mutation in the first exon that results in a relatively strong phenotype similar to *pi-1* (Figure 3A; Jack et al., 1992). F_2 progeny homozygous for the *ap3* mutant alleles and carrying the *DEF* transgene (Ap3/Tdef) were identified genetically, and the floral phenotype was characterized. All Ap3/Tdef plants produced flowers that were intermediate in phenotype between Ap3 and Tdef flowers (Figures 3B and 3C). These modified Tdef flowers had petals or sepal–petal organs in the second whorl, normal stamens or carpel–stamens in the third whorl, and stamens or stamen–carpels in the fourth whorl. The floral phenotype varied, depending on the position of the flower on the inflorescence, so that as one moves along the inflorescence in an acropetal direction, the degree of fourth-whorl staminody and second- and third-whorl complementation increases. Later flowers had some completely normal petals and stamens in the second and third whorl (Figure 3D). These results show that the *DEF* gene can replace the *AP3* gene in making normal petals and stamens and can also cause the Tdef fourth-whorl phenotype in the absence of *AP3* activity. The degree to which *DEF* can complement depends on the position of the flower in the inflorescence. The TDF3 line, which had no floral phenotype, could not complement even the weak *ap3-1* allele (data not shown).

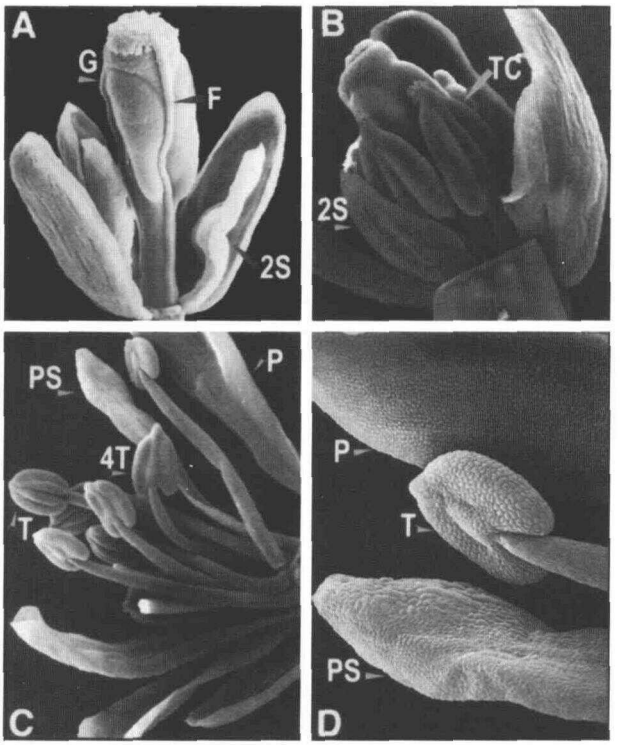

Figure 3. Scanning Electron Microscopy of Ap3-3 Flowers Complemented by *DEF*.

Some perianth organs were removed to show more clearly the reproductive organs.

(A) An Ap3-3 flower with second-whorl sepaloid organs (2S) and third-whorl filaments (F) fused to the fourth-whorl gynoecium (G). Magnification is ×20.

(B) An Ap3-3/Tdef flower with second-whorl sepaloid organs and third-whorl stamens with stigmatic papillae (TC).

(C) An Ap3-3/Tdef flower with second-whorl petals (P), second-whorl petals with sectored sepaloid tissue (PS), third-whorl stamens (T), and additional fourth-whorl stamens (4T) replacing the gynoecium. Magnification is ×16.

(D) Detailed view of the section from **(C)**, showing second-whorl petals (P), petal-sepals (PS), and normal third-whorl organs (T). Magnification is ×53.

Maintenance of the *AP3* Transcript Is Independent of Tissue Type and Dependent on the AP3 Protein

As shown in the previous section, *DEF* can replace *AP3* in making normal petals and stamens. We also wanted to determine whether *DEF* could replace *AP3* in the maintenance of *AP3* transcript levels. For this purpose, we looked at the distribution of *AP3* transcript in Ap3-3, Pi-1, and Ap3-3/Tdef plants. As previously described (Goto and Meyerowitz, 1994; Jack et al., 1994), the pattern of *AP3* transcript distribution in both mutants (Ap3-3, Figures 4A and 4B; Pi-1, Figures 4C and 4D) is similar to that in the wild type until stage 5. At later stages, continued high expression of the *AP3* transcript in a subset of cells at the base of the first and second organs can clearly be seen. Thus, unlike in previous reports (Goto and Meyerowitz, 1994; Jack et al., 1994), we could not see a clear difference in *AP3* expression between the two class B mutants. Our data suggest that some cells do not require functional class B proteins to continually express the *AP3* transcript.

