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The *Arabidopsis* homeotic genes *APETALA3* and *PISTILLATA* are sufficient to provide the B class organ identity function

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SUMMARY

The class B organ identity genes, *APETALA3* and *PISTILLATA*, are required to specify petal and stamen identity in the *Arabidopsis* flower. We show here that the activities of these two genes are sufficient to specify petals and stamens in flowers, in combination with the class A and C genes, respectively. Flowers of plants constitutively expressing both *PISTILLATA* and *APETALA3* under the control of the 35S promoter from cauliflower mosaic virus consist of two outer whorls of petals and inner whorls of stamens. These plants also exhibit vegetative phenotypes that are not present in either of the singly (*APETALA3* or *PISTIL-*

LATA) overexpressing lines. These phenotypes include leaf curling and the partial conversion of later-arising cauline leaves to petals. The presence of additional floral whorls in flowers ectopically expressing *APETALA3* and *PISTILLATA* and the rescue of missing organs in class A mutants by ectopic B function suggest that *APETALA3* and *PISTILLATA* play an additional role in proliferation of the floral meristem.

Key words: *Arabidopsis*, flower development, MADS box, *APETALA3*, *PISTILLATA*

INTRODUCTION

A wild-type *Arabidopsis thaliana* flower consists of four organ types that are arranged spatially in four concentric whorls. The organ type that develops in a particular whorl has been proposed to result from the activity of three different classes of organ identity genes (the ABC model) (Bowman et al., 1991; Coen and Meyerowitz, 1991; Meyerowitz et al., 1991; reviewed in Weigel and Meyerowitz, 1994). The combination of class A genes (including *APETALA1*; *AP1* and *APETALA2*; *AP2*), class B genes (including *APETALA3*; *AP3* and *PISTILLATA*; *PI*) and class C genes (including *AGAMOUS*; *AG*) that are active in each whorl determines the identity of the organs that will later develop in that whorl. The outermost whorl of sepals is specified by A activity, the second whorl petals are specified by the combination of A and B activities, the third whorl stamens are specified by the combination of B and C activities, and the fourth whorl carpels are specified by C activity. Two additional postulates of the model are that the A and C functions repress each other, such that a mutation in either of these functions results in the expansion of the other function into all four floral whorls, and that the A, B and C activities are capable of functioning in all whorls of the flower.

All five of these organ identity genes have now been cloned, and four of them *AP1*, *AP3*, *PI* and *AG* belong to the MADS box family of transcription factors. Consistent with their proposed activities, these four genes are expressed in spatially restricted domains (Yanofsky et al., 1990; Jack et al., 1992; Mandel et al., 1992b; Goto and Meyerowitz, 1994). The fifth organ identity gene, *AP2*, is a member of another family of

proteins that share a domain (the AP2 domain), which has been shown to bind DNA in other members of this family (Jofuku et al., 1994; Weigel, 1995). *AP2* is expressed throughout floral and vegetative tissues (Jofuku et al., 1994).

Although each of these genes is clearly required for the specification of floral organs, the ABC model does not address whether they will be sufficient to specify a particular organ. Ectopic expression of *AG* (p35S-*AG*) has shown that this gene is sufficient to repress A function in the outer two floral whorls (Mandel et al., 1992a; Mizukami and Ma, 1992). Flowers of these plants contain carpelloid first whorl organs and staminoid or missing second whorl organs; flowers which closely resemble *ap2* mutants. Flowers of plants ectopically expressing *AP3* exhibit a partial conversion of fourth whorl carpels to stamens (Jack et al., 1994). Ectopic expression of *AP3* results in the persistent fourth whorl expression of the endogenous *PI* gene, and an active fourth whorl B function.

In this paper we show that ectopic expression of both class B genes together (p35S-*PI* p35S-*AP3*) results in flowers containing two outer whorls of petals and inner whorls of stamens, demonstrating that the activities of these two genes together are sufficient to provide B function. These plants also exhibit vegetative phenotypes including early flowering and leaf curling which are not found when either *AP3* or *PI* is ectopically expressed alone. The lack of any vegetative phenotype in either of the singly overexpressing lines results from the posttranscriptional regulation of *AP3* (Jack et al., 1994) and perhaps a similar posttranscriptional regulation of *PI*. Surprisingly, the ectopic expression of *AP3* and *PI* causes some of the later cauline leaves produced by the inflorescence to become petaloid. That the rosette leaves and early

cauline leaves do not undergo this transformation suggests that there may be a gradient of floral character in the plant. Ectopic B function also results in the formation of extra whorls of stamens in the center of the flower and restores the initiation of second whorl primordia which are missing in strong *ap1* and *ap2* mutants. These results reveal a role for the *Arabidopsis* B class genes in the proliferation of the floral meristem.

MATERIALS AND METHODS

Construction of p35S-PI

*Bam*HI linkers were ligated to a 960 bp *Eco*RI/*Kpn*I *PI* cDNA fragment (Goto and Meyerowitz, 1994). This piece of DNA was cloned into the *Bam*HI site of pCGN18 (constructed by Leslie Sieburth). pCGN18 contains a 1.6 kb fragment of the 35S promoter and a 1.2 kb fragment of 3' NOS cloned into the *Hind*III/*Bam*HI and *Bam*HI/*Kpn*I sites respectively of pCGN1547 (McBride and Summerfelt, 1990).

Scanning electron microscopy

Samples were fixed, dried, coated and dissected as described previously (Bowman et al., 1989, 1991). The images were photographed on Kodak TMAX film.

In situ hybridization

Flowers were fixed, embedded, sectioned, hybridized and washed as described previously (Drews et al., 1991) except that the tissue was fixed for only 1-2 hours. *AP3* and *PI* antisense probes were made by digestion of pD793 and pCPINX (Jack et al., 1994; Goto and Meyerowitz, 1994).

Immunolocalization

Flowers were fixed, embedded, sectioned and incubated as described previously (Jack et al., 1994).

GUS staining

Flowers containing pAP3-GUS (Jack et al., 1994) were stained for β -glucuronidase in a protocol adapted from Jefferson et al. (1987). The tissue was fixed for 15 minutes on ice with cold 90% acetone. The tissue was rinsed with 50 mM phosphate buffer pH 7.2 containing 0.5 mM $K_3Fe(CN)_6$ and 0.5 mM $K_4Fe(CN)_6$ and was subsequently incubated in 2 mM X-gluc in the above rinse solution for several hours at 37°C. The tissue was embedded and sectioned similarly to tissue prepared for in situ hybridization and was observed under dark-field illumination. Tissue for whole mounts was stained by incubating in 2 mM X-gluc overnight at 37°C.

Strain constructions

p35S-PI p35S-AP3 plants were crossed to various mutants by manual cross-pollination using homozygous mutant strains, except for *ag-3* and *sup-1* (for which heterozygous parents were used). The resulting p35S-PI p35S-AP3 F₁ plants were crossed again to homozygous (or heterozygous in the case of *ag-3* and *sup-1*) mutant strains. Triples (p35S-PI p35S-AP3 and the mutant) were identified in the F₂. The presence of both p35S lines could be detected (in almost all cases) by the curled leaf phenotype. The presence of the mutant in the doubly transgenic lines was confirmed by crossing of F₂ plants to homozygous mutants and/or sequencing (or digestion) of PCR reactions of leaf tissue (Klimyuk et al., 1993) that amplified the relevant gene.

RESULTS

Floral phenotype resulting from ectopic expression of *PI*

Flowers of plants ectopically expressing *PI* (p35S-PI) show a

partial first whorl conversion of sepals to petals (Fig. 1, compare A with B). The first whorl organs of p35S-PI are mosaic organs with both sepal and petal tissue. Cells at the base and margins of the organ are characteristic of petals while cells in the upper central region of the organ are characteristic of sepals. The partial first whorl transformation conferred by ectopic expression of *PI* is similar to that reported for ectopic expression of the *Petunia* class B gene, *GREEN PETAL* (Halfter et al., 1994).

Floral phenotype resulting from ectopic expression of *PI* and *AP3*

Plants ectopically expressing both class B genes were generated by crossing plants containing the p35S-AP3 construct (Jack et al., 1994) to p35S-PI plants. Flowers of these plants (p35S-PI p35S-AP3) have two outer whorls of petals and inner whorls of stamens (Figs 1C, 2E, compare with wild type, Figs 1A, 2A). These first and fourth whorl transformations are more complete than the corresponding partial transformations exhibited by p35S-PI (first whorl) or p35S-AP3 (fourth whorl). The first whorl petals of p35S-PI p35S-AP3 have no sepal tissue (Fig. 2F,G compare with wild type B,C). However, stomata, which are not found on wild-type petals, are occasionally present on these petals. The primordia of the first and second whorl organs arise as in wild type, with the second whorl primordia arising later in development, interior and alternate with the first whorl petals (Fig. 2J). The third whorl usually consists of six stamens, with additional whorls of stamens located interior to this whorl (Fig. 2K). Only rarely is carpelloid tissue present in these inner whorls (Fig. 2L). The average number of stamens in p35S-PI p35S-AP3 is 15.6 (flowers 1-20 counted on five plants, with a range from 11-23) and is greater than that found in flowers homozygous for the p35S-AP3 construct (9.1) (Jack et al., 1994). The number of stamens decreases slightly in an acropetal manner such that the average stamen number in the first five flowers is 17.5 while that in flowers 16-20 is 14.8.

