The Plant Cell, Vol. 12, 1607–1618, September 2000, www.plantcell.org © 2000 American Society of Plant Physiologists

Regulation of *SUP* Expression Identifies Multiple Regulators Involved in Arabidopsis Floral Meristem Development

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During the course of flower development, floral homeotic genes are expressed in defined concentric regions of floral meristems called whorls. The *SUPERMAN* (*SUP,* also called *FLO10*) gene, which encodes a C2H2-type zinc finger protein, is involved in maintenance of the stamen/carpel whorl boundary (the boundary between whorl 3 and whorl 4) in Arabidopsis. Here, we show that the regulation of *SUP* expression in floral meristems is complex, consisting of two distinct phases, initiation and maintenance. The floral meristem identity gene *LEAFY* (*LFY*) plays a role in the initiation phase through at least two pathways, which differ from each other in the involvement of two homeotic genes, *APETALA3* (*AP3*) and *PISTILLATA* (*PI*). *AP3*, *PI*, and another homeotic gene, *AGAMOUS* (*AG*), are further required for *SUP* expression in the later maintenance phase. Aside from these genes, there are other as yet unidentified genes that control both the temporal and spatial patterns of *SUP* expression in whorl 3 floral meristems. *SUP* appears to act transiently, probably functioning to trigger a genetic circuit that creates the correct position of the whorl 3/whorl 4 boundary.

INTRODUCTION

Flowers of angiosperms develop from floral meristems, small groups of undifferentiated cells derived from shoot apical meristems. One of the characteristics that often separate floral meristems from shoot meristems is the pattern of organ initiation. In Arabidopsis, shoot apical meristems form organs in a spiral pattern, whereas floral meristems form organs in a whorled pattern. Four types of floral organs are produced in four concentric whorls: sepals in the outermost whorl (whorl 1), petals in whorl 2, stamens in whorl 3, and carpels in the innermost whorl (whorl 4) (Smyth et al., 1990). Boundaries of these whorls are established very early in flower development, as evidenced by the distinct expression pattern of the homeotic genes *APETALA3* (*AP3*), *PISTILLATA* (*PI*), and *AGAMOUS* (*AG*) in whorl-specific patterns (Yanofsky et al., 1990; Jack et al., 1992; Goto and Meyerowitz, 1994). The initial expression of these genes is detected in early stage 3 flowers formed after 3 days of floral meristem development. Accordingly, the pattern of whorls (the prepattern of homeotic gene expression) has to be set before this stage. Several genes are involved in whorl patterning. Floral meristem identity genes appear to be partly responsible for the establishment of these boundaries. Loss of function of the *LEAFY* (*LFY*) gene, for instance, changes the floral whorl structure to a partially spiral pattern (Huala and Sussex, 1992; Weigel et al., 1992). The *lfy* phenotype is synergistically enhanced by mutations in *APETALA1* (*AP1*), another floral meristem identity gene. In the double mutant *ap1 lfy*, the whorl pattern and other floral meristem characteristics are severely disrupted (Weigel et al., 1992). A similar but less severe phenotype is observed in flowers lacking the function of the *UNUSUAL FLORAL ORGANS* (*UFO*) gene, which is required for the proper identity of floral meristems (Ingram et al., 1995; Levin and Meyerowitz, 1995; Wilkinson and Haughn, 1995).

Several mutations are known to alter the expression domains of the floral homeotic genes. Recessive mutations in the *SUPERMAN* (*SUP*) gene specifically affect the boundary between whorls 3 and 4 (Schultz et al., 1991; Bowman et al., 1992). In the *sup* mutant, this boundary is shifted toward the center of the floral meristem, resulting in ectopic expression of *AP3* and *PI* in the whorl 4 region and the formation of extra stamens at the expense of carpels (Bowman et al., 1992). This alteration of the whorl 3/4 boundary in the *sup* mutant occurs progressively from stage 4 onward, after the initial expression of *AP3* and *PI* has been established (Sakai et al., 1995). From these observations, *SUP* was thought to be a factor in maintaining this boundary after the whorl prepattern had been established.

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The cloning of *SUP* has enabled us to study the function of this gene at the molecular level. We report here studies of *SUP* expression and genetic interactions between *SUP* and other floral regulatory genes, which reveal the presence of multiple genetic pathways that control the patterning of floral whorl boundaries. Our results show that *SUP* expression is regulated by floral meristem identity genes as well as by floral organ identity genes. By analyzing *SUP* expression in different floral mutants and in transgenic plants that ectopically express homeotic genes, we found that one or more additional, as yet unidentified whorl-specific factors act to define *SUP* expression in the inner part of whorl 3. Further, genetic analyses of double mutants revealed that only a transient period of *SUP* expression is required for setting the right pattern of the whorl 3/4 boundary in stage 4 and older floral meristems. This indicates that *SUP* functions as a trigger of balanced whorl proliferation that is later inherited without further *SUP* RNA input.

RESULTS

Genetic Interaction between *sup* and the Floral Meristem Identity Mutants

The floral meristem identity genes *LFY* and *AP1* are involved in early flower development, partly establishing the whorl pattern (Irish and Sussex, 1990; Huala and Sussex, 1992; Mandel et al., 1992; Weigel et al., 1992; Bowman et al., 1993). To examine a possible interaction between these genes and *SUP*, we analyzed double mutants of *sup* with *lfy* as well as of *sup* with *ap1*. In the strong *lfy* mutant (*lfy-6*), floral organs exhibit sepal/carpel–like characteristics with various degrees of mosaic features. The number of organs in *lfy* flowers was reduced, and the whorled floral structure was partially converted to a spiral pattern. The phenotype of the flowers of the double mutant *sup-1 lfy-6* was very similar to

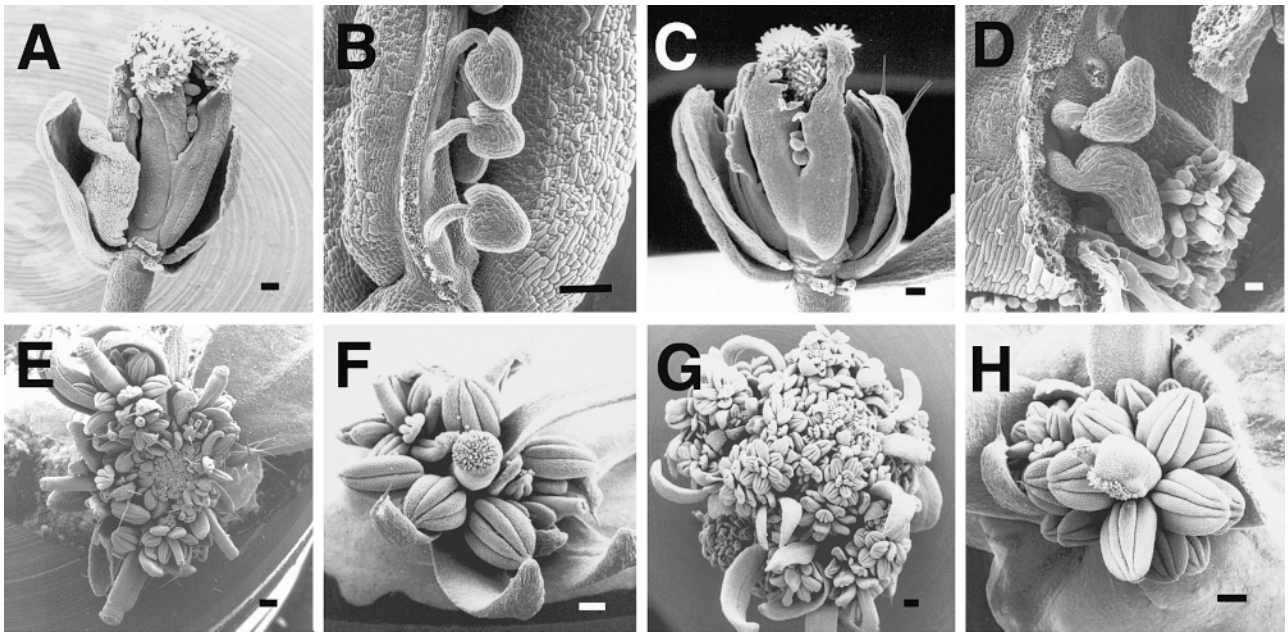

Figure 1. Scanning Electron Microscopy of *sup lfy* and *sup ap1* Double Mutants.