The expression pattern of *ap3-3* in most Ap3-3/Tdef flowers (Figures 4E to 4L) was similar to that seen in Ap3-3 flowers. At late stages of flower development, the transcript was clearly seen in a subset of cells in developing petals but not in the developing stamens (Figures 4G to 4I). Morphologically normal stamens devoid of *ap3-3* transcript (Figure 4J) developed. Rarely were third-whorl stamens showing limited sectors of *ap3-3* expression found (Figure 4J). These results suggest that *DEF* and *PI* activity is not sufficient to maintain normal *AP3* transcription in the absence of functional *AP3* gene product.

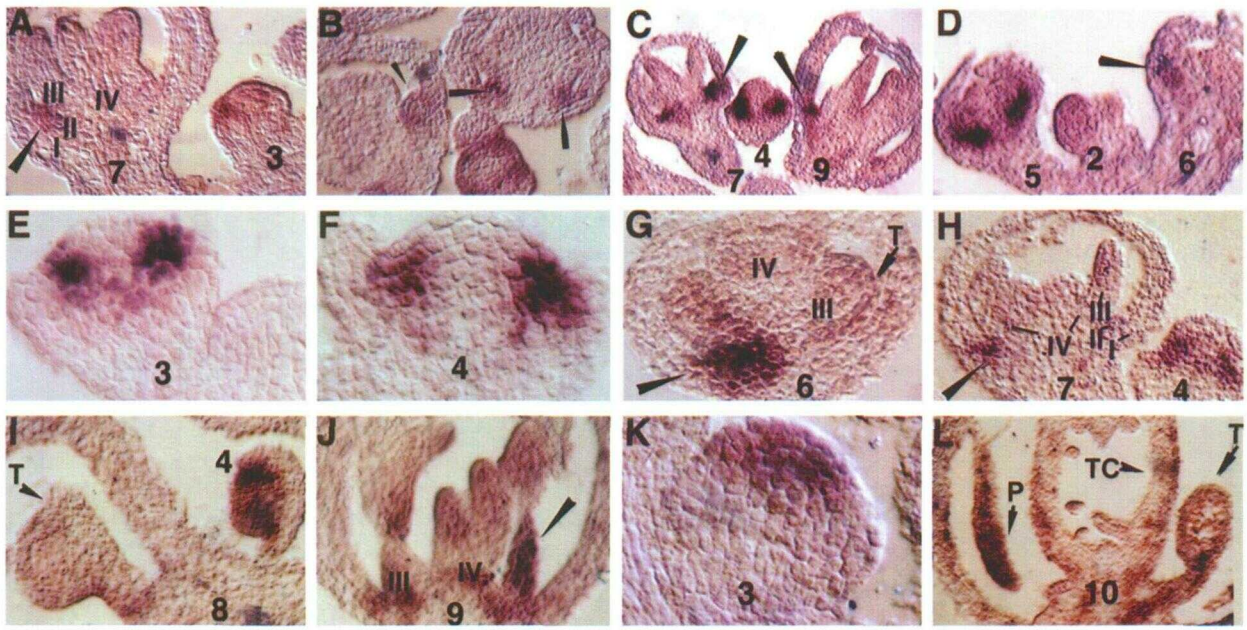

Figure 4. Distribution of *AP3* and *PI* mRNA in Mutant Backgrounds.

Sections were hybridized with digoxigenin-labeled antisense *AP3* (**[A]** to **[J]**) or *PI* (**[K]** and **[L]**) RNA probes and photographed under differential interference contrast optics. The transcript signal is dark brown. All sections except for the section shown in **(B)** (transverse) are longitudinal.

(A) and **(B)** Ap3-3 inflorescences. Expression of *AP3* is similar to that of the wild type in a stage 3 flower. Mature flowers maintain the *AP3* transcript in a subset of cells (arrowheads).