The first whorl organs of p35S-PI p35S-AP3 are noticeably different from wild type by stage 8 (stages according to Smyth et al., 1990), when the surfaces of these organs consist of cells of similar shapes and sizes, while the epidermal cells of wild type show more irregularity in shape and size (Fig. 2H compare with wild type, D). The longer epidermal cells found in these wild-type flowers are the probable progenitors of the elongate cells characteristic of sepals (Bowman, 1994). The fourth whorl of p35S-PI p35S-AP3 flowers is morphologically distinguishable from wild type at stage 6. At this developmental stage in wild type, a central dome of cells is visible in the middle of the flower, which by stage 7 forms a slotted tube that will develop into the gynoecium (Fig. 2I) (Smyth et al., 1990). In a stage 6 p35S-PI p35S-AP3 flower, cells in the center of the floral meristem have already initiated small bulges (usually three or four) that will develop into another whorl of stamens (Fig. 2J). Additional whorls of stamens may form interior to these fourth whorl stamens at a later time in the development of the flower (Fig. 2K).

Vegetative phenotypes resulting from ectopic expression of *PI* and *AP3*

Ectopic expression of both class B genes causes vegetative phenotypes that are not present in p35S-AP3 or p35S-PI. p35S-

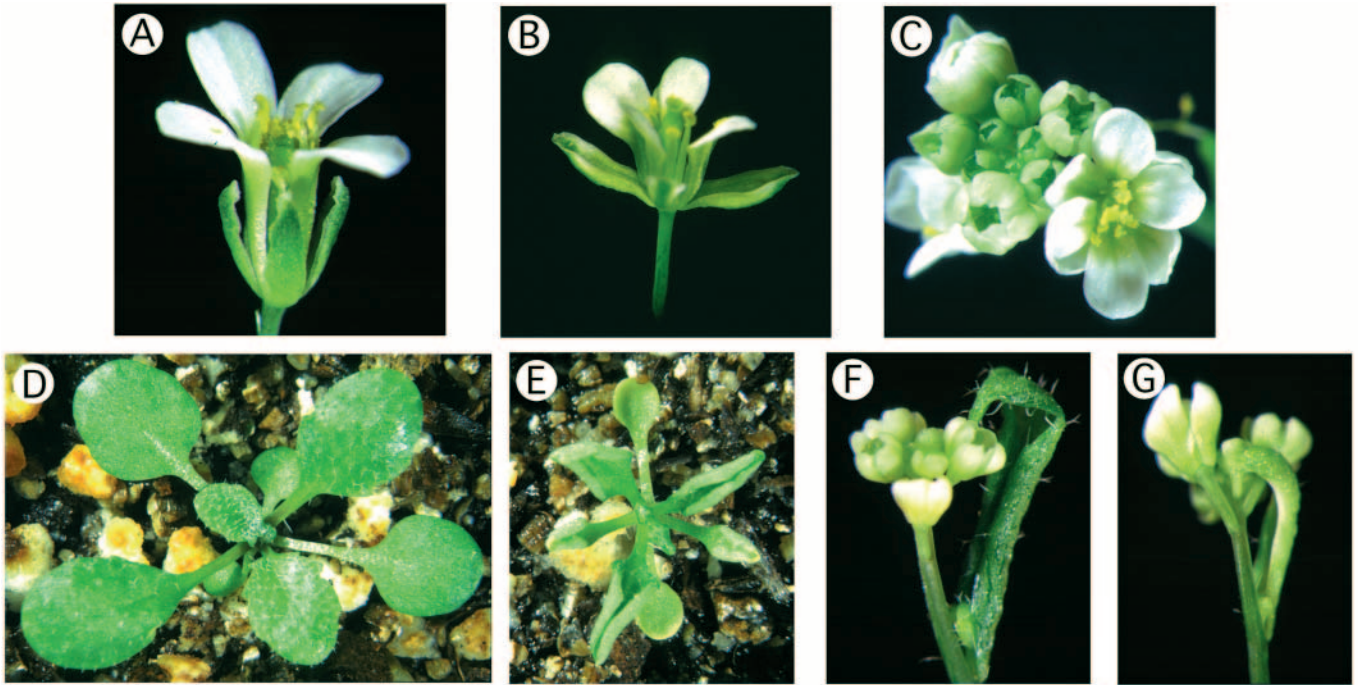


Fig. 1. Phenotypes of wild-type, p35S-PI, and p35S-PI p35S-AP3 plants. (A) Wild-type flower. (B) Flower of p35S-PI. (C) Inflorescence of p35S-PI p35S-AP3. (D) p35S-PI plant which has a normal vegetative appearance. (E) p35S-PI p35S-AP3 plant showing the curled leaf phenotype. This plant is the same age as that shown in D. (F) Normal cauline leaf and inflorescence of p35S-PI p35S-AP3. (G) Petaloid cauline leaf found on p35S-PI p35S-AP3. These leaves are smaller and more elongate than normal cauline leaves and often have white tissue on the margins of the organ.

PI p35S-AP3 plants are spindly and early flowering, and the leaves of these plants (both rosette and cauline) are smaller and curled around the midrib (Fig. 1, compare D with E). This curled leaf phenotype is similar to that observed in plants that ectopically express *AG* (p35S-*AG*) (Mizukami and Ma, 1992) and in mutations in *curly leaf* (*clf*), which result in ectopic expression of *AG* in both leaves and flowers (Coupland et al., 1993; J. Goodrich, G. Coupland, and E.M.M., unpublished data). This leaf curling appears to be at least somewhat environmentally controlled since reduced leaf curling is observed at lower temperatures (16°C) and in short days (8 hours of light) (data not shown).

An additional leaf phenotype caused by p35S-PI p35S-AP3 is the partial transformation of some of the cauline leaves to petals. The petaloid cauline leaves are smaller and thinner than normal cauline leaves, and often have a white fringe of tissue along the curled edges of the leaf (Fig. 1, compare F with G). The morphology of cells on the abaxial surface of these organs are leaf-like (Fig. 2M) except for the cells along the leaf edges which are small and round in appearance with the cuticular thickenings characteristic of the abaxial surface of a petal blade (Fig. 2N,O). The adaxial surface of these organs consists of small roundish cells which do not resemble the puzzle-shaped cells of cauline leaves and are somewhat similar to cells on the adaxial surface of a petal blade, although they are not ridged and the cuticular thickenings are less pronounced (Fig. 2P).

AP3 RNA and protein and PI RNA are detected throughout p35S-PI p35S-AP3 plants

To confirm that both *AP3* and *PI* are constitutively expressed throughout p35S-PI p35S-AP3 plants, in situ hybridization and

immunolocalization studies were performed. *AP3* RNA (Fig. 3, compare A with B) and *PI* RNA (Fig. 3, compare C with D) were detected throughout the flower and inflorescence stem of p35S-PI p35S-AP3. Immunolocalization using an AP3-specific antibody shows that AP3 protein (Fig. 3, compare E,F with G,H) is distributed throughout the flowers and stem of p35S-PI p35S-AP3 plants, analogous to the distribution of *AP3* RNA. This correlation between the *AP3* RNA and protein distributions in p35S-PI p35S-AP3 is in contrast to that found in p35S-AP3 (Jack et al., 1994). Although *AP3* RNA was detected throughout the inflorescence and vegetative tissues of p35S-AP3, AP3 protein was only detected at high levels in the second, third, and fourth whorls of the flowers, suggesting that *AP3* was posttranscriptionally regulated in p35S-AP3 (Jack et al., 1994). A similar posttranscriptional regulation of *AP3* was observed in the second whorl of a *pi-1* mutant, suggesting that *PI* was involved not only in the transcriptional regulation of *AP3* but also posttranscriptionally (Jack et al., 1994). Here we show that upon addition of ectopic *PI* expression (in p35S-PI p35S-AP3), AP3 protein is now detectable in all places where *AP3* RNA is expressed, including the cauline leaves, the inflorescence stem, and in all regions of the flowers. This result provides evidence that *PI* is sufficient for the posttranscriptional control of AP3, perhaps by stabilizing AP3 protein in an AP3/PI heterodimer, since AP3 and PI are known to interact in solution (Goto and Meyerowitz, 1994).

