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Genes Directing Flower Development in *Arabidopsis*

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We describe the effects of four recessive homeotic mutations that specifically disrupt the development of flowers in *Arabidopsis thaliana*. Each of the recessive mutations affects the outcome of organ development, but not the location of organ primordia. Homeotic transformations observed are as follows. In *agamous-1*, stamens to petals; in *apetala2-1*, sepals to leaves and petals to staminoid petals; in *apetala3-1*, petals to sepals and stamens to carpels; in *pistillata-1*, petals to sepals. In addition, two of these mutations (*ap2-1* and *pi-1*) result in loss of organs, and *ag-1* causes the cells that would ordinarily form the gynoecium to differentiate as a flower. Two of the mutations are temperature-sensitive. Temperature shift experiments indicate that the wild-type *AP2* gene product acts at the time of primordium initiation; the *AP3* product is active later. It seems that the wild-type alleles of these four genes allow cells to determine their place in the developing flower and thus to differentiate appropriately. We propose that these genes may be involved in setting up or responding to concentric, overlapping fields within the flower primordium.

INTRODUCTION

Flowers develop from groups of undifferentiated cells that grow from the flanks of shoot apical meristems. The cells in these floral primordia divide and then differentiate into appropriate numbers of floral organs, in appropriate places. During this process of development, each cell must somehow determine its position relative to others, and must differentiate accordingly. Nothing is known about the mechanisms by which the cells of a developing flower establish their positions and subsequently give rise to appropriate cell types. Several processes do not seem to be involved: there is no cell migration in higher plants, and the totipotency of many higher plant cells indicates that floral primordia do not rely on deposition of positional cues in the egg cell, as is sometimes the case in aspects of animal development. As an approach to revealing the mechanisms by which cells in developing flowers choose an appropriate developmental fate, we are studying genes whose wild-type products seem to play a central role in these mechanisms (Pruitt et al., 1987; Bowman et al., 1988).

In this paper we describe four homeotic mutations in the flowering plant *Arabidopsis thaliana*, each of which appears to affect fundamental processes in floral development. The mutations are *agamous* (*ag*), *apetala2* (*ap2*), *apetala3* (*ap3*), and *pistillata* (*pi*) (Koornneef et al., 1983). All of them are recessive mutations in single genes.

These particular mutations were chosen for detailed study because each appears to cause cells in the developing flower to misinterpret their position, and thus differentiate into inappropriate cell types. At the same time, none of the mutations appears to cause any abnormal phenotype outside of the flower. The adult phenotypes of some alleles of all of them have been described before, although not in detail (Koornneef et al., 1983; Pruitt et al., 1987; Bowman et al., 1988; Haughn and Somerville, 1988). In addition to a detailed description of the adult phenotype, we give here a description of the development of each mutant type, based on scanning electron microscopy. Furthermore, we show that mutant alleles of two of the genes are temperature sensitive, and we determine the temperature-sensitive developmental stage. Finally, the phenotypes and development of double mutants are described as a means of understanding the interactions of the gene products.

As a background to the descriptions of these mutant plants, we must describe briefly the appearance of wild-type flowers. A mature *Arabidopsis* flower is typical of the flowers of plants in the Brassicaceae. It is composed of four concentric whorls. The first whorl is occupied in wild-type flowers by four sepals. We will ignore the two-whorl interpretation of the four sepals in the Brassicaceae (see Lawrence, 1951) because there seems no compelling reason to treat the two pairs of sepals differently, and because we wish to avoid the old controversies about whether the lateral or the medial sepals belong to the outermost whorl (Arber, 1931). Inside and alternate to the sepals are four

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petals, which occupy the second whorl. Historically, the stamens of flowers in the mustard family have been considered as two whorls, the outer containing the two lateral stamens, and the inner the four medial ones (see Lawrence, 1951). Because no studies have revealed fundamental differences between different stamens, we will for convenience and simplicity refer to the region containing the stamens as the third whorl. The center of the flower is occupied by a gynoecium made up of an ovary that contains two chambers separated by a septum. The ovary is topped by a short style and a papillate stigma. Within the ovary there develop approximately 50 ovules, attached in rows to the margins of the carpels. The region occupied by the gynoecium in the wild-type flower will be referred to as the fourth whorl. In describing the mutants, we will use whorl to indicate a region of the flower, regardless of the nature of the organs contained within it.