(C) and **(D)** Pi-1 inflorescences. Expression of *AP3* is similar to that of the wild type in a stage 4 flower. Mature flowers maintain the *AP3* transcript in a subset of cells (arrowheads), as occurred in AP3-3 inflorescences.

(E) to **(J)** Ap3-3/Tdef inflorescences probed with the *AP3* antisense RNA probe. Expression of *AP3* is similar to that of the wild type in stage 3 and stage 4 flowers. Mature flowers maintain the *AP3* transcript in a subset of cells (arrowheads), as occurred in AP3-3 inflorescences. In **(I)**, notice a stage 8 flower with a developing stamen devoid of *AP3* expression. A stage 4 flower with signal is shown beside it for comparison. In **(J)**, notice that in this stage 9 flower, *AP3* transcripts are also seen in a subset of cells of the third-whorl stamens (arrowhead). Unlike this flower, most Ap3-3/Tdef flowers showed a pattern similar to **(I)**.

(K) and **(L)** Ap3-3/Tdef inflorescences probed with the *PI* antisense RNA probe. In **(K)**, a stage 3 flower shows normal expression of *PI* in the central dome of the flower primordia. A mature flower with normal *PI* expression in petals and stamens is shown in **(L)**.

I, first-whorl organ; II, second-whorl organ; III, third-whorl organ; IV, fourth-whorl organ; P, petal; T, stamen; TC, staminoid–carpel. Numbers refer to stages of floral development. Magnifications are as follows: **(A)** ×150; **(B)** ×148; **(C)** ×90; **(D)** ×144; **(E)** ×365; **(F)** ×378; **(G)** ×284; **(H)** ×160; **(I)** ×136; **(J)** ×81; **(K)** ×396; **(L)** ×91.

PI transcript was not detected in developing organs of an Ap3-3 mutant (Goto and Meyerowitz, 1994). The *DEF* gene could successfully replace *AP3* in restoring normal *PI* expression in the second- and third-whorl organs (Figures 4K and 4L).

DISCUSSION

Comparison of the *DEF* and *AP3* Genes in a Common Genetic Background

The transfer of floral regulators from one plant species to a distantly related one has provided important information on gene function. In previous studies, a cDNA clone under the control of either a constitutive viral promoter (Mandel et al., 1992; Weigel and Nilsson, 1995; Davies et al., 1996b) or a promoter from the recipient species (Irish and Yamamoto, 1995) was used as a transgene. In contrast, we have compared the function of two analogous class B organ identity genes from diverse angiosperm species by introducing them into the same genetic background. A genomic clone of the Antirrhinum class B organ identity gene *DEF* complete with its endogenous promoter was introduced into Arabidopsis, and its expression and function were compared with that of the Arabidopsis *AP3* gene in wild-type and mutant backgrounds. Like *AP3*, transcription of the *DEF* gene in Arabidopsis is floral specific and maintained at high levels in petal and stamen primordia. The DEF protein can restore normal

petal development in whorl two and stamen development in whorl three of strong Ap3 mutant flowers. Like *AP3*, *DEF* function depends on a functional product made by the second Arabidopsis class B organ identity gene *PI*. Thus, for the most part, the *DEF* gene functions like the *AP3* gene. However, there were also differences between *AP3* and *DEF* function and expression in Arabidopsis. Unlike *AP3*, *DEF* is also expressed in fourth-whorl primordia, where it promotes *PI* expression and development of stamen tissue. *DEF* is also incapable of substituting for *AP3* in maintaining *AP3* transcript. In the following discussion, we propose that both of these differences result from the fact that the *DEF* gene has regulatory elements more similar to the *PI* gene than to the *AP3* gene.

Comparison of *DEF* Expression and Function in Arabidopsis Versus Antirrhinum

Both *DEF* in Antirrhinum and *PI* in Arabidopsis are expressed initially in the second-, third-, and fourth-whorl floral primordia of stage 3 and stage 4 wild-type flowers. Expression of these genes in the fourth whorl is transient because their class B partners, required for transcript maintenance and protein stability, are absent from this tissue. Our data are consistent with the idea that the *DEF* gene in Arabidopsis is regulated, as it is in Antirrhinum, with an expression pattern more similar to *PI* than to *AP3*. In Tdef Arabidopsis plants, initial expression of both *DEF* and *PI* in the fourth whorl enables persistent expression of both genes in this tissue and homeotic transformation of carpels to stamens. *DEF* expression in Arabidopsis differs from its expression in Antirrhinum in two ways. Unlike Antirrhinum, we can only detect clear expression of the *DEF* gene in Tdef plants when second- and third-whorl primordia begin to form (stage 5 flowers). Although expression in early stages was not detectable above background levels by in situ hybridization, we suspect that sufficient functional product was made by stage 4. That is because others have shown that *AP3* is required in these early stages (Bowman et al., 1989), and we know that *DEF* can successfully replace *AP3*. A simple explanation for the difference in levels of expression is that the efficiency of the *DEF* promoter in Arabidopsis is lower. We cannot rule out that not all regulatory elements required for *DEF* expression are included in the genomic fragment used.