Phenotypes of p35S-PI p35S-AP3 in floral mutants

Class A mutants

ap1-1

Flowers of *ap1-1* exhibit both inflorescence and floral defects.

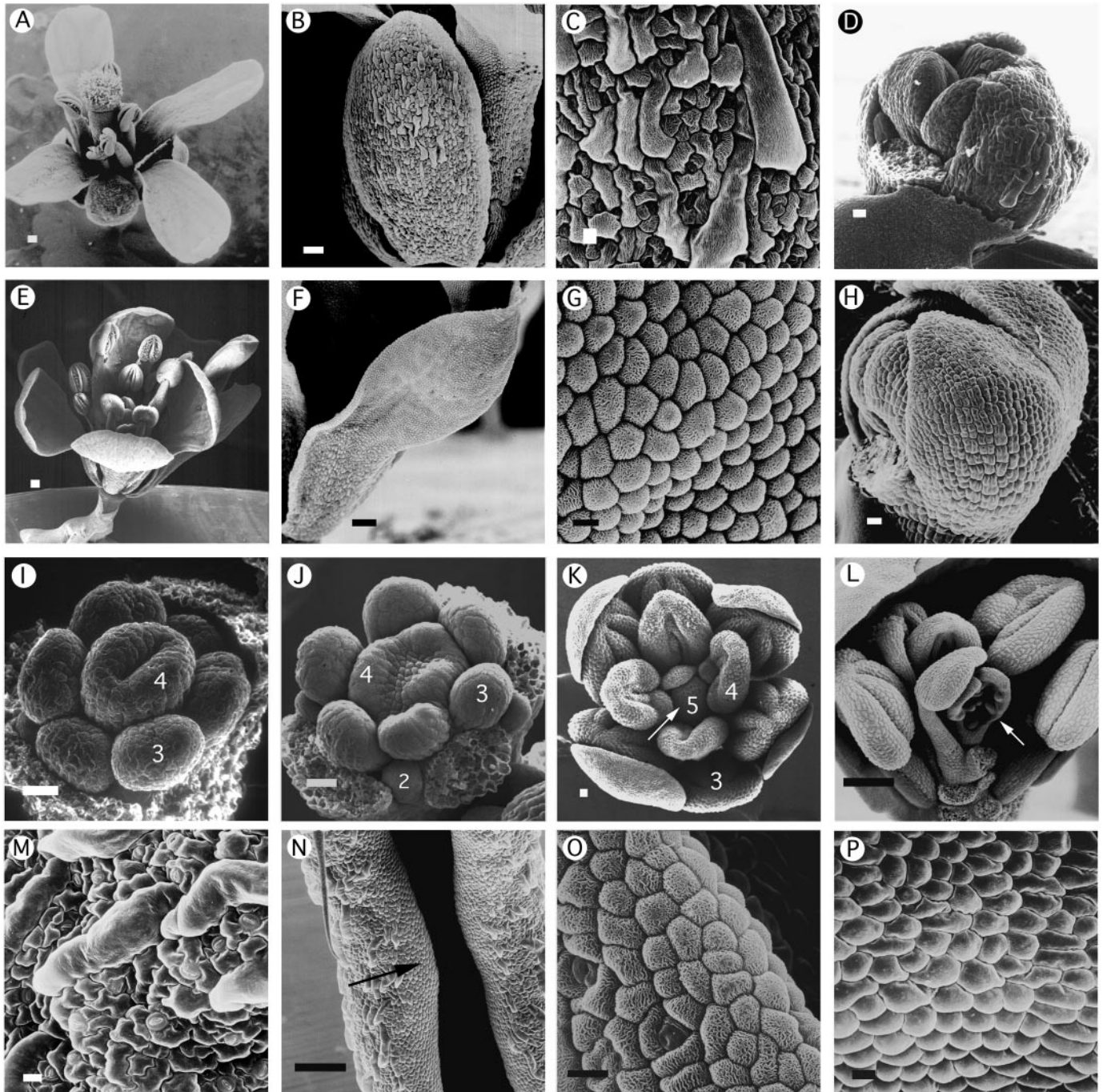


Fig. 2. Development of wild-type (A-D,I) and p35S-PI p35S-AP3 (E-H, J-P) plants viewed by scanning electron microscopy. Flowers staged according to Smyth et al. (1990). Size bars represent 100 μ m for (A,B,E,F,N) and 10 μ m for (C,D,G-M,O,P). Numbers indicate the whorl. (A) Mature wild-type flower. (B) Wild-type sepal. (C) High magnification of sepal showing the characteristic epidermal morphology. (D) Stage 8 wild-type flower. (E) Mature p35S-PI p35S-AP3 flower. (F) p35S-PI p35S-AP3 first whorl petal. (G) High magnification of p35S-PI p35S-AP3 petal showing characteristic petal epidermal morphology. (H) Side view of a first whorl organ of p35S-PI p35S-AP3 stage 8 flower. The first whorl organs of p35S-PI p35S-AP3 do not have the characteristic elongate sepal cells found in wild type as shown in D. (I) Wild-type stage 7 flower. A slotted cylinder has formed in the center of the floral meristem which will develop into the gynoecium. (J) A stage 6 p35S-PI p35S-AP3 flower. Three bulges are visible in the center of the flower which will give rise to fourth whorl stamens. (K) Top down view of p35S-PI p35S-AP3. Three whorls of stamens are visible consisting of 6 St, 4 St, and 2 St. The 2 fifth whorl stamens are indicated with a white arrow. (L) Carpelloid structures (indicated by white arrow) which are rarely found in p35S-PI p35S-AP3 flowers. (M) Leaf-like abaxial surface of a petaloid cauline leaf. (N) Curled margins of a petaloid cauline leaf showing small round cells on the edge of the organ (indicated by black arrow). (O) High magnification of these small round cells showing a similar cellular morphology as the abaxial surface of a petal blade. (P) Adaxial surface of petaloid cauline leaf. The cells are not leaf-like and somewhat resemble petal cells.

These include a transformation of the first whorl sepals to leaf-like organs, the almost complete absence of second whorl petals, and the presence of secondary flowers that arise in the axils of the first whorl leaf-like organs (Irish and Sussex, 1990; Bowman et al., 1993). Flowers of p35S-PI p35S-AP3 *ap1-1* (Figs 4A, 5E) consist of petaloid leaf-like organs in the first whorl with an epidermal morphology intermediate between that of leaves and petals (Fig. 5, compare A with F). The first whorl organs of later-arising flowers are petals with few leaf-like characteristics. Second whorl organ primordia are often initiated (Fig. 5, compare B with G,H) and these primordia develop into petals, stamen/petal mosaic organs, and stamens. Stamens are found in the third and fourth whorls. The number of organs in each whorl is variable. Axillary flowers are also found, especially in the early flowers, but the number is reduced compared to *ap1-1*. The average number of flowers per pedicel in *ap1-1* is 9.1 for flowers 1-5, 3.7 for flowers 6-10, and 2.1 for flowers 11-15 (Bowman et al., 1993). The corresponding numbers for p35S-PI p35S-AP3 *ap1-1* flowers are 3.3 (flowers 1-5), 1.4 (flowers 6-10), and 1.2 (flowers 11-15).

ap2-2

Strong *ap2* mutants have medial first whorl carpels, lateral first whorl organs that are missing or leaf-like, no second whorl organs, a reduction in the number of third whorl stamens, and carpels which are sometimes abnormal in the fourth whorl (Bowman et al., 1989, 1991; Kunst et al., 1989). Ectopic expression of both *PI* and *AP3* in an *ap2-2* background results in flowers consisting almost entirely of stamens, with some petal/stamen organs (Figs 4B, 5I). The first whorl medial organs are stamens while the first whorl lateral organs have an epidermal morphology intermediate between petals and stamens (Fig. 5, compare C with J). These lateral organs are sometimes leaf-like with stellate trichomes on their surface and stipules at their base (Fig. 5K). Stamens develop in the second (Fig. 5L), third, fourth and any interior whorls. Ectopic B function in an *ap2-2* background results in the initiation and development of second whorl organs and in an increased number of third whorl organs as compared to *ap2-2*.