(A) Mature *lfy-6* flower. Two sepal-like organs were removed to show the organs formed inside.
(B) Ovules of a *lfy-6* flower, with the amphitropous configuraton seen in wild type.
(C) Mature *sup-1 lfy-6* flower. Several sepal-like organs were removed to show the organs formed inside.
(D) Ovules of a *sup-1 lfy-6* flower. They exhibit an elongated structure seen in *sup* mutants (Gaiser et al., 1995).
(E) *ap1-1* inflorescence.
(F) *ap1-1* flower. Extra flowers are formed in the axils of the first whorl organs.
(G) *sup-1 ap1-1* inflorescence. Most of the floral organs formed on the inflorescence are stamens.
(H) *sup-1 ap1-1* flower. Stamens are formed interior to whorl 2. A very reduced carpelloid structure with some staminoid characteristics is formed in the center of the flower, similar to the structure found in *sup-1* flowers (Bowman et al., 1992).
Bars in **(A)** to **(C)** and **(E)** to **(H)** = 100 μm; bar in **(D)** = 10 μm.

Table 1. Organ Number of *lfy-6* and *sup-1 lfy-6* Flowers[a]

Genotype	Leaf- or Sepal-like Organs[b]	Carpels or Sepal/Carpel Mosaic Organs	Total No. Organs[c]	
lfy-6	9.6 ± 1.5	4.7 ± 1.1	14.4 ± 1.3	$n = 30$
sup-1 lfy-6	9.7 ± 2.6	4.4 ± 1.3	14.1 ± 2.9	$n = 37$

[a] Numbers given are the mean ±SD.
[b] The leaf-like or sepal-like class includes organs that are mainly leaf-like but had some petaloid or staminoid character.
[c] Flowers 5 and 14 were analyzed for organ numbers. *n*, number of flowers counted.

that of the flowers of *lfy-6* (Figures 1A and 1C). The same number and types of organs were formed in *sup-1 lfy-6* and *lfy-6* flowers (Table 1), demonstrating that *lfy* is epistatic to *sup*. However, ovules produced in the double mutant were often elongated, like those seen in *sup* mutants (Gaiser et al., 1995), indicating an additive phenotype with regard to ovule development (see Figures 1B and 1D).

Another gene that controls the establishment of floral meristem identity is *AP1*. The strong loss-of-function mutation *ap1-1* shows transformation of the two outer whorls into inflorescence-like structures, often forming leaves with axillary flowers in whorl 1 and no petals (Figures 1E and 1F). Whorls 3 and 4 are not affected by the mutation. The double mutant *sup-1 ap1-1* exhibited phenotypes characteristic of both single mutants; whorls 1 and 2 were *ap1*-like, and whorls 3 and 4 were *sup*-like (Figures 1G and 1H). The number of stamens formed in the double mutant flower was the same as the number formed in the *sup* mutant flower (Table 2). The genetic interaction between *sup* and *ap1* thus appears to be additive.

SUP Expression in Floral Meristem Identity Mutants

To further characterize the interaction between *SUP* and the floral meristem identity genes, we examined *SUP* expression in these mutants. *SUP* expression is detected from late stage 3/early stage 4 in the inner part of the ring of floral meristem that will later give rise to stamen primordia (Figures 2A and 2B; Sakai et al., 1995). By stage 8, when stamen primordia are completely separated from the adjacent carpel primordia, *SUP* expression is no longer detectable. In stage 9 flowers, *SUP* expression is detected in developing ovule primordia. This expression later becomes restricted to the stalks of the ovules, called funiculi. We refer to this expression as late *SUP* expression and to that in floral meristems as early *SUP* expression.

In the strong *lfy-6* mutant, early *SUP* expression was not detected in floral meristems at any stage (Figures 3H and 3I). However, later *SUP* expression was seen in ovules that occasionally formed in *lfy* flowers (data not shown). This result is in agreement with the phenotype of the double mutant *sup lfy*, which produces flowers with a floral whorl structure indistinguishable from *lfy* single mutants but produces ovules that are *sup*-like (Figures 1A to 1D).

Consistent with the phenotypes observed in double mutants with *sup* (Figures 1E to 1H; Levin and Meyerowitz, 1995), the strong loss-of-function mutants of two other genes controlling floral meristem identity (*ap1-1* and *ufo-2*) cause no alteration of early *SUP* expression (data not shown).

SUP Expression in Floral Homeotic Mutants

The expression of the homeotic genes *AP3*, *PI*, and *AG* precedes early *SUP* expression by approximately half a day. This observation raises the possibility that these floral homeotic genes act upstream of *SUP* and regulate *SUP* expression in whorl 3 (Sakai et al., 1995). To explore this, we analyzed *SUP* expression in *ap3*, *pi*, and *ag* mutant flowers. To be as quantitative as possible, all floral tissues were fixed and processed in the same way, and the mutant and wild-type sections were placed on the same slides. The tissues on the slides were then hybridized with the ^{35}S-labeled *SUP* antisense probe.

In strong *ap3-3* and *pi-1* mutants with nonsense mutations (Jack et al., 1992; Goto and Meyerowitz, 1994), decreased early *SUP* expression was detectable in late stage 3/early stage 4 flowers (Figures 2C and 2E). The spatial expression pattern, however, was not affected in these young mutant flowers. At stage 5 and later, *SUP* expression was further reduced, to an undetectable level (Figures 2D and 2F). In contrast to the early expression, late *SUP* expression in funiculi of ovules was not affected by these mutations (data not shown). In the strong *ag-3* mutant, no reduction of the initial *SUP* expression in stage 4 flowers was observed; however, no *SUP* expression was detected in whorl 3 primordia at stage 5 and older (Figures 2G and 2H). *SUP* was subsequently and repeatedly expressed in the stage 4 floral meristems that formed in the interior region of the indeterminate *ag* flowers. These results show that the class B homeotic genes *AP3* and *PI* positively regulate early *SUP* expression from its initial stage, whereas the class C gene

Table 2. Stamen and Axillary Flower Numbers in *ap1-1*, *sup-1*, and *sup-1 ap1-1* Flowers[a]

Genotype	Stamens		Axillary Flowers[b]	
ap1-1	ND[c]		1.8 ± 0.7	$n = 20$
sup-1	11.3 ± 1.7	$n = 25$	0 ± 0	$n = 25$
sup-1 ap1-1	12.4 ± 1.6	$n = 25$	2.2 ± 1.0	$n = 20$

[a] Numbers given are the mean ±SD; *n*, number of flowers counted.
[b] Flowers 5 and 14 were analyzed for organ numbers.
[c] ND, not determined.