In Tdef and Ap3/Tdef inflorescences, as one moves along the inflorescence in an acropetal direction, the degree of fourth-whorl staminody and second- and third-whorl complementation increases. This observation suggests that in Arabidopsis, *DEF* function increases throughout inflorescence development and that such an increase can overcome any deficiencies in the intrinsic ability of DEF protein to function. Similar correlations between floral phenotype and position on the inflorescence have been noted for a wide variety of floral homeotic mutants (reviewed in Haughn et al., 1995; see also Krizek and Meyerowitz, 1996a) and are consistent with the idea that there is a gradual increase in the activation of genes in the floral program throughout inflorescence development.

Maintenance of Class B Expression

Previous studies have indicated that in the absence of functional class B activity, class B transcripts fail to be properly maintained in developing second- and third-whorl organs (Schwarz-Sommer et al., 1992; Tröbner et al., 1992; Goto and Meyerowitz, 1994; Jack et al., 1994; Zachgo et al., 1995). However, our results (Figure 4) and those of others (Goto and Meyerowitz, 1994) clearly show that in Arabidopsis, expression of class B transcripts persists in some cells of the developing second and third whorls of class B mutants. Typically, in class B mutants, cells expressing *AP3* are found in the second whorl, whereas cells expressing *PI* are located in the third whorl, as if such cells have been pushed aside by those proliferating to form the second- and third-whorl organs. Such data demonstrate that in some cells, class B activity is not required for class B gene expression in the later stages of Arabidopsis floral development. It is possible that the same phenomenon is true for Antirrhinum, but in that case, the cells that keep on expressing class B genes in the mutant background are either dispersed or harder to notice because they occupy a relatively smaller part of much bigger organs.

A simple explanation for persistent class B gene expression in some cells of class B mutants is as follows. The transcription factors that initiate class B gene expression in cells of stage 3 flowers are capable of maintaining class B gene expression as long as such cells do not divide. Division of these cells dilutes the initial factor to a point at which class B expression is not maintained without the activation by a functional class B heterodimer. There is increasing evidence that class B function promotes cell division (Tröbner et al., 1992; Krizek and Meyerowitz, 1996a). In wild-type flowers, cells initially expressing both AP3 and PI would both proliferate and continue to maintain class B gene expression, ultimately giving rise to a domain occupying the second and third whorls. In Ap3 or Pi mutants, many of the cells initially expressing class B transcripts may not divide. Such cells would continue to express class B genes even in the absence of class B heterodimer but be pushed aside by cells expressing only class A or class C organ identity genes that are actively proliferating.

Ap3 and *DEF* Transcripts Are Maintained by Different Factors

We have shown that in fourth-whorl stamens of Tdef plants and second-, third-, and fourth-whorl organs of Ap3/Tdef plants, the *AP3* transcript is not maintained. Only on rare occasions can patches of *AP3* expression be detected in these organs. How can the DEF protein replace the AP3 protein in

activating petal and stamen development and yet not be capable of substituting for AP3 in the maintenance of *AP3* transcription? A simple explanation for these data would be that AP3 can bind to a site within its own promoter that DEF cannot recognize. We do not favor this hypothesis for two reasons. First, different MADS box proteins seem to bind similar CarG box sequences in vitro (Riechmann et al., 1996). Second, switching the DNA binding domain of AP3 with that of other MADS box proteins did not affect its function (Krizek and Meyerowitz, 1996b). The current model must be refined to take into account our results and the following recent observations. (1) Maintenance of class B transcript levels requires additional floral factors (Schwarz-Sommer et al., 1992; Krizek and Meyerowitz, 1996a). (2) Class B functional specificity is attributed to regions required for protein–protein interactions (Krizek and Meyerowitz, 1996b). (3) Sequence comparisons suggest that it is unlikely that DEF would be able to replace PI more successfully than would AP3 in interactions with other proteins. (4) A sequence comparison of the *DEF* and *AP3* promoters shows no similarities besides the CarG box (Irish and Yamamoto, 1995).