Class B mutants

pi-1

pi-1 flowers consist of two outer whorls of sepals and an abnormally large gynoecium in the center of the flower (Fig. 5M; Bowman et al., 1989; Hill and Lord, 1989). Ectopic expression of *PI* and *AP3* does not fully rescue *pi-1*. p35S-PI p35S-AP3 *pi-1* flowers consist of two outer whorls of petals, a third whorl which contains stamens, carpelloid stamens, filaments, or the absence of organs, and a fourth whorl staminoid/carpelloid structure (Figs 4C, 5N,O). Organs from the third and fourth whorls are often fused (Fig. 5N). p35S-PI p35S-AP3 plants heterozygous for *pi-1* exhibit a similar but less pronounced phenotype (Fig. 4D). The outer three whorls of these flowers, consist of four petals, four petals, and six stamens. The fourth whorl organ has features of both stamens and carpels. SEM analyses of these fourth whorl structures show that they arise in a carpel-like fashion as a slotted tube (Fig. 5P). A wild-type gynoecium consists of two carpels whose place of fusion is marked by a false septum characterized by vertical files of

cells. The carpels are topped by a short style and stigmatic papillae (Fig. 5Q). The fourth whorl structures of p35S-PI p35S-AP3 *pi-1* and p35S-PI p35S-AP3 *pi-1/+* are different in overall appearance from wild-type carpels; the fused carpelloid structures (usually two but sometimes three or four) are wider at the top and become narrower near their base (Fig. 5, compare Q with S). Files of cells along the length of their fusion sites resemble those of a wild-type septum, and stigmatic papillae are present on the top of the organ much as in a wild-type gynoecium, but no style is present. Ovules are present inside the carpelloid structures, but the plants do not produce seed. The epidermal cellular morphology of the fourth whorl organs is quite different from that characteristic of ovaries; no epicuticular wax is evident (Fig. 5, compare R with T) and the shape of the cells is reminiscent of epidermal anther cells. Cells at the narrow base of these fourth whorl structures are arranged in files like those of a stamen filament. As these organs senesce, the upper 'ovary-like' part turns yellow (similar to an anther) but the filament-like basal part does not. Thus, these organs have both carpel features (stigmatic papillae, ovules) and stamen features (epidermal morphology, color).

pi-2

Flowers of the intermediate *pi-2* allele have sepals in the second whorl and carpelloid organs, filaments, or an absence of organs in the third whorl (Bowman et al., 1991). Ectopic expression of both *PI* and *AP3* fully rescues a *pi-2* mutant with the flowers of p35S-PI p35S-AP3 *pi-2* indistinguishable from those of p35S-PI p35S-AP3.

pi-3

Flowers of the weaker *pi-3* allele contain four sepals in the second whorl and carpels, staminoid carpels, or filaments in the third whorl (Bowman et al., 1991). Flowers of p35S-PI p35S-AP3 *pi-3* have a phenotype similar to that described above for p35S-PI p35S-AP3 *pi-1* flowers. There is often a fourth whorl staminoid/carpelloid structure with the same morphology as seen in p35S-PI p35S-AP3 *pi-1/+* flowers. The third whorl is often wild type with six stamens, however, the stamens are occasionally missing or replaced by filaments. *pi-3* also becomes partially dominant in p35S-PI p35S-AP3 plants. The fourth whorl of p35S-PI p35S-AP3 *pi-3/+* flowers sometimes contain the novel staminoid/carpelloid structure or stamens with carpelloid characteristics.

ap3-3

Flowers mutant for *ap3-3* have two outer whorls of sepals and two inner whorls of carpels (Jack et al., 1992). Ectopic expression of both *PI* and *AP3* fully rescues an *ap3-3* mutant. The flowers of p35S-PI p35S-AP3 *ap3-3* are indistinguishable from those of p35S-PI p35S-AP3.

ap3-1

Flowers containing the *ap3-1* temperature sensitive mutation have sepals in the second whorl; stamens, carpelloid stamens, or carpelloid organs in the third whorl, when grown at 25°C (Bowman et al., 1989). Ectopic expression of both B class genes rescues the second and third whorl defects of the weak *ap3-1* allele.

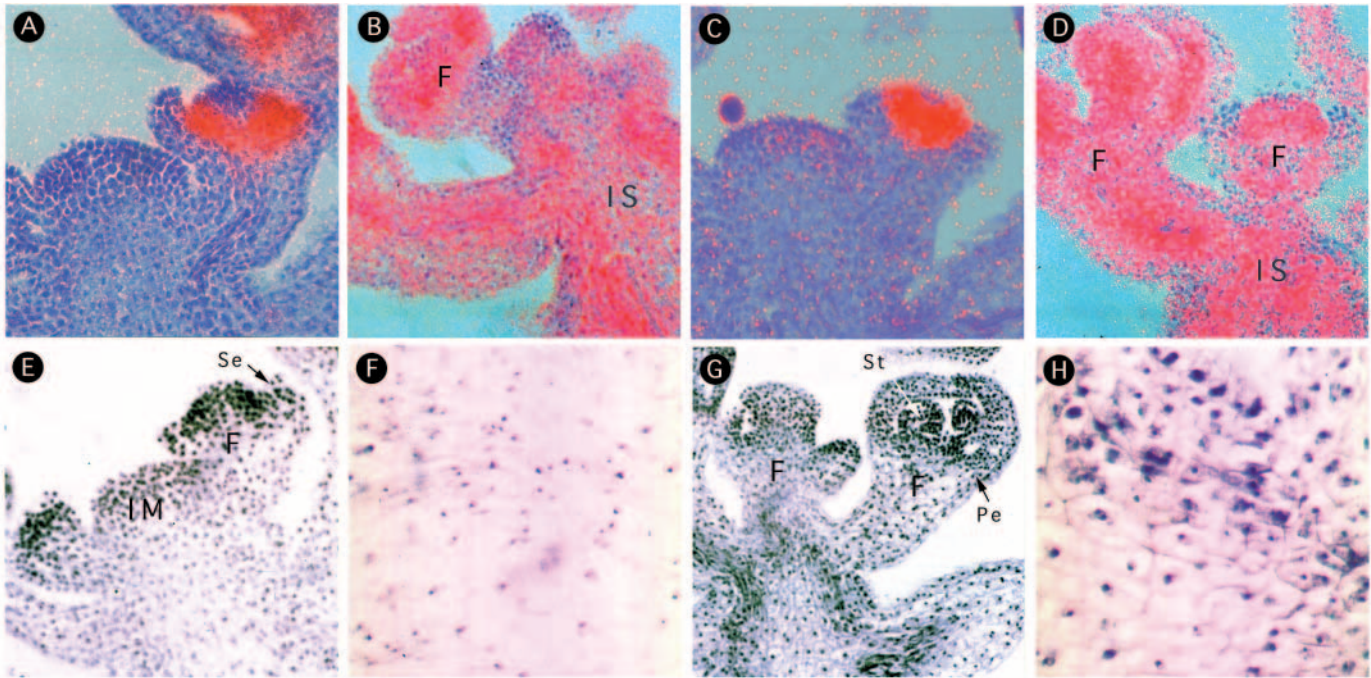


Fig. 3. Expression of *AP3* and *PI* in wild-type and p35S-PI p35S-AP3 flowers. Abbreviations: F, flower, IM, inflorescence meristem, IS, inflorescence stem, Se, sepal, St, stamen, and Pe, petal. The immunolocalization experiments on wild-type and p35S-PI p35S-AP3 tissues were done in parallel and the slides were incubated in the staining solution for equivalent lengths of time. (A) Section of wild-type inflorescence hybridized with *AP3*. (B) Section of a p35S-PI p35S-AP3 inflorescence hybridized with *AP3*. *AP3* RNA is found throughout the section. (C) Section of wild-type inflorescence hybridized with *PI*. (D) Section of p35S-PI p35S-AP3 hybridized with *PI*. *PI* RNA is detected in all regions of the inflorescence. (E) Immunolocalization of *AP3* antibody on wild-type tissue. *AP3* protein is detected in the young flower primordia but not in the inflorescence meristem. (F) Close-up of *AP3* immunolocalization in the inflorescence stem of wild type. There is some background staining in the nucleolus. (G) Immunolocalization of *AP3* antibody on p35S-PI p35S-AP3 tissue. *AP3* protein is detectable in all whorls of the flower and in the inflorescence stem. (H). Close-up of *AP3* immunolocalization in the inflorescence stem of p35S-PI p35S-AP3, showing strong staining throughout each nucleus.