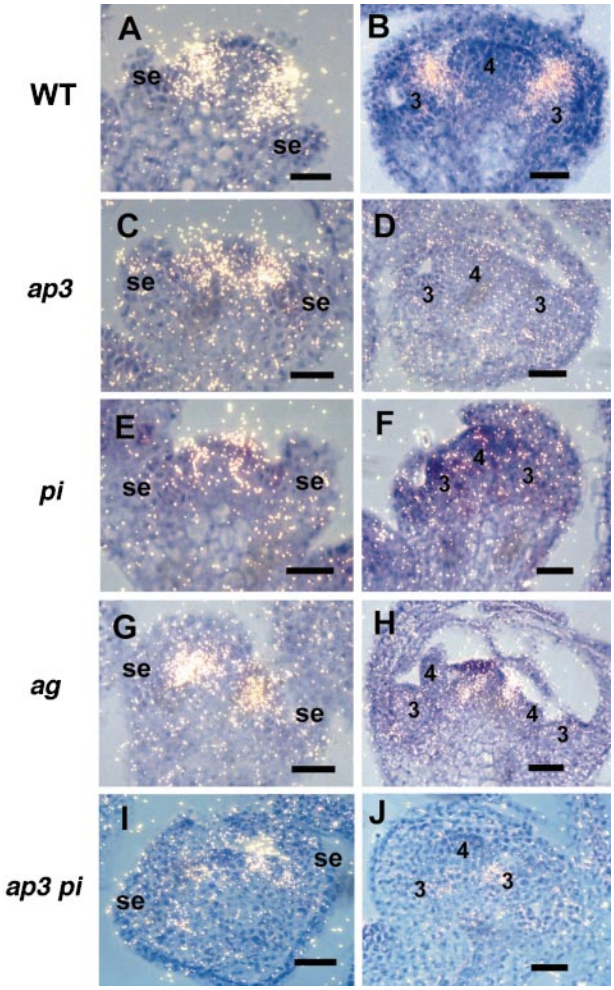

Figure 2. Expression of *SUP* RNA in Wild-Type (Landsberg *erecta*), *ap3, pi, ag*, and *ap3 pi* Mutant Flowers.

(A) and **(B)** Wild type (WT).
(C) and **(D)** *ap3-3*.
(E) and **(F)** *pi-1*.
(G) and **(H)** *ag-3*.
(I) and **(J)** *ap3-3 pi-1*.
SUP expression is shown in late stage 3/stage 4 in **(A)**, **(C)**, **(E)**, **(G)**, and **(I)** and in stage 5/6 in **(B)**, **(D)**, **(F)**, **(H)**, and **(J)**. Flowers were sectioned longitudinally. In situ hybridization with the *SUP* antisense probe was performed as described by Sakai et al. (1995). To detect weakly expressed *SUP* RNA, sections were exposed on film for 2 months after hybridization. se, sepal primordium; 3, whorl 3 primordium; 4, whorl 4 primordium. Bars = 10 μm.

AG is involved only in maintenance of *SUP* expression from stage 5 onward.

We further examined *SUP* expression in the class A floral homeotic mutant *ap2-2*, which is a strong allele with a splice junction alteration that acts to truncate the encoded protein. In *ap2-2*, *SUP* was expressed in the area of the floral meristem corresponding to the third whorl (data not shown). Later, *SUP* was ectopically expressed in the carpelloid organs formed in whorl 1 of the *ap2-2* mutant flower, which correlated with late *SUP* expression in ovule primordia. These results show that the class A homeotic gene *AP2* is not required for the normal regulation of early *SUP* expression.

Strong Class B Mutations Are Not Epistatic to *sup*

Although the loss of *AP3* and *PI* function reduced early *SUP* expression, *SUP* RNA was still detectable in stage 4 floral meristems (Figures 2C and 2E). To investigate whether this residual expression retains any function, we examined double mutants between *sup-1* and the strong class B mutants in detail. Previously, flowers of the double mutants had been shown to exhibit a phenotype very similar to those of *ap3* and *pi* single mutants (Schultz et al., 1991; Bowman et al., 1992). Our detailed analysis, however, revealed that several features of double mutant flowers were distinctly different from single mutant flowers. In strong *ap3-3* and *pi-1* mutants, filamentous organ structures frequently formed in whorl 3 (Figures 4A and 4H). The organs occasionally showed carpelloid characteristics and were sometimes fused to the central gynoecium in whorl 4. The formation of these structures was reduced acropetally such that these filamentous organs were more frequently found in early-forming flowers compared with late-forming flowers (Table 3). The late-forming flowers of *ap3-3* and *pi-1* often had no solitary organs in whorl 3 but instead formed a central gynoecium that consisted of several unfused carpels. In the double mutants *sup-1 ap3-3* and *sup-1 pi-1*, no formation of the filamentous organs in whorl 3 was observed (Table 3 and Figures 4D and 4K). Moreover, flowers of the double mutants produced only one gynoecium interior to whorl 2 sepals, and there was no obvious acropetal difference in floral organ formation. The gynoecium of the double mutants was more uniformly fused than that of the *ap3-3* and *pi-1* single mutants (Figures 4C, 4F, and 4G). When developing flowers were examined by scanning electron microscopy, the gynoecium of the double mutants appeared to have originated from six fused organ primordia—two lateral and four medial—a pattern that resembled the ontogeny of the wild-type whorl 3 organs (Figure 4F). Formation of any other organ primordia distinct from the central gynoecium was not observed inside the gynoecium at or near stage 7 (Figure 4G), whereas organ primordia were formed from two whorls in *ap3-3* and *pi-1* and displayed some irregularity in the numbers and their fusions (Figure 4C; Jack et al., 1992).

To further investigate the developmental changes of double mutant flowers, we analyzed histological features of *sup-1 pi-1* and *sup-1 ap3-3*. As early as stage 6, when the carpel primordia were formed from the floral meristem, we observed a difference in the development of the central region. In the single mutants *pi-1* and *ap3-3*, the center of the floral meristem developed into carpel primordia (Figures 5A to