A revised model for maintenance of class B gene expression that is consistent with all of the information presented above is presented in Figure 5. We suggest that class B proteins indeed bind to their own promoters as heterodimers, yet maintenance of transcript levels requires an additional interaction of one of the class B proteins with a third DNA binding transcription factor (DNA binding protein). Different factors would be required for *AP3* and *PI* maintenance. *AP3* maintenance would require a factor that binds only to the *AP3* promoter at a site near the CarG box and interacts specifically with the AP3 protein (Figure 5A). A different factor, required for *PI* maintenance, would bind to a site in the *PI* promoter and interact with the PI protein. In Tdef transgenic plants (Figure 5B), the DEF protein cannot interact efficiently with the Arabidopsis *AP3*-specific factor. The *DEF* transcript is expressed and maintained in Arabidopsis in a pattern similar to *PI*. We predict that the *PI*-specific factor recognizes and binds to a site in the *DEF* promoter and interacts with PI protein to allow maintenance of *DEF*. Interestingly, a mutation (*DEF-chlorantha*) in the *DEF* promoter close to the CarG box causes severely reduced DEF transcription. In support of our hypothesis, it was proposed that this mutation defines a target sequence of the additional factor required for maintenance of *DEF* transcription (Schwarz-Sommer et al., 1992). Our model predicts that in addition to the class B heterodimer recognition element, the *DEF* and *PI* promoters should have a second *cis*-acting element in common. Such a prediction can be tested easily once the *PI* promoter sequence has been published.

The inability of *DEF* to properly control expression from the *AP3* promoter provides an explanation for results from Irish and Yamamoto (1995). These investigators showed that unlike the *DEF* genomic clone, an *AP3–DEF* cDNA fusion gene under the control of the *AP3* promoter was unable to produce any wild-type petals or stamens in an *ap3-3* background. In this case, the lack of full complementation could be explained by the DEF protein's inefficiency in activating its own transcription via the *AP3* promoter.

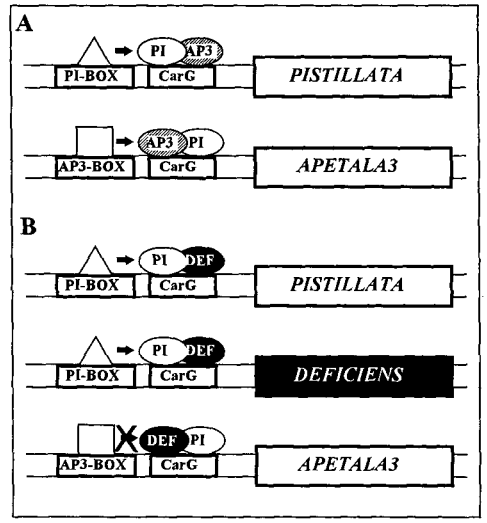

Figure 5. A Possible Mechanism for Class B Gene Maintenance.

The class B genes, with their promoters and coding regions, are depicted schematically. *cis*-Acting binding sites are depicted as boxes in the promoter regions. Open, hatched, and black circles represent the PI, AP3, and DEF proteins, respectively.

(A) Autoregulation of class B genes in wild-type Arabidopsis. Different factors bind to the *PI* and *AP3* promoters (triangle and box, respectively). Each factor interacts specifically with just one of the class B proteins of the heterodimer.

(B) Autoregulation of class B genes in transgenic Ap3-3/Tdef Arabidopsis plants. The *DEF* gene is autoregulated by the PI-specific factor. The *AP3* transcript is not maintained because DEF cannot interact efficiently with the AP3-specific factor.

A direct association between the AP3 protein and *AP3* promoter and the DEF protein and *DEF* promoter has been suggested by in vitro binding experiments (Schwarz-Sommer et al., 1992; Tröbner et al., 1992; Riechmann et al., 1996). However, it has been difficult to rule out the possibility that in vivo class B autoregulation is not direct but is controlled by a separate factor downstream of the class B genes. We have shown here that developing stamen tissue can be devoid of the *AP3* transcript, providing in vivo evidence that autoregulation is not an indirect consequence of stamen development.