Class C mutants

ag-3

Mutations in *ag* result in the formation of indeterminate flowers which produce sepals, petals and petals in a repeating pattern (Bowman et al., 1991). Ectopic expression of *PI* and *AP3* in an *ag-3* background results in indeterminate flowers consisting entirely of petals (Fig. 4E).

superman (sup-1)

Mutations at the *superman (sup-1)* locus result in the production of extra stamens and a reduction in carpel tissue (Schultz et al., 1991; Bowman et al., 1992). One explanation of this phenotype is that *SUP* increases cell division in the third whorl, while repressing it in the fourth (Sakai et al., 1995). p35S-PI p35S-AP3 *sup-1* flowers consist of two outer whorls of petals and an indeterminate number of stamens (Fig. 4F). A central dome of cells continues to proliferate and produce stamens until the flower senesces (Fig. 6I,J). This central dome of undifferentiated cells is visible after the fourth whorl stamen primordia have initiated (Fig. 6K). p35S-PI p35S-AP3 *sup-1* flowers average more than 18 stamens (although this number is somewhat difficult to determine accurately, since the meristem continues to produce stamens even as the flowers are dying), which is greater than the number found in *sup-1* flowers (14.6) (Bowman et al., 1992) or p35S-PI p35S-AP3 flowers (15.6). The enhancement of the *sup-1* phenotype by p35S-PI

p35S-AP3 is greater than that exhibited by p35S-AP3, since p35S-PI p35S-AP3 *sup-1* flowers are completely indeterminate while p35S-AP3 *sup-1* flowers have extra whorls but do not proliferate indefinitely (Jack et al., 1994). An additional observation in p35S-PI p35S-AP3 *sup-1* flowers is the delayed initiation of the fourth whorl primordia as compared to the development of the fourth whorl in p35S-PI p35S-AP3 or *sup-1* flowers (compare Fig. 6L with Fig. 2J).

Ectopic expression of both *PI* and *AP3* rescues the floral organ identity defects of *lfy* and *ufo*

Ectopic expression of both class B genes rescues the floral organ identity defects of *lfy* and *ufo*. *LFY* and *UFO* are involved in positive regulation of the B function in flowers, as demonstrated by the reduction or elimination of petals and stamens in *lfy* and *ufo* mutants and a corresponding decrease in the expression of *PI* and *AP3* (Huala and Sussex, 1992; Weigel et al., 1992; Weigel and Meyerowitz, 1993; Levin and Meyerowitz, 1995). Strong *lfy* alleles (such as *lfy-6*) have flowers consisting of sepals, carpels, and sepal/carpel mosaic organs. Strong *ufo* alleles (such as *ufo-2*) show a reduction in the number of petals and stamens; in general these organs are replaced by sepals, filaments, carpels and mosaic organs. Flowers of p35S-PI p35S-AP3 *lfy-6* consist of sepal-like organs, petals, stamens and mosaic organs consisting of various combinations of sepal, petal and stamen tissue (Figs 4I, 6A,B). The outer whorls usually consist of sepals,

sepal/petal mosaic organs and petals. Interior to these organs are petal/stamen mosaic organs and stamens. The flowers of p35S-PI p35S-AP3 *ufo-2* have petaloid organs in the outer two whorls and stamens in the inner whorls with the occasional appearance of filaments (Figs 4G, 6E). The rescue of the organ identity defects of *lfy* and *ufo* by ectopic expression of both B class genes is much greater than that resulting from ectopic expression of either *PI* or *AP3* alone (Jack et al., 1994; Krizek and Meyerowitz, unpublished data).

LFY and *UFO* are also involved in the promotion of floral meristem identity, and mutations in these genes cause various inflorescence defects. One example is the presence of subtending leaf-like bracts on flowers (Weigel et al., 1992; Levin and Meyerowitz, 1995). Similar leaf-like organs are found below p35S-PI p35S-AP3 *lfy-6* and p35S-PI p35S-AP3 *ufo-2* flowers, except that they now have petal characteristics. These organs are narrower than those found in *lfy-6* or *ufo-2*, tapering at their base, and are pale green or white in color (Fig. 4H). The surface of these organs is quite petal-like; the cells are small and round with petal cuticular morphology (Fig. 6H). The observation that these leaf-like bracts can be converted to petaloid organs is consistent with the presence of *LFY* RNA in these organs, which apparently indicates that they are floral (Weigel et al., 1992).

The spiral pattern of floral organ initiation found in *lfy-6* flowers is also present in some p35S-PI p35S-AP3 *lfy-6* flowers (Fig. 6C). The severity of this defect seems to vary, perhaps with position of the flower on the inflorescence; some flowers exhibit a nearly whorled pattern of organ primordia (Fig. 6D). Defects found in *ufo* that are still present when *PI* and *AP3* are ectopically expressed are the disproportionately faster growth rate of the second whorl organs (Fig. 6F) and the presence of filaments on the inflorescence stem and squamule-like filaments at the base of the bracts (Fig. 6G). Thus, ectopic expression of *PI* and *AP3* is unable to rescue inflorescence defects such as bract formation or defects in floral organ positioning and growth of *lfy* and *ufo*.

AP3 and PI are sufficient to autoregulate in floral tissue

Much evidence has accumulated that *AP3* and *PI* work together in an autoregulatory loop to maintain each other's expression (Goto and Meyerowitz, 1994; Jack et al., 1994). Experiments with an *AP3* promoter-reporter gene (β -glucuronidase) construct showed that *AP3* and *PI* appear to be sufficient for activation of the *AP3* promoter

in flowers (Jack et al., 1994). In a wild-type background this construct directs GUS staining in the petals, stamens and at the base of the first whorl sepals (Fig. 7A,C), a pattern similar to that seen for *AP3* RNA. Staining of p35S-AP3 pAP3-GUS flowers showed GUS expression in the second, third and fourth whorls, consistent with the distribution of AP3 protein, and thus indicates that *AP3* is able to regulate its own transcription in regions where *PI* is active (Jack et al., 1994). In p35S-PI pAP3-GUS flowers, GUS activity is detected in the first whorl (Krizek and Meyerowitz, unpublished data), providing additional evidence that *PI* is involved in the autoregulation of *AP3*.

p35S-PI p35S-AP3 plants containing the pAP3-GUS construct showed GUS activity in all four whorls of the flower and parts of the pedicel, but not in the inflorescence stem or cauline leaves (Fig. 7B,D). Since p35S-PI p35S-AP3 plants show a vegetative phenotype, and neither p35S-PI nor p35S-AP3 show one, we would expect that both AP3 and PI proteins are present in these vegetative tissues, and this has been confirmed for AP3 protein (Fig. 3G,H). However, *AP3* and *PI* are not able to activate the pAP3-GUS gene here, indicating that they are not sufficient for their autoregulation in nonfloral tissue. *PI* and *AP3* may require an additional flower-specific