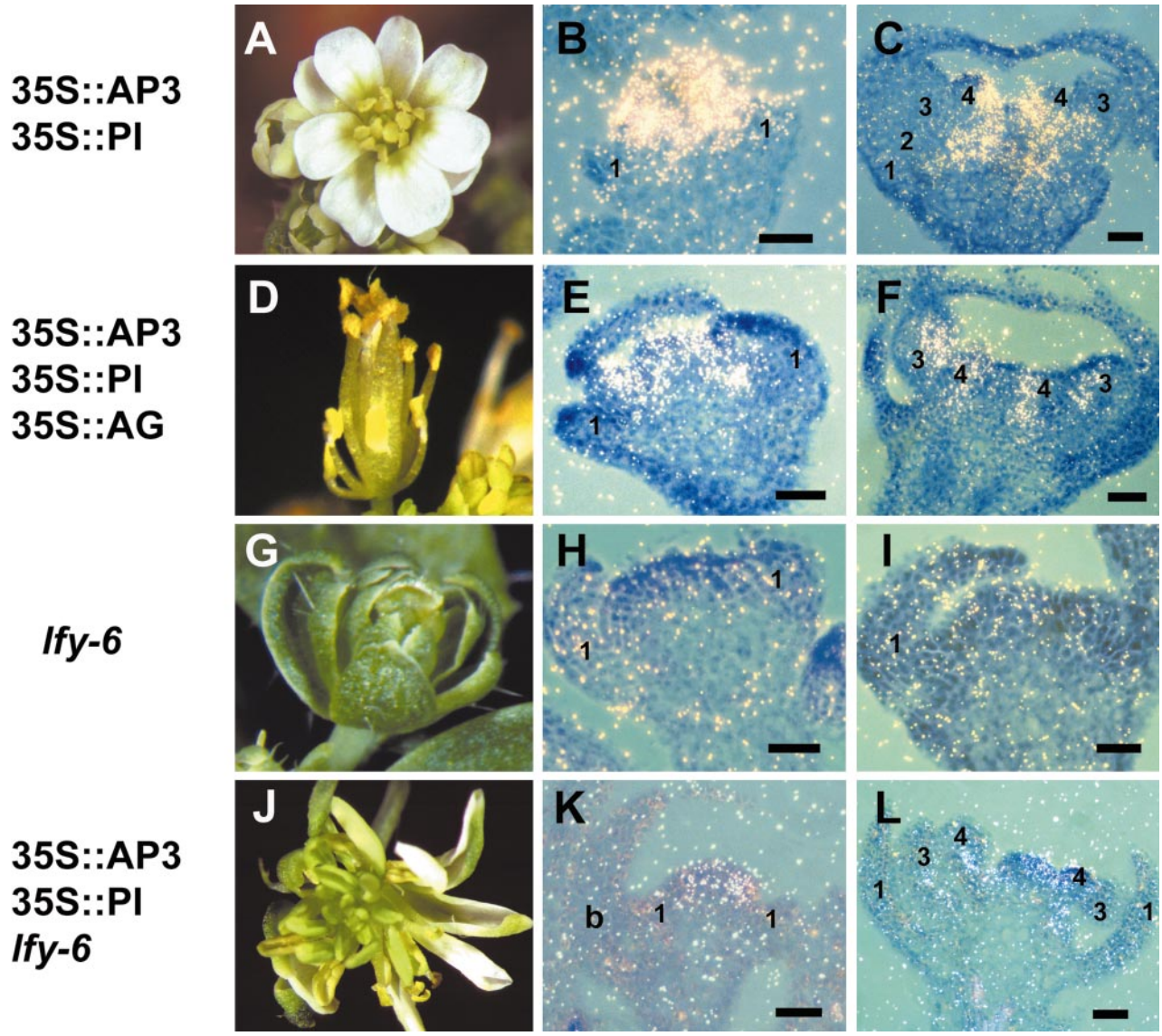

Figure 3. Expression of *SUP* RNA in Flowers of the *lfy* Mutant and Transgenic Plants.

Flowers were sectioned longitudinally.
(A) to **(C)** 35S::AP3 35S::PI transgenic plants. Flowers ectopically expressing *AP3* and *PI* have transformation of sepals to petals in whorl 1 and carpels to stamens in whorl 4 with indeterminate characteristics (Krizek and Meyerowitz, 1996). **(A)** A mature flower; **(B)** Stage 4 flower; **(C)** a stage 6 flower.
(D) to **(F)** 35S::AP3 35S::PI 35S::AG transgenic plants. All floral organs are transformed to stamens or staminoid organs in the triple transgenic lines expressing *AP3*, *PI*, and *AG* ectopically. **(D)** A mature flower; **(E)** a stage 4 flower; **(F)** a stage 6 flower.
(G) to **(I)** Floral organs of the strong *lfy-6* mutant carrying a nonsense mutation are mostly sepal- or carpel-like. **(H)** A stage 4 flower; **(I)** a stage 5 flower.
(J) to **(L)** 35S::AP3 35S::PI *lfy-6* transgenic plants. Ectopic expression of *AP3* and *PI* partially rescues the organ identity defect of *lfy-6*, producing more stamen- and petal-like organs (Krizek and Meyerowitz, 1996). **(J)** A mature flower; **(K)** a stage 4 flower; **(L)** a stage 6 flower.
1, whorl 1 primordium; 2, whorl 2 primordium; 3, whorl 3 primordium; 4, whorl 4 primordium; b, bract. Bars = 10 μm.

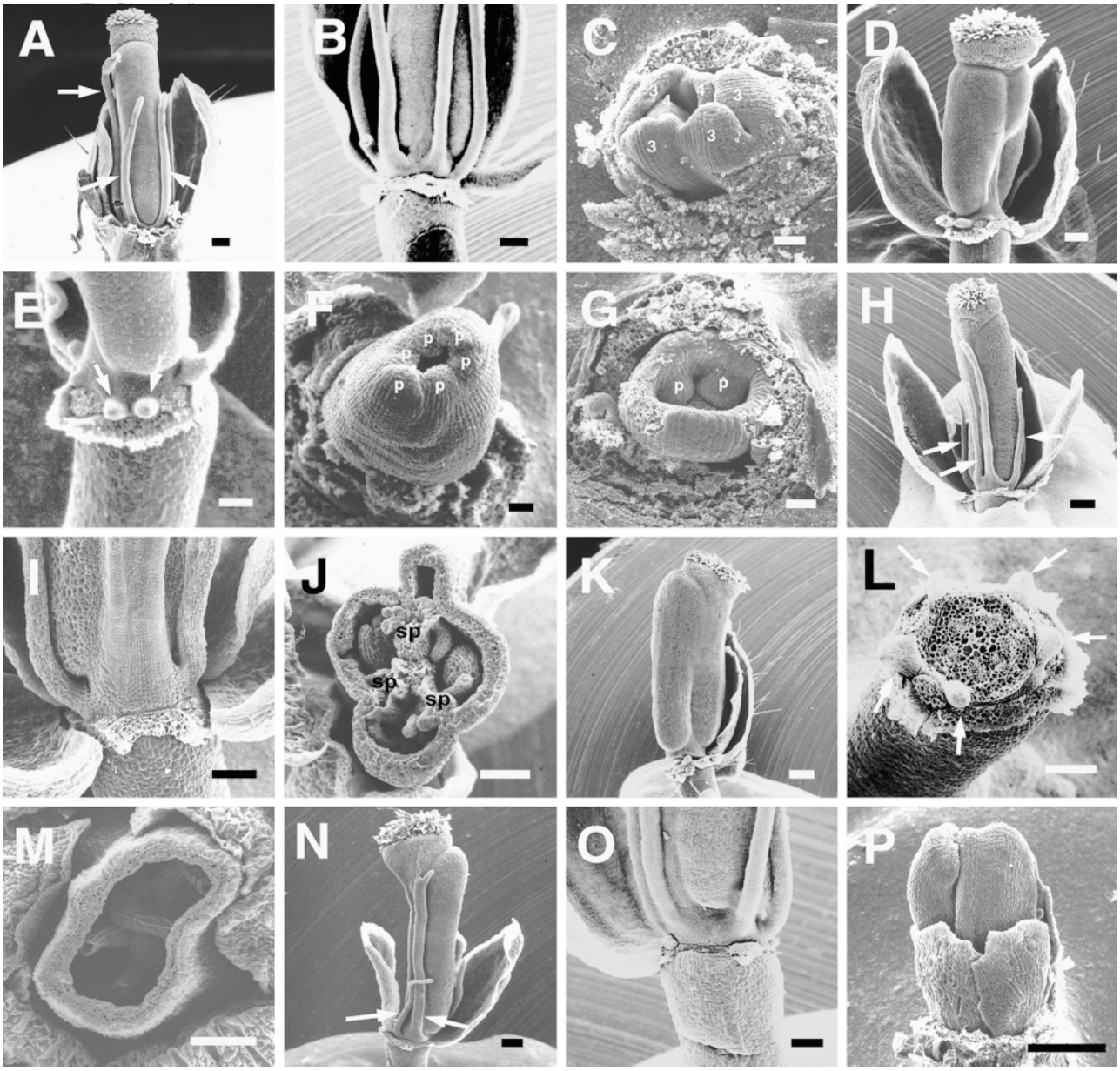

Figure 4. Scanning Electron Microscopy of the Double Mutants *sup ap3*, *sup pi*, and *ap3 pi*.

(A) to **(C)** *ap3-3* flowers. **(A)** Mature flower with solitary filamentous organs in the third whorl (indicated by arrows). Several sepals in whorl 1 and 2 were dissected. **(B)** The receptacle region of a flower. Mature nectaries are not formed at the base of the third whorl organs. **(C)** Young flower in stage 7. Organ primordia are formed in whorl 3 (3) as well as in whorl 4 (4). All sepal primordia were removed from the flower bud.