Evolution of the Class B Expression Pattern

In young flowers (stages 3 and 4) of both Arabidopsis and Antirrhinum, one of the class B genes is expressed in the second-, third-, and fourth-whorl progenitor cells, whereas the other gene is expressed primarily in the second- and third-whorl progenitor cells. Expression of class B genes in fourth-whorl organs has also been found in tobacco (Davies et al.,

1996b) and tomato (Pnueli et al., 1991, 1994). The *DEF*-like petunia gene *pMADS1* appears to be an exception to this rule (Angenent et al., 1995), although spatial expression before late stage 4 has not been reported.

To date, there is no known function for class B gene expression in the center of the young floral primordium. Indeed, somatic reversions of *DEF* in the third whorl of Antirrhinum also restored fourth-whorl organs (Tröbner et al., 1992), suggesting that *DEF* transcript is not required in the fourth whorl. How could such an expression pattern have evolved? The expression domain of class B organ identity genes in ancestral angiosperm plants may have been broad and loosely regulated, with transcript levels declining gradually toward the center of the floral meristem, leading to the production of carpels. Such an expression pattern would result in flowers with many reproductive organs that vary in phenotype from functional stamen in the outermost whorls to functional carpels in the innermost whorls, with whorls of nonfunctional stamen–carpel organs in between, as has been observed in some members of the Magnoliaceae. A mutation in one of the class B genes, causing its expression domain to be restricted to the second and third whorls, would result in a flower with only one whorl of stamens followed immediately by carpel formation, as has been observed in the simple complete flowers of Arabidopsis and Antirrhinum.

Oddly, the class B organ identity genes in Arabidopsis and Antirrhinum expressed primarily in the second and third whorls (*AP3* and *GLO*) are not orthologous. This difference could be explained by the possibility that the restricted expression pattern of a class B gene evolved more than once among the angiosperms after the progenitors of these two species were separated. Alternatively, the evolution of heterogeneous expression patterns could have occurred in a common progenitor, and later on, a recombination event (illegitimate) occurred between the 5′ ends of the two class B genes, switching regulatory regions between them. Such a recombination event would result in a partially sterile plant with a Tdef-like phenotype (stamen–carpels in the fourth whorl); however, those of its progeny that were homozygous for either the parental or recombinant chromosomes would be perfectly normal. Therefore, this recombinant would not only represent a divergence in transcript patterns but could also be the beginning for the formation of new species (producing progenitors of Antirrhinum and Arabidopsis) because a cross between the parental plant and the recombinant would lead again to partial sterility. Our results suggest that the *DEF* and *PI* genes share common upstream regulatory elements, causing us to favor this second hypothesis.

METHODS

Growth Conditions

Arabidopsis thaliana plants were grown in 5-inch-diameter pots containing prepared soil mix (Terra-Lite Redi Earth; W.R. Grace & Co. Canada Ltd., Ajax, Ontario, Canada) and then transferred to growth chambers at 22°C and continuous light (90 to 120 $\mu E\ m^{-2}\ sec^{-1}$).

Plant Transformation and Strain Construction

A 7-kb EcoRI genomic fragment from Antirrhinum contains the *DEF* gene with ~4 kb upstream and 0.25 kb downstream of the transcribed sequences (Schwarz-Sommer et al., 1992). This fragment was cloned into the EcoRI site of the binary transformation vector RD1 (R. Datla, unpublished data). This vector contains a T-DNA that also encodes kanamycin resistance and glucuronidase (GUS) activity. The T-DNA was transformed into wild-type Arabidopsis (T_1) by using *Agrobacterium tumefaciens*–mediated transformation methods. TDF1 was a result of in-the-plant transformation (Katavic et al., 1994) in ecotype Columbia, and TDF2 and TDF3 were a result of root transformation (Valvekens et al., 1988) in the Landsberg *erecta* and RLD ecotypes, respectively.

TDF1 was outcrossed to Columbia, and one of the F_1 plants was designated TDF1-1. TDF1-1, segregating for two loci, was used in crosses to the following homozygous mutant strains by manual cross-pollination: Ap3-1 and Pi-1 (Bowman et al., 1989; gifts of M. Koornneef, Wageningen Agricultural University, Wageningen, The Netherlands), Ap3-3 (Jack et al., 1992; gift of E. Meyerowitz, California Institute of Technology, Pasadena, CA), and Flo10-1 (Schultz et al., 1991). The resulting kanamycin-resistant F_1 plants were allowed to self-fertilize, and the F_2 plants were analyzed for floral morphology. Genotypes were confirmed by test crosses and polymerase chain reaction amplification (to detect *DEF*). Both loci of TDF1-1 produced independent plants with a strong Tdef phenotype.