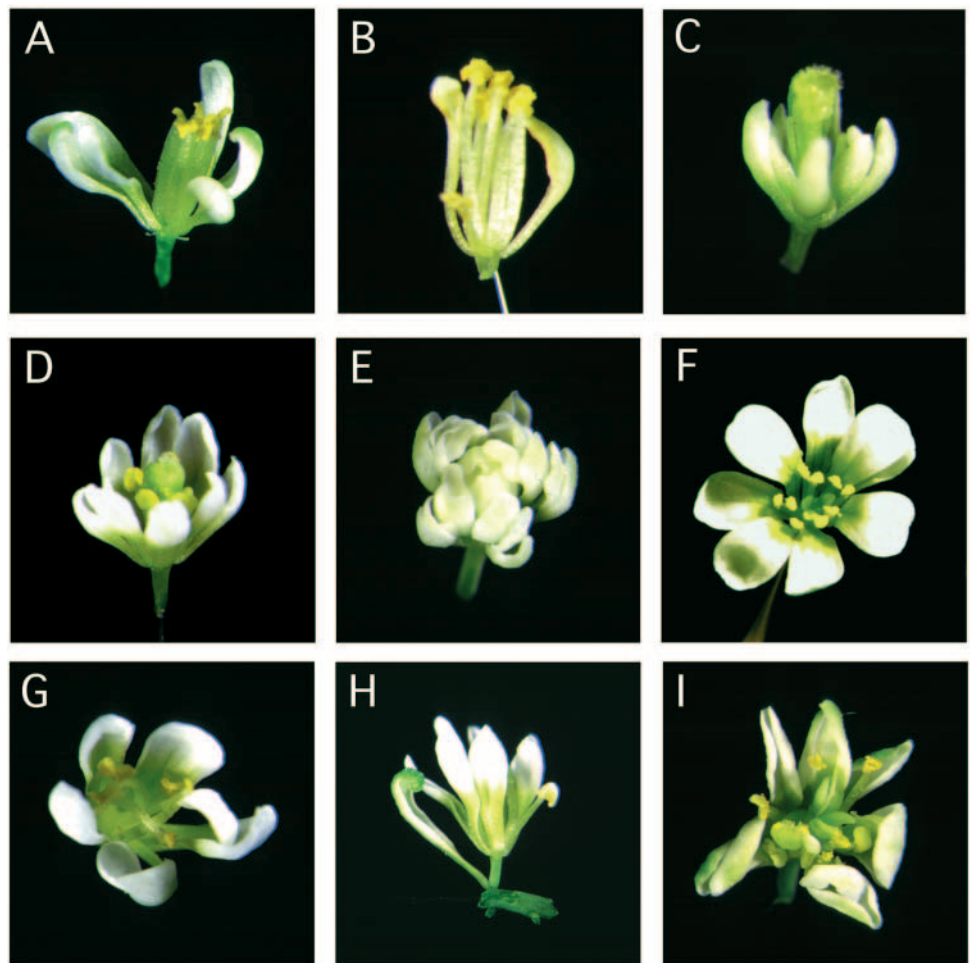


Fig. 4. Phenotypes of floral mutants containing p35S-PI p35S-AP3. (A) p35S-PI p35S-AP3 *ap1-1*. (B) p35S-PI p35S-AP3 *ap2-2*. (C) p35S-PI p35S-AP3 *pi-1*. (D) p35S-PI p35S-AP3 *pi-1/+*. (E) p35S-PI p35S-AP3 *ag-3*. (F) p35S-PI p35S-AP3 *sup-1*. (G) p35S-PI p35S-AP3 *ufo-2*. (H) p35S-PI p35S-AP3 *lfy-6* flower subtended by petaloid bract. (I) p35S-PI p35S-AP3 *lfy-6*.

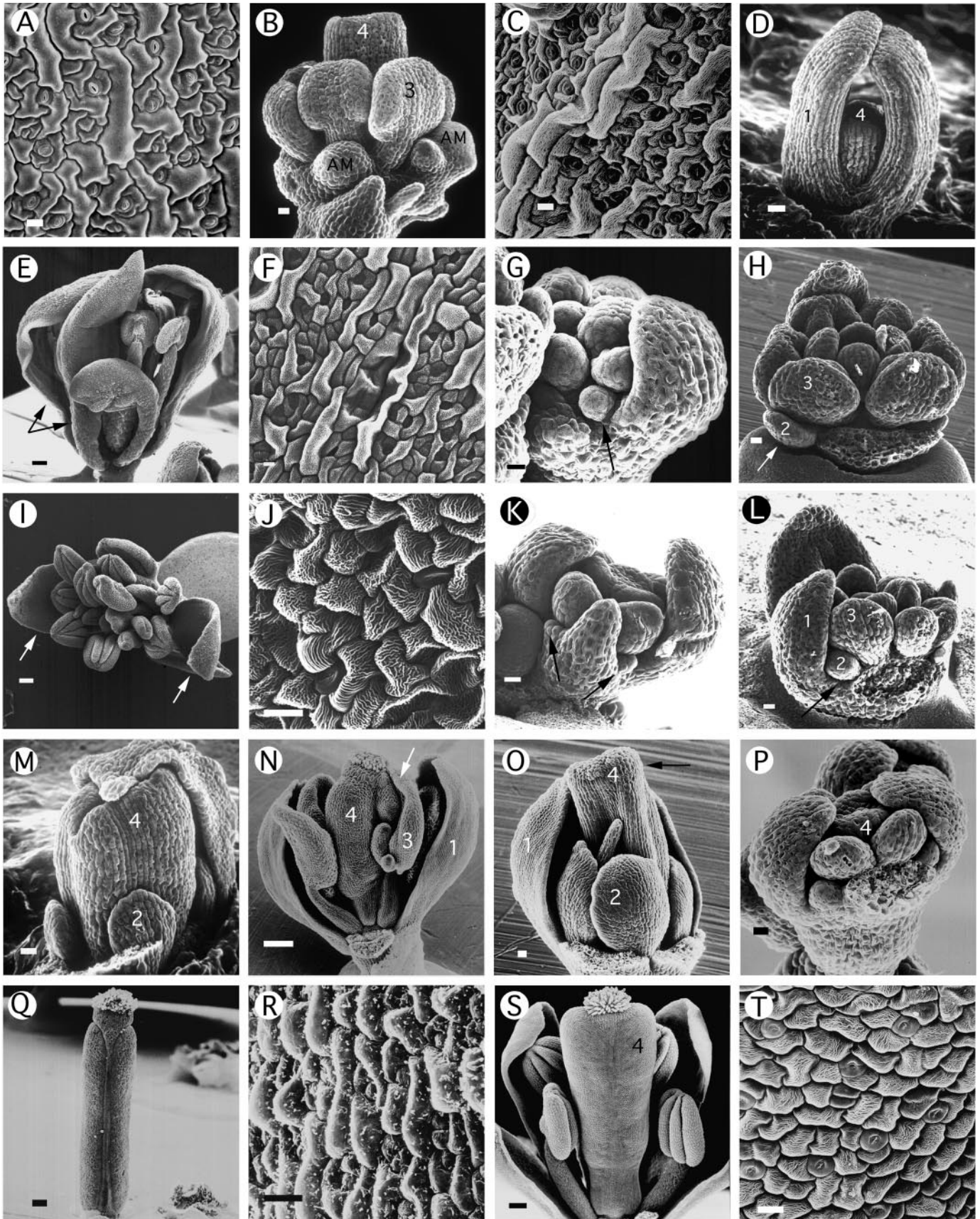


Fig. 5. SEM characterization of floral homeotic mutants containing p35S-PI p35S-AP3. Scanning electron micrographs of *ap1-1* (A,B), *ap2-2* (C,D), *pi-1* (M), wild-type (Q,R), and p35S-PI p35S-AP3 flowers mutant for *ap1-1* (E-H), *ap2-2* (I-L), *pi-1* (N-P), and *pi-1/+* (S,T). Bars represent 100 μ m for (E,I,N,Q,S), and 10 μ m for (A-D,F-H,J-M,O,P,R,T). AM, axillary meristem. The numbers indicate whorls. (A) Surface morphology of a first whorl organ of *ap1-1*. (B) *ap1-1* flower showing the absence of second whorl organs. (C) Surface morphology of a first whorl organ of *ap2-2*. (D) *ap2-2* flower showing the absence of second and third whorl organs. (E) p35S-PI p35S-AP3 *ap1-1* flower. The outer whorl contains leaf-like petals, indicated by black arrows. (F) Surface morphology of a first whorl leaf-like petal. (G) Stage 6 p35S-PI p35S-AP3 *ap1-1* flower. A black arrow points to a probable second whorl primordia. Since this is a later flower, it is unlikely that this outgrowth of cells is the floral meristem of an axillary flower. (H) Stage 9 p35S-PI p35S-AP3 *ap1-1* flower showing a second whorl organ (white arrow). (I) p35S-PI p35S-AP3 *ap2-2* flower. These flowers consist entirely of stamens except for the lateral first whorl organs which develop into petaloid stamens, indicated by white arrows. (J) Surface of a first whorl petaloid stamen. (K) The first whorl petaloid stamens sometimes have leaf-like qualities such as stipules (indicated by arrows). (L) A stage 7 p35S-PI p35S-AP3 *ap2-2* flower showing that second whorl primordia are sometimes initiated (indicated by arrows). (M) *pi-1* flower. (N) p35S-PI p35S-AP3 *pi-1* flower. Stamens or carpelloid stamens are visible in the third whorl. One of these carpelloid stamens (marked with arrow) is fused to the fourth whorl carpelloid/staminoid structure. (O) p35S-PI p35S-AP3 *pi-1* flower showing the absence of stamens in the third whorl. One filament is present and the large fourth whorl carpelloid/staminoid structure (indicated by black arrow) is probably due to incorporation of third whorl cells. (P) A stage 6 p35S-PI p35S-AP3 *pi-1* flower. The fourth whorl appears to develop as in wild type with the invagination of cells in the center of the meristem. (Q) Wild-type mature gynoecium consisting of two fused carpels. The carpels are topped by a short style and stigmatic tissue. (R) Surface morphology of wild-type ovary epidermal cells. (S) p35S-PI p35S-AP3 *pi-1/+* flower. A carpelloid/staminoid structure has formed in the fourth whorl. (T) Surface morphology of this fourth whorl carpelloid/staminoid structure.

factor to autoregulate or alternatively, there could be a vegetative factor that represses the ability of *AP3* and *PI* to autoregulate.