(D) to **(G)** *sup-1 ap3-3* flowers. **(D)** Mature flower with no filamentous organs formed between the central gynoecium and the sepals in whorls 1 and 2. **(E)** The receptacle region. Nectaries with a mature appearance are formed at the base of the central gynoecium (indicated by arrows). **(F)** Young stage 7 flower with the central gynoecium developed as six fused lobes in the distal region, indicating that it consists of six organ primordia (p). All sepal primordia were dissected from the flower. **(G)** Young stage 7 flower with the central gynoecium formed as a fused structure of several primordia (p) from a very early developmental stage. All sepal primordia were removed.

(H) to **(J)** *pi-1* flowers. **(H)** Mature flower with solitary, filamentous organs in whorl 3 (indicated by arrows). Several sepals in whorls 1 and 2 were removed. **(I)** The receptacle region of a flower. Mature nectaries were not formed at the base of the third whorl organs. **(J)** Transverse section through a mature *pi-1* gynoecium. sp, septa between carpels.

(K) to **(M)** *sup-1 pi-1* flowers. **(K)** Mature flower with several sepals in whorls 1 and 2 dissected. No organs were formed between the central gynoecium and the sepals in whorls 1 and 2. The surface of the gynoecium does not show any clear septum along its whole length. **(L)** The receptacle region of a mature flower. All distal floral organs were removed to show the nectaries (indicated by arrows), which look well developed. **(M)** Transverse section of a mature gynoecium. Note that the septum is not evident within the central gynoecium.

(N) to **(P)** *ap3-3 pi-1* flowers. **(N)** Mature flower showing solitary or fused filamentous organs formed between whorls 2 and 4 (indicated by arrows), similar to the organs seen in the single mutants *ap3-3* and *pi-1*. **(O)** The receptacle region of a mature flower with immature nectaries. **(P)** Young stage 7 flower. The carpel primordia develop as separate organs, as seen in *ap3-3* and *pi-1*.

Bars in **(C)**, **(F)**, and **(G)** = 10 μm; all other bars = 100 μm.

5C, 5J, and 5K), whereas in *sup-1 ap3-3* and *sup-1 pi-1*, the region remained as an undeveloped meristematic structure (Figures 5D to 5F, 5M, and 5N). This structure was further detected through the carpel development of the double mutants. A similar meristematic structure has also been observed in *sup* single mutants (Schultz et al., 1991; Bowman et al., 1992; Sakai et al., 1995). For *sup pi* double mutants, similar ontogeny has been noted in *sup-2* (*flo10*) *pi-1* double mutants (Schultz et al., 1991). The phenotypic correlation between *sup* and the double mutants is observed in the further floral organ development. In *sup-1 ap3-3* and *sup-1 pi-1*, the base of the inner gynoecium surface often formed several bulges, which later developed into placental structures with ovules (Figures 5F and 5N). Occasionally, the outgrowth of the inner surface was so extensive that a new gynoecium structure was later formed inside of the original gynoecium (Figure 5O). Such overproliferation of the placental region was not detected in the single mutants *ap3-3* and *pi-1* (Figures 5C and 5K). Although the bulged structure was often congenitally fused with the gynoecium, it resembled the formation of extra whorls of stamen primordia in *sup* mutants, which also differentiated after the development of organ primordia in whorl 3. The subsequent gynoecium development of the double mutants also differed from single mutants and wild type. In double mutants, the placental region was restricted to the bulged structure, which did not extend along the entire length of carpels (data not shown). In accordance with the irregular placentation, a complete septum did not form in the gynoecium of double mutants, and often no clear evidence of the region between the valves on the outer epidermis of gynoecium was detected (Figures 4K and 5I).

Flowers of the double mutants further differed from the *ap3* and *pi* single mutant flowers in the formation of nectaries. In wild-type flowers, nectaries form at the outer bases of stamen filaments. In *ap3-3* and *pi-1*, nectaries rarely developed, and those that did usually appeared small and immature (Figures 4B and 4I). On the other hand, in double mutants, nectaries morphologically indistinguishable from those of wild type frequently formed at the base of the central gynoecium (Figures 4E and 4L). Although the ontogenetic linkage of nectaries to whorl 3 is not proven, the formation of well-developed nectaries suggests that the *sup ap3* and *sup pi* double mutants have restored the structure of the whorl 3 region, which is reduced in *ap3* or *pi* mutants.

These phenotypes strongly indicate that the gynoecium of the double mutants forms from whorl 3 organ primordia and that the development of the whorl 4 organs is arrested even in the background of *pi* and *ap3* loss of function. Accordingly, these strong class B mutations are not epistatic to *sup*. Rather, they exhibit an additive phenotype with the *sup* mutation.

Loss of Both *AP3* and *PI* Functions Does Not Completely Eliminate *SUP* Expression

To investigate whether the residual expression of *SUP* in *ap3-3* or *pi-1* single mutants depends on the activity of the partner gene (*PI* or *AP3*, respectively), we examined *SUP* expression in the *ap3-3 pi-1* double mutant. F_2 double mutant plants were identified by polymerase chain reaction (PCR)–based genotyping (see Methods). For comparison, sections of the wild type and the *pi-1* and *ap3-3* single mutants were placed on the same slides with sections from the *ap3-3 pi-1* double mutant and processed simultaneously. *SUP* expression in whorl 3 was weakly detected in the double mutant with an intensity similar to that observed in the single mutants (Figures 2I and 2J). This further shows that an additional gene or genes aside from *AP3* and *PI* are involved in induction of *SUP* expression at the initial phase.

The phenotype of *ap3-3 pi-1* also demonstrates that *SUP* has a function even in the absence of both *AP3* and *PI* activity. The isolated *ap3-3 pi-1* double mutant plants exhibited a phenotype indistinguishable from the single mutant *ap3-3* and *pi-1* plants (Figures 4N to 4P). In the *ap3-3 pi-1* flowers, filamentous organs were often produced in whorl 3, like those produced in the single mutants. The development of nectaries

Table 3. Number of Organs between Whorl 2 and the Central Gynoecium in *ap3*, *pi*, *sup ap3*, and *sup pi* Flowers

	No. of Filamentous Organs in Whorl 3[b] (Solitary Organ No./Fused Organ No.)[c]							
Floral Order[a]	*ap3-3*		*sup-1 ap3-3*		*pi-1*		*sup-1 pi-1*	
1–4	2.7 ± 1.9/1.0 ± 1.1	$n = 44$	0 ± 0/0 ± 0	$n = 51$	2.0 ± 1.8/1.2 ± 1.3	$n = 33$	0 ± 0/0 ± 0	$n = 36$
5–8	1.0 ± 1.3/0.7 ± 1.0	$n = 42$	0 ± 0/0 ± 0	$n = 60$	0.6 ± 1.0/0.6 ± 1.0	$n = 36$	0 ± 0/0 ± 0	$n = 36$
9–12	0.5 ± 0.9/0.3 ± 0.5	$n = 44$	0 ± 0/0 ± 0	$n = 60$	0.1 ± 0.2/0.3 ± 0.8	$n = 36$	0 ± 0/0 ± 0	$n = 36$
13–16	0.2 ± 0.5/0.4 ± 0.6	$n = 44$	0 ± 0/0 ± 0	$n = 60$	0.2 ± 0.6/0.2 ± 0.6	$n = 36$	0 ± 0/0 ± 0	$n = 36$
17–20	0.1 ± 0.4/0.1 ± 0.3	$n = 44$	0 ± 0/0 ± 0	$n = 60$	0.1 ± 0.5/0.2 ± 0.5	$n = 36$	0 ± 0/0 ± 0	$n = 36$

[a] Four flowers were grouped together for organ counting according to their ages, that is, floral formation along the inflorescence (the earliest produced flower is counted as 1 of the floral order).