Scanning Electron Microscopy

Samples were fixed, dried, coated, and dissected as described previously (Wilkinson and Haughn, 1995).

RNA in Situ Hybridization

Gene-specific antisense probes were prepared from pD793 for *AP3* (digested with BglII; Jack et al., 1992), pcPINX for *PI* (digested with NsiI; Goto and Meyerowitz, 1994), and a plasmid containing the 3′ end of *DEF* cDNA (Sommer et al., 1990). Preparation, hybridization, and detection of ^{35}S-labeled antisense RNA were done as given in Huijser et al. (1992). Preparation of digoxigenin-labeled probes was according to the Boehringer Mannheim nucleic acid labeling kit. Tissue was fixed in FAA (3.7% paraformaldehyde, 5% acetic acid, and 50% ethanol), according to Huijser et al. (1992), and embedded in paraffin (Paraplast Plus; Sigma). Sections (8 μm) were prepared using a microtome. Sections were transferred to slides pretreated with Vectabond (Dimension Labs, Mississauga, Canada), dried at 40°C overnight, and affixed to the slides by raising the temperature of the hot plate to 56°C for 4 hr.

The in situ hybridization protocol used was a modified procedure based on that of Coen et al. (1990) and G. Drews (personal communication). Paraffin was removed by immersing slides in 100% xylene, 50% xylene–50% ethanol, and 100% ethanol for 5 min each. Sections were hydrated by immersion in 95, 85, 70, 50, and 25% ethanol and H_2O for 5 min each, treated with 2 × SSPE (300 mM NaCl, 20 mM NaH_2PO_4, 2 mM EDTA, pH 7) at 70°C for 20 min, and incubated for 20 min at 37°C with 1 μg/mL proteinase K in 100 mM Tris-HCl, pH

8, and 50 mM EDTA. Slides were then dehydrated in 25, 50, 75, 85, 95, and 100% ethanol for 5 min each and air dried at 52°C for 10 min. Hybridization was done overnight at 52°C with a digoxigenin-labeled RNA probe (2 to 20 ng) in 100 μL of hybridization buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 300 mM NaCl, 50% formamide, 7% dextran sulfate, 1 × Denhardt's solution [1 × Denhardt's solution is 0.02% Ficoll type 400, 0.02% polyvinylpyrrolidone, 0.02% BSA], 500 μg/mL tRNA, and 250 μg/mL poly(A) RNA). Slides were washed in 2 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate) for 5 min and twice in 0.2 × SSC at 52°C for 30 min.

Immunological detection of the hybridized probe was performed according to Coen et al. (1990), with a few modifications. Slides were covered for 20 min with 1 mL of 1% blocking reagent (Boehringer Mannheim) in 100 mM maleic acid, pH 7, and 150 mM NaCl. Slides were then covered for 30 min in 1 mL of buffer A (1% BSA [Sigma], 0.3% Triton X-100 [Sigma], 100 mM Tris-HCl, pH 7.5, and 150 mM NaCl). The slides were incubated for 4 hr with 1 mL of dilute (1:1200) antibody conjugate (Boehringer Mannheim) in buffer A, followed by two washes in buffer A (each for 20 min).

For the color reaction, slides were immersed twice for 5 min in substrate buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, and 50 mM $MgCl_2$) and incubated overnight with 0.5 mL of 0.34 mg/mL nitro blue tetrazolium salt and 0.175 mg/mL 5-bromo-4-chloro-3-indolyl-phosphate *p*-toluidine salt in substrate buffer in the dark. The color reaction was stopped with 10 mM Tris-HCl, pH 8, and 5 mM EDTA, and slides were viewed before (brown color) or after (blue color) ethanol dehydration, 100% xylene immersion, and coverslip mounting with Entellen (Merck). Sections were photographed under differential interference contrast optics by using a light microscope (Leitz DRB; Leica, Wetzlar, Germany) with Ektachrome 160 ASA film (Eastman Kodak).

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