DISCUSSION

PI and *AP3* are sufficient in flowers to specify petals and stamens in combination with class A and C genes

Ectopic expression of both class B genes gives rise to flowers with petals in whorls one and two and stamens in the interior whorls. This result demonstrates that *AP3* and *PI* are sufficient to provide the B organ identity function in flowers (Fig. 8). Flowers of p35S-PI p35S-AP3 *ap2-2* plants consist entirely of stamens and staminoid organs as predicted by the ABC model when B and C functions are active in all four whorls. Similarly, flowers of p35S-PI p35S-AP3 *ag-3* plants consist entirely of petals as predicted when A and B functions are active throughout the flower. Thus, the results of ectopic expression of the B function genes provide new experimental evidence in support of the hypotheses of the ABC model. These are that: the identity of an organ is dependent upon the combination of

organ identity genes expressed by the cells giving rise to that organ and that the effects of the organ identity genes are not restricted to a particular region of the flower. This last premise predicts that any type of organ can occur in any whorl of a flower, and this has now been demonstrated. For example, all four types of organs can be found in whorl one: sepals (wild type), petals (p35S-PI p35S-AP3), stamens (p35S-PI p35S-AP3 *ap2-2*), and carpels (*ap2-2*).

Ectopic *PI* and *AP3* expression causes partial transformation of later developing leaves to petals

Ectopic expression of the B class genes results in the partial transformation of some of the later-produced cauline leaves into petaloid leaves. The presence of stigmatic papillae on some cauline leaves of p35S-AG plants shows that C function genes can also act in cauline leaves (Mizukami and Ma, 1992). It is not reported, however, if this phenotype is only observed in more apical cauline leaves. Our results suggest that there is a gradient of floral character during the life of the plant that could arise either by increasing amounts of floral activators or by decreasing amounts of repressors of flowering. This is supported by the acropetally decreasing severity of the floral meristem identity defects of *lfy-6* and *ap1-1* (Irish and Sussex, 1990; Weigel et al., 1992). The presence of a gradient of floral quality has also been previously suggested by work on tobacco (McDaniel and Hartnett, 1993). In these studies, explants of axillary buds were attached to stem cuttings, and the number of nodes produced by the bud before flowering were counted. Buds attached to more apical stem cuttings produced fewer nodes before flowering than buds of an equivalent position attached to a more basal stem cutting.

Our results would seem to indicate that ectopic expression of the organ identity genes is not sufficient to change the developmental fate of a leaf to that of a floral organ unless these organs possess a sufficient degree of floral character. This can seemingly be acquired by primordia in at least two ways: being specified as floral or alternatively, being produced late in the development of an inflorescence. The first case is exemplified by the floral leaves or bracts of *lfy* and *ufo* mutants which become petaloid in the presence of ectopic B function and by the carpelloid bracts produced in tobacco plants overexpressing the tobacco *AG* homolog (p35S-NAG) (Mandel et al., 1992a). The second case describes the later cauline leaves of p35S-PI p35S-AP3 plants, which are partially transformed to petals. This floral character does not appear to be specified by either *LFY* or *API* alone, since petaloid bracts are found in p35S-PI p35S-AP3 *lfy* null mutants, and petaloid cauline leaves are found in p35S-PI p35S-AP3 *ap1* mutants. Furthermore, it seems unlikely that this floral character is specified by *LFY* and *API* together since *API* is not expressed in the bracts of *lfy-6* (D. Weigel, personal communication), which become petaloid when B function is ectopically expressed. Therefore, it appears that the factors responsible for the partial petaloid transformations of bracts and cauline leaves are as yet unidentified.

The earlier expression of *AP3* and *PI* in floral primordia does not affect the time course of organ development

For all of the homeotic mutants except *AG*, floral organs develop on a schedule corresponding to their whorl identity and not their organ identity (Bowman et al., 1989). The earliest

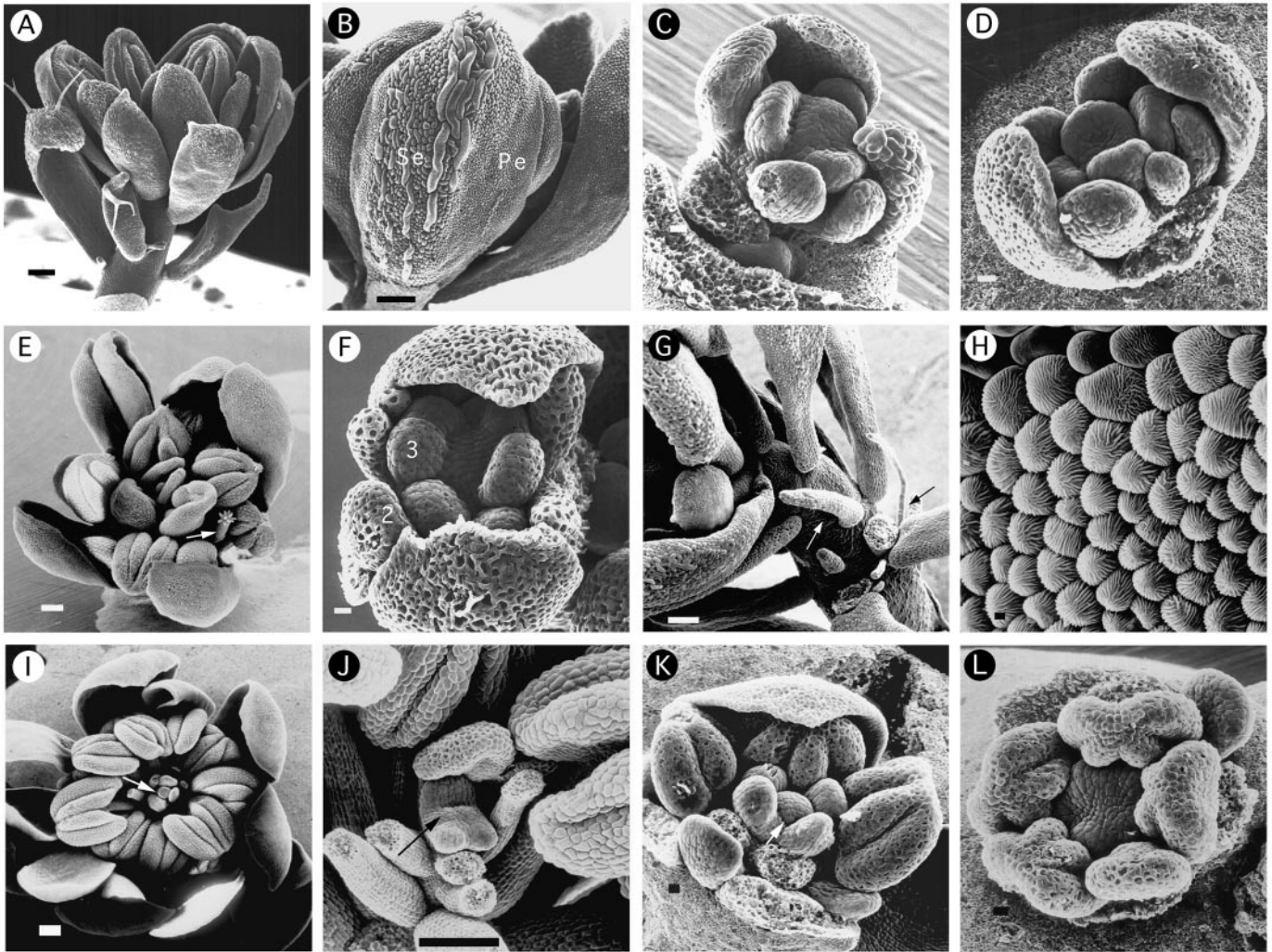


Fig. 6. SEM characterization of other floral mutants containing p35S-PI p35S-AP3. Scanning electron micrographs of p35S-PI p35S-AP3 flowers mutant for *lfy-6* (A-D), and *ufo-2* (E-H), and *sup-1* (I-L). Bars represent 100 μ m for (A,B,E,G,I); 10 μ m for (C,D,F,J-L), and 1 μ m (H). Abbreviations: Se, sepal, Pe, petal. Numbers indicate whorls. (A) p35S-PI p35S-AP3 *lfy-6* flower consisting of mostly petal and stamen-like organs. (B) High magnification of a mosaic sepal/petal organ of p35S-PI p35S-AP3 *lfy-6*. (C) A young p35S-PI p35S-AP3 *lfy-6* flower exhibiting spiral phyllotaxis. (D) A young p35S-PI p35S-AP3 *lfy-6* flower in which the organ primordia seem to have arisen in a nearly whorled pattern. (E) p35S-PI p35S-AP3 *ufo-2* flower consisting of petals and stamens. A filament topped with stigmatic tissue is indicated by a white arrow. (F) A young p35S-PI p35S-AP3 *ufo-2* flower showing second whorl primordia which are abnormal in number, shape and relative size compared to the third whorl primordia. (G) Inflorescence stem of p35S-PI p35S-AP3 *ufo-2*. A filament (indicated by white arrow) and squamule-like organs (indicated by black arrow) are visible. (H) Inner petaloid surface of a p35S-PI p35S-AP3 *ufo-2* bract. (I) p35S-PI p35S-AP3 *sup-1* flower showing a dome of cells in the center of the flower, as indicated by a white arrow. Some stamens were dissected to make this visible. (J) Higher magnification of the central dome (indicated by black arrow) of a p35S-PI p35S-AP3 *sup-1* flower. (K) A stage 9 p35S-PI p35S-AP3 *sup-1* flower. A large floral meristem (indicated by a white arrow) is visible after the fourth whorl stamen primordia have been initiated. (L) A stage 8 p35S-PI p35S-AP3 *sup-1* flower showing the delayed development of the fourth whorl organs.