[b] Numbers given are the mean ±SD; *n*, number of flowers counted.

[c] Solitary organs were counted separately from organs that were fused to the central gynoecium along more than half of their length.

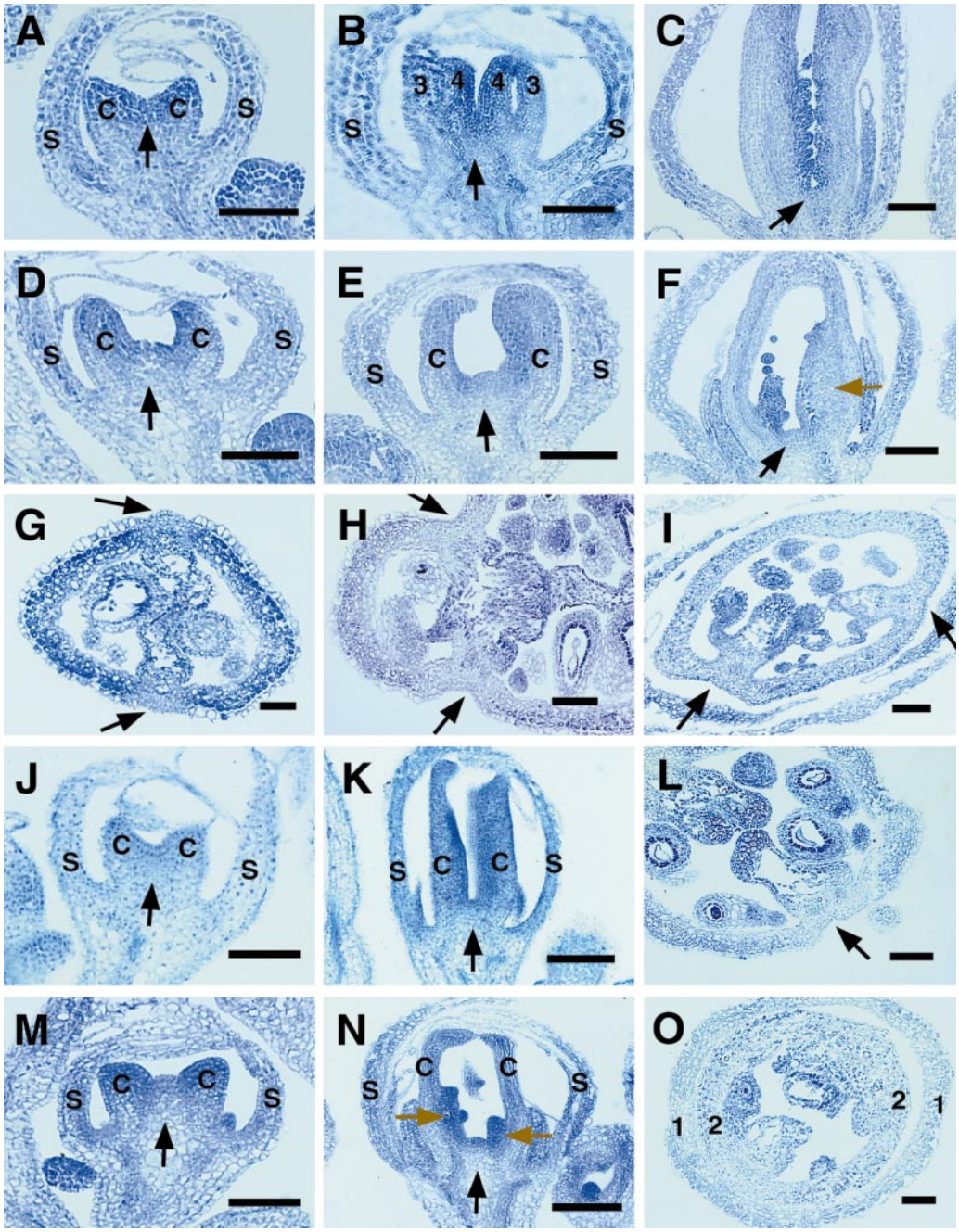

Figure 5. Histological Analyses of *ap3-3*, *pi-1,* and the Double Mutants *sup-1 ap3-3* and *sup-1 pi-1*.

(A) to **(C)** and **(H)** *ap3-3*.
(D) to **(F)** and **(I)** *sup-1 ap3-3*.
(G) Wild type (Landsberg *erecta*).
(J) to **(L)** *pi-1.*
(M) to **(O)** *sup-1 pi-1*.
(A), **(D)**, **(J)**, and **(M)** Developing flowers at stage 6; **(B)** and **(E)** developing flowers at stage 7; **(C)** and **(F)** young flowers at stage 9; **(K)** and **(N)** young flowers at stage 8; and **(G)** to **(I)**, **(L)**, and **(O)**, transverse sections of gynoecium. The central region (indicated with black arrows) shows an undeveloped meristematic structure in double mutants. Brown arrows on **(F)** and **(N)** show the region formed inside of the carpel primordia. Arrows on **(G)** to **(I)** and **(L)** indicate the valval area of gynoecia. In wild type **(G)**, the valval area forms a distinct tissue structure with small epidermal cells. Similar structures are seen in *ap3-3* **(H)** and *pi-1* **(L)** gynoecia but are not obvious in double mutants **(I)**. Occasionally, a gynoecium (designated 2) is formed within a gynoecium (designated 1) in double mutants **(O)**.
C, carpel primordium; S, sepal; 3, whorl 3 organ primordium; 4, whorl 4 organ primordium. Bars = 50 μm.

was also impaired, similar to what was observed in *ap3-3* and *pi-1* single mutants (Figure 4O). Given how this phenotype differs from that of *sup-1 ap3-3* or *sup-1 pi-1*, *SUP* appears to have a function, even in the *ap3-3 pi-1* double mutant.

AP3, *PI*, and *AG* Are Not Sufficient to Activate *SUP* Expression in Whorls 1 and 2

Our expression analysis showed that the homeotic genes specifying stamen identity, *AP3*, *PI*, and *AG*, positively regulate *SUP* expression in the adaxial part of whorl 3. To determine whether these homeotic genes were sufficient to induce *SUP* expression, we further examined *SUP* expression in flowers constitutively expressing these homeotic genes. Transgenic plants constitutively expressing both *AP3* and *PI* under the 35S promoter of cauliflower mosaic virus form petals in whorls 1 and 2 and stamens in all interior whorls (Krizek and Meyerowitz, 1996). The initial *SUP* expression in these flowers was found in a pattern very similar to that seen in wild type but at a higher level (Figures 3B and 3C). Later, *SUP* expression was detected in the stamen primordia that formed interior to whorl 3. This expression pattern was very similar to that seen in the 35S::AP3 transgenic plants (Sakai et al., 1995). Although *SUP* expression was observed in the stamen primordia formed interior to whorl 3, it was not detected in the central region of the floral meristem, where all three of the *AP3*, *PI*, and *AG* genes were expressed. This indicates that another factor is present that prevents *SUP* expression in the center of whorl 4.

SUP was expressed in a similar pattern in flowers that ectopically expressed all three of these homeotic genes (*AP3*, *PI*, and *AG*) under the control of the constitutive 35S promoter from cauliflower mosaic virus. These transgenic plants produced flowers with stamens and staminoid organs in all floral whorls (Figure 3D). In the flowers of these transgenic plants, *SUP* was expressed in the whorls interior to whorl 3 but not in the outer two whorls and not in the very center of the floral meristem (Figures 3E and 3F). The expression pattern was indistinguishable from that seen in the 35S::AP3 35S::PI double transgenic flowers. Similar results were obtained in experiments using two different transgenic plants: 35S::AG, constitutively expressing *AG* under the 35S promoter, and pAP3::AG, expressing *AG* in whorl 2 under the *AP3* promoter (data not shown). These results demonstrate that *AP3*, *PI*, and *AG* are not sufficient to induce *SUP* expression in whorls 1, 2, and the center of floral meristems—a further indication that another factor is restricting *SUP* expression to whorl 3.

LFY Regulates *SUP* through Two Pathways

LFY positively regulates the class B homeotic genes *AP3* and *PI* (Weigel and Meyerowitz, 1993). To elucidate possible interactions between *LFY* and *AP3*/*PI* in *SUP* regulation, we analyzed *SUP* expression in the *lfy* mutant with constitutive

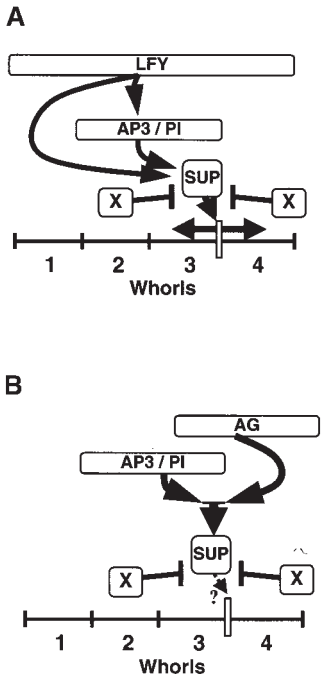

Figure 6. Schematic Presentation of *SUP* Gene Regulation.

The regulation of early *SUP* expression has two distinct phases, the initiation phase (stage 3/4) **(A)** and the maintenance phase (stage 5 and later) **(B)**. In the first phase, *SUP* is controlled through at least three pathways; two pathways are regulated by *LFY* (one mediated through *AP3*/*PI*), and another pathway restricts the spatial and temporal pattern, which is regulated by one or more as yet unidentified genes (X). The role of X can also be filled by an activator specific for whorl 3. *SUP* expression at this phase is sufficient to fulfill the function of maintaining the whorl 3/4 boundary. In the maintenance phase, *AP3*, *PI*, and *AG* control *SUP* expression. The activity of each homeotic gene is absolutely required for the maintenance of *SUP* expression. The *SUP* function in this phase is as yet undetermined.

AP3 and *PI* expression (35S::AP3 35S::PI *lfy-6*). Flowers of these plants formed organs with more staminoid characteristics than seen in *lfy-6* (Figure 3J; Krizek and Meyerowitz, 1996). In this background, *SUP* expression was detected in the whorl 3 region of floral meristems (Figures 3K and 3L), although this expression was substantially lower than in the 35S::AP3 35S::PI plants. This result shows that early *SUP* expression can be partially recovered through activation of *AP3* and *PI* in the absence of *LFY* function; further, at least two pathways of *SUP* regulation are controlled by *LFY*, one mediated through *AP3* and *PI*, and the other mediated not through these class B genes but in some other way. Given that *SUP* is expressed in whorl 3 from stage 3/4 in this transgenic *lfy* mutant background, as was seen in wild type, the whorl-specific factor that restricts early *SUP* expression temporally and spatially appears not to be affected by *LFY* activity.

DISCUSSION

Role of *LFY* in *SUP* Expression

Our results show that the floral meristem identity gene *LFY* regulates *SUP* expression in floral meristems. The double mutant between *sup-1* and the strong nonsense mutant allele *lfy-6* is indistinguishable from *lfy-6* with regard to whorl structure and floral organ numbers. However, the double mutant produces ovules similar to those of *sup-1*. This phenotype is in agreement with the *SUP* expression pattern; that is, early *SUP* expression but not late expression is repressed by the loss of *LFY* activity. Previously, the class B homeotic genes were shown to be partly regulated by *LFY* (Weigel and Meyerowitz, 1993). Examination of *SUP* expression in *lfy-6* transgenic plants ectopically expressing *AP3* and *PI* showed partial recovery of *SUP* early expression. This demonstrates that *LFY* regulates early *SUP* expression via two pathways, one of which is mediated through *AP3*/*PI* (see Figure 6A). The full requirement of *LFY* for *SUP* early expression is rather remarkable, considering that the loss of *LFY* function alone does not fully eliminate the floral whorl structure. This may reflect that the genetic circuit establishing the whorl structure is not the same as the one maintaining it. Recently, *LFY* was shown to act on downstream genes through direct binding to target sites (Parcy et al., 1998; Busch et al., 1999). The 6.7-kb-long *SUP* genomic fragment that complements the *sup* mutation also contains three sites matching the consensus sequence for LFY binding (Sakai et al., 1995; H. Sakai, unpublished data). Future analyses of the *SUP* promoter will determine whether the non–*AP3*/*PI LFY* pathway is mediated by direct LFY binding.

Floral Homeotic Genes *AP3*, *PI*, and *AG* Activate Early *SUP* Expression in Two Phases

By analyzing the expression pattern in corresponding mutants, we showed that the floral homeotic genes *AP3*, *PI*, and *AG* are positive regulators of *SUP*, although their requirement differs temporally. Early *SUP* expression is substantially lessened and becomes undetectable by stage 5 in the loss-of-function mutants of *AP3* and *PI*. Thus, these class B homeotic genes are partly required for the initiation and fully required for the maintenance of *SUP* expression in floral meristems. Their function in control of *SUP* expression appears to be redundant. Flowers of *ap3-3 pi-1* are indistinguishable from *ap3-3* and *pi-1* flowers, forming both third and fourth whorl organs. This is in agreement with the functioning of AP3 and PI proteins as a DNA binding heterodimer (Riechmann et al., 1996). The amount of *SUP* mRNA is strongly reduced but yet detectable in *ap3-3 pi-1* as in *ap3-3* or *pi-1*. Again, this shows that the initial *SUP* expression is positively regulated by factors aside from these homeotic genes.

On the other hand, the class C homeotic gene *AG* functions in a different way. In the strong *ag* mutant, *SUP* expression is unaffected in stage 4 but is not detectable later in the whorl 3 organ primordia. This shows that the role of *AG* is to maintain *SUP* expression in the floral meristem rather than to control initial expression. Phenotypically, *ag* and *sup* mutations exhibit an additive interaction, producing flowers with an indeterminate number of petals interior to whorl 2 in the double mutant (Schultz et al., 1991; Bowman et al., 1992). In *ag* flowers, despite the lack of *SUP* expression at stage 5 and later, the structure of whorl 3 is well maintained and *AP3* is not ectopically expressed in the interior to whorl 3 (Bowman et al., 1991; Jack et al., 1992). This shows that the very brief *SUP* expression in *ag* stage 4 flowers is sufficient to fulfill *SUP* function in whorl boundary maintenance.

The requirement of *AP3*, *PI*, and *AG* for *SUP* expression in the maintenance phase is absolute. *SUP* expression is not maintained when any of these genes loses function. Apparently these homeotic genes coordinate together such that each gene is indispensable for the activation of *SUP* (see Figure 6B). The role of *LFY* in this phase appears to be minimal because *SUP* expression is maintained in stage 5/6 *lfy* flowers when *AP3* and *PI* are overexpressed (35S::AP3 35S::PI *lfy-6*).