primordia to differentiate from the floral meristem are the first whorl organs, independent of the type of organ that will differentiate in this region. The second and third whorl primordia arise about the same time in development, with the second whorl primordia alternate in position with the first whorl primordia. Last to differentiate are cells in the center of the meristem which form the fourth whorl organs. The results of ectopic expression of *AP3* and *PI* provide further evidence that the identity of an organ and the temporal development of that organ are distinct processes. The second whorl petals of p35S-PI p35S-AP3 arise developmentally later than the first whorl petals even though all cells in the first and second whorls

express the A and B class genes from their inception. Similarly, the fourth whorl stamens arise later than those in the third whorl. Thus, the earlier expression of the B class genes in a floral meristem does not alter the relative temporal development of the floral organs produced by that meristem. These experiments also demonstrate that the organ identity and the whorl or positional identity of floral organ primordia are distinct.

p35S-PI p35S-AP3 Makes *pi-1* and *pi-3* partially dominant

A surprising result from these studies is that ectopic *PI* and *AP3* expression does not rescue *pi-1* or *pi-3* and makes these

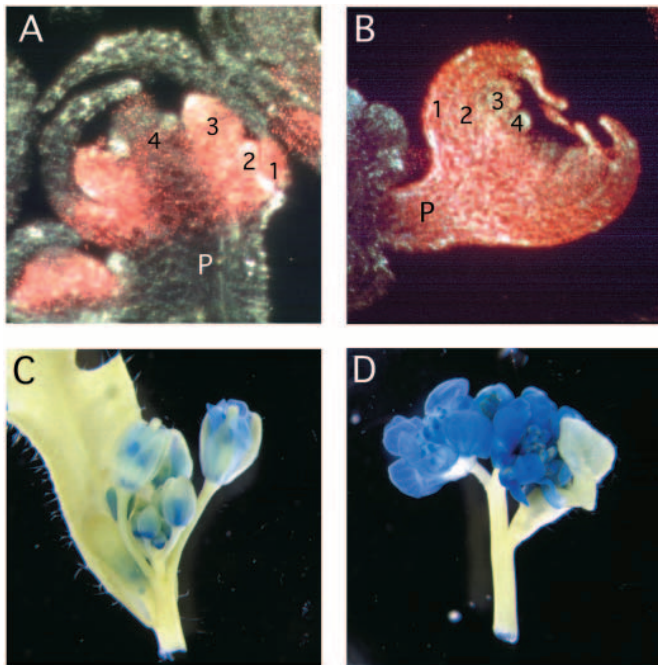


Fig. 7. GUS staining of pAP3-GUS and p35S-PI p35S-AP3 pAP3-GUS flowers. P, pedicel. The numbers indicate the whorl. (A) 10 µm section of a GUS stained wild-type flower containing the pAP3-GUS construct. GUS staining is detected in the petals (2) and stamens (3) and at the base of the first whorl sepals (1). (B) 10 µm section of a p35S-PI p35S-AP3 pAP3-GUS flower stained for GUS activity. GUS staining is found in all parts of the flower and in the pedicel. (C) GUS stained inflorescence of a wild-type plant containing pAP3-GUS. (D) p35S-PI p35S-AP3 pAP3-GUS inflorescence stained for GUS activity. GUS activity is detected in the flowers but not in the stem or cauline leaves.

recessive alleles partially dominant. Ectopic B function does rescue the *pi-2* mutant. One explanation for the dominant phenotypes of these flowers is that *pi-1* and *pi-3* may be acting in a dominant negative manner. The *pi-1* mutation results in a stop codon 22 amino acids after the MADS domain. The mutation in *pi-3* is a substitution of Ala₄₉ by Ser in the MADS domain of the protein. It is possible that these genes could encode proteins of sufficient length to interact with both the endogenous and transgenic AP3 protein. The AP3/PI(*pi-1*, *pi-3*) protein complexes might be inactive due to an inability to bind DNA or perform some other function. It is possible that quite high levels of the mutant gene products could be produced since PI and AP3 overproduction from the transgenes would be expected to activate the endogenous *pi* gene.

An alternative explanation is that the development of stamens is sensitive to the levels of PI protein. A mutation in one copy of *PI* (p35S-PI p35S-AP3 *pi-1*+) would result in decreased levels of active PI protein relative to p35S-PI p35S-AP3 in a wild-type background. If the production of stamens, particularly in the fourth whorl, is sensitive to the level of AP3/PI complex, this decrease in the amount of PI could result in the observed phenotype.

Role of AP3 and PI in cellular proliferation in the floral meristem

That AP3 and PI play a role in the determinacy of the floral

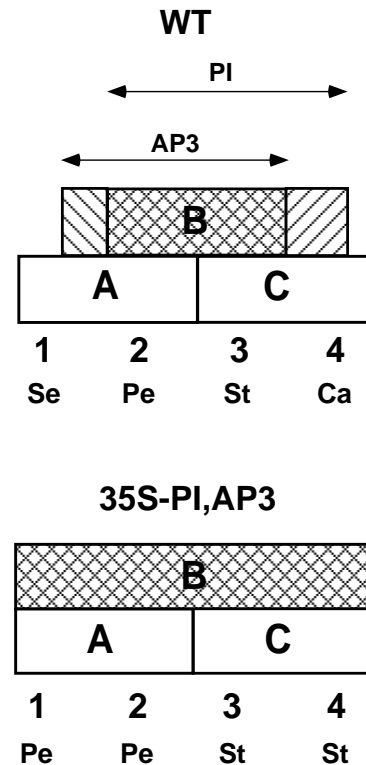


Fig. 8. ABC model and ectopic B function. In wild-type flowers the B function results from the overlap of the expression of the two B class genes, AP3 and PI. As determined by in situ hybridization, AP3 is expressed at the base of the first whorl organs and throughout the second and third whorl organs (Jack et al., 1992; Weigel and Meyerowitz, 1993). PI is initially expressed in the second, third and fourth whorl organs (Goto and Meyerowitz, 1994). In wild-type flowers, transcriptional and posttranscriptional interactions between these two genes cause B activity to exist only in whorls two and three (Goto and Meyerowitz, 1994; Jack et al., 1994; Krizek and Meyerowitz, unpublished observations). When both PI and AP3 are expressed throughout the flower, B activity is present in all four whorls. The combined activities of A and B in the outer two whorls (one and two) lead to the development of petals and the combined activities of B and C in the inner two whorls (three and four) lead to the development of stamens.

meristem is indicated by the presence of additional whorls of stamens in p35S-PI p35S-AP3 flowers. The rescue of second whorl primordia in class A mutants by ectopic expression of PI and AP3 also indicates a role for the B class genes in proliferation. This second whorl defect of *ap1* and *ap2* mutants is believed to result from AG, since second whorl primordia are present in *ap1-1 ag-2* and *ap2-2 ag-2* double mutant flowers (Bowman et al., 1991). The expression of PI and AP3 is reduced in *ap2* mutants (Jack et al., 1992; Goto and Meyerowitz, 1994), and the elimination of the second whorl organs may be due in part to the reduced activity of the B function and in part by ectopic AG. The B and C class genes work in opposition in the growth of the floral meristem: AP3/PI act to promote cell division while AG acts to suppress cell division. The decreased number of third whorl organs in *pi* and *ap3* mutants are consistent with this role (Bowman et al., 1989; Jack et al., 1992). A similar function has been proposed for DEFA and GLO, the *Antirrhinum* homologs of AP3 and PI, (Tröbner et al., 1992) based on the observation that mutations

in *DEF* and *GLO* (unlike those in *AP3* and *PI*) result in an absence of fourth whorl organs.

The results from these ectopic expression experiments have demonstrated not only that the class B genes *AP3* and *PI* are sufficient to direct the floral development of petals and stamens with the class A and C genes respectively, but have also provided evidence that these genes play an additional role in cellular proliferation in the floral meristem. Furthermore, these experiments suggest that there are additional unknown factors that signal cells that they are floral, thereby allowing both the organ identity and autoregulatory activities of the B function genes. Identification of these factors and their function should provide an important clue to the molecular mechanism of floral induction.

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