Role of as yet Unidentified Genes in *SUP* Expression

The requirement of other factors for early *SUP* expression is further demonstrated by expression analyses in transgenic flowers ectopically expressing homeotic genes. In flowers constitutively expressing *AP3* and *PI*, *SUP* is not activated in the whorl 4 region of the stage 4 floral meristem, where these homeotic genes as well as *AG* are expressed. Moreover, constitutive expression of *AP3*, *PI*, and *AG* does not induce *SUP* expression in whorls 1 and 2, although the transgenic plants form stamens in all floral whorls. The genetic activity required for stamen identity is thus not sufficient to induce *SUP* expression ectopically in floral meristems. These results again show the requirement of an additional whorl-specific factor to activate *SUP* expression in whorl 3 or to prevent *SUP* expression in whorls 1 and 2 as well as in the center of the flower (see Figure 6).

As seen in 35S::AP3 35S::PI *lfy-6* flowers, ectopic expression of *AP3* and *PI* partly restores *SUP* expression in the third whorl region of floral meristem, even in the *lfy* mutant background. This suggests that the whorl-specific factor delimiting *SUP* expression to whorl 3 is still active in the absence of *LFY* function. Because no other known mutants of floral genes, including genes controlling floral meristem identity (*AP1* and *UFO*), affect early *SUP* expression, the third whorl–specific pathway appears to be mediated by at least one as yet unidentified gene. Such whorl-specific factors might not necessarily be flower-specific factors, as was proposed for factors interacting with LFY (Parcy et al., 1998).

Transient Requirement of *SUP* in Whorl Boundary Maintenance

As shown by *SUP* activity in the *ag* mutant, the temporal window of the *SUP* requirement appears to be very brief. This is further demonstrated by the interaction between *sup* and class B homeotic mutations. In the strong mutants *ap3-3* and *pi-1*, early *SUP* expression is detected only in stage 4 flowers and at a reduced amount. However, the flowers of the double mutants *sup ap3* and *sup pi* are not identical to those of *ap3* and *pi* single mutants. In *ap3-3* and *pi-1*, sepals are formed in two outer whorls, whereas carpels and often filamentous organs are formed in two inner whorls. The structure of the two inner whorls is distinguishable in these flowers by the pattern of emerging organ primordia, especially in early forming flowers. On the other hand, *sup ap3-3* and *sup pi-1* double mutants produce flowers with one central gynoecium interior to whorl 2. Based on its morphology and its ontogeny (developing from more than two primordia), the gynoecium appears to be formed from whorl 3 carpel primordia, and the formation of whorl 4 is arrested. The further development of the double mutant gynoecium shows characteristics seen in *sup* mutants: proliferating extra tissues inside of whorl 3 that occasionally form an extra whorl structure, yet undeveloped meristematic structures present at the floral center. These phenotypic correlations reveal that *SUP* is functional in *ap3* and *pi* mutants. Because early *SUP* expression is detectable only at stage 4 in *ap3* and *pi* mutants, *SUP* appears to be required for a short period of time during flower development (possibly $<$12 hr).

Possible *SUP* Function

Gradual alteration of *AP3* expression in *sup* mutant flowers suggests two possible functions for *SUP* in floral meristems (Sakai et al., 1995). In one model, *SUP* regulates *AP3* expression by inhibiting the possible spread of the capability to induce *AP3* mRNA from the third whorl into the fourth whorl region. Alternatively, *SUP* could control the balance of cell proliferation activity in the third and fourth whorl region of the floral meristem. In the latter model, progressive ectopic expression of the class B homeotic genes in the whorl 4 region of *sup* flowers is the consequence of overproliferation of the third whorl cells and of reduced cell division in fourth whorl cells. The results presented here support the latter model. A nonepistatic relationship between *ap3-3*/*pi-1* and *sup-1* demonstrates that *SUP* function is not simply to control the activity of *AP3* and *PI*. The cell division model is also consistent with the phenotype of 35S::AP3 35S::PI *sup-1*. Flowers of these transgenic plants constitutively expressing *AP3* and *PI* in the *sup* mutant background exhibit a more enhanced indeterminate fate of floral meristems than do the flowers of 35S::AP3 35S::PI in the wild-type background (Krizek and Meyerowitz, 1996). One plausible role of *SUP* is therefore to coordinate proliferation of stamen- and carpel-specific meristematic cells, keeping the proper structure of whorls and maintaining the boundary between whorls 3 and 4 at the right position. Because it is expressed in the inner part of whorl 3 but not in whorl 4, *SUP* appears to control the cell division activity of whorl 4 nonautonomously. *SUP* also might induce a cell–cell communication mechanism such as that mediated by *CLAVATA1* and *CLAVATA3* (Fletcher et al., 1999), which allows whorl 3 and 4 cells to receive information from the adjacent floral region. Given that *SUP* is required for a very short time before the cells in floral whorls undergo extensive divisions to produce organ primordia, possibly the role of *SUP* is to trigger a cascade of genetic actions that maintain balanced proliferation of two adjacent floral whorls for a long developmental period.

METHODS

Plant Material

All plants were grown as described previously (Bowman et al., 1989). To obtain the *ap3-3 pi-1* double mutants, homozygous *pi-1* flowers were pollinated by pollen from heterozygous *ap3-3* mutants. F_2 plants from the selfed F_1 were genotyped by polymerase chain reaction (PCR) with the primer sets 5′-AGAGGATAGAGAACCAGACAAATCGA-3′/5′-GTTTAGAGAGATGGTGTACGTGG-3′ for *ap3-3* (Sablowski and Meyerowitz, 1998), and 5′-GATTACTGTTGTCCTTCCATGG-3′/5′-ATCTAGGGTTAAAGATTCAAGGG-3′ for *pi-1* (Riechmann and Meyerowitz, 1997).

In Situ Hybridization

Flowers were fixed, embedded, sectioned, hybridized with the ^{35}S-labeled *SUP* antisense probe, and exposed for 8 to 10 weeks as described previously (Sakai et al., 1995).

The experiments were repeated at least three times for each mutant background.

Scanning Electron and Bright-Field Microscopy

Flowers were fixed, dried, dissected, and coated for scanning electron microscopy as described previously (Bowman et al., 1989). For histological analyses, flowers were fixed and embedded in Paraplast as they were for in situ hybridization. Floral tissues were sectioned in 6- or 4-μm-thick sections and stained with toluidine blue. The sections were analyzed with a Nikon Eclipse-800 microscope, and the images were captured through the charge-coupled device camera system and processed with the Adobe Photoshop 5.0 program (Adobe Systems, Inc., San Jose, CA).

Strain Construction

The transgenic line 35S::AP3 was derived from Jack et al. (1994), and 35S::PI was derived from Krizek and Meyerowitz (1996). The AG open reading frame corresponding to ATG-1 (Mizukami and Ma, 1992) was amplified by PCR and cloned into pCGN1547 containing a

35S promoter and a 3′-nos sequence (Krizek and Meyerowitz, 1996). The construct was transformed into ecotype No-0 by standard root method (Valvekens et al., 1988) and using the Agrobacterium ASE strain to generate the 35S::AG line. The 35S::AP3 35S::PI double mutant was constructed by the crossing described by Krizek and Meyerowitz (1996). The triple transgenic line (35S::AP3 35S::PI 35S::AG) was constructed by fertilizing 35S::AG carpels with pollen from 35S::AP3 35S::PI. Triple transgenics were selected in F_1 plants based on their phenotype.

ACKNOWLEDGMENTS

We thank Catherine Baker, Chiou-Fen Chung, Toshiro Ito, Carolyn Ohno, Doris Wagner, and Eva Ziegelhofer for comments on the manuscript and Pat Koen for assistance with the scanning electron microscope. This work was supported by National Science Foundation Grant No. MCB-9603821 to E.M.M.

Received March 10, 2000; accepted July 24, 2000.

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