The flowers develop in a raceme, so that a single stem can have a series of flowers in different stages of development, from primordia at the top to mature fruits nearer the bottom. The development of individual flowers is much like that described for *Cheiranthus cheiri* by Payer (1857), and *Brassica napus* by Polowick and Sawhney (1986). The earliest and latest stages of flower development in *Arabidopsis* have been described (Vaughan, 1955; Müller, 1961).

RESULTS

Development of Wild-Type Flowers

Scanning electron microscope observations of developing *A. thaliana* flowers has allowed their early development to be divided into 12 stages. Flower initiation begins when the cells that will develop into the flower form a buttress on the flank of the florally induced shoot apical meristem (stage 1). As this group of cells grows, an indentation arises that separates it from the adjacent meristem, at which time stage 2 begins. After this, sepal buttresses form on the primordium (stage 3 begins) and grow to form distinct ridges (stage 4). The abaxial and adaxial (medial) sepals form before the lateral ones. The primordia of petals and stamens then appear, and the continued growth of the medial sepals causes them to meet and cover these developing inner organs, marking the start of stage 5. Stage 6 begins when the lateral sepals meet. The primordia of the petals do little until later in flower development; the stamens develop first. Stage 7 begins when the filament and anther precursors become distinct; stage 8 when locules appear in the anthers. Petal elongation accelerates as stage 9 starts; the length of the petals equals that of the short stamens when stage 10 begins. During these stages, the gynoecium is developing from the cells interior to the stamens: An initial dome of cells becomes a cylinder as the cells of the periphery grow; the stigmatic papillae first appear on the rim of the cylinder at the beginning of stage 11. This is also the stage at which the lateral nectaries appear at the base of the lateral filaments; the development of nectaries at the base of the other filaments occurs later. Stage 12 begins when petals reach the height of the long stamens. Figure 1A shows a mature *Arabidopsis* flower; Figure 2A shows some of its early developmental stages. A detailed description of early flower development in *Arabidopsis* is being prepared (D.R. Smyth, J.L. Bowman, and E.M. Meyerowitz, manuscript in preparation).

agamous (*ag*)

agamous flowers consist of many sepals and petals, and of chimeric organs consisting partly of sepal and partly of petal tissue. There are no stamens or carpels. The mutant flowers have an outer whorl of four sepals, then a series of 10 petals, and in the place of the gynoecium a variable number of sepals, petals, and intermediate organs (Figure 1B). The mutant allele of *AG* used (Koornneef et al., 1980) is here designated *ag-1*; its locus is on the fourth chromosome (Koornneef et al., 1983). Observations of developing *ag* flowers make clear the developmental basis of the phenotype: The sepals form normally in the first whorl; the primordia of the second and third whorls also form in their wild-type positions. The remaining cells, which would normally develop into the ovary, behave, however, as if they constituted a new floral primordium (Figure 2B). Four new sepals form at its margins, and apparent petal and stamen primordia develop inside of them. The central cells of this second flower also develop as a new flower, which itself has a new flower develop inside of it, and so on for enough rounds to result in a mature flower with more than 70 organs.

In addition to the development into new flowers of the cells that would ordinarily form an ovary, the third whorl primordia of each flower develop into petals, not stamens; their development is similar in its time course with that of petals, and the organs they form are petals. Although the number of primordia that form petals in the outer flower is a uniform 10, the inner flowers have irregular numbers and positions of primordia that will become petals, and thus irregular numbers and positions of the inner petals. One other irregularity is also apparent. The organs that develop at the margin of each of the internal flower primordia are not perfect sepals, instead they are mosaics of sepal and petal tissue. The mosaic sectors always extend from the base to the apex of the organs, with sepal tissue in the center and petal tissue at the margins (Figure 3). Table 1 summarizes the *ag* phenotype.

apetala2 (*ap2*)

apetala2 is a fourth chromosome gene, mapping more than 25 centimorgans from *ag* (Koornneef et al., 1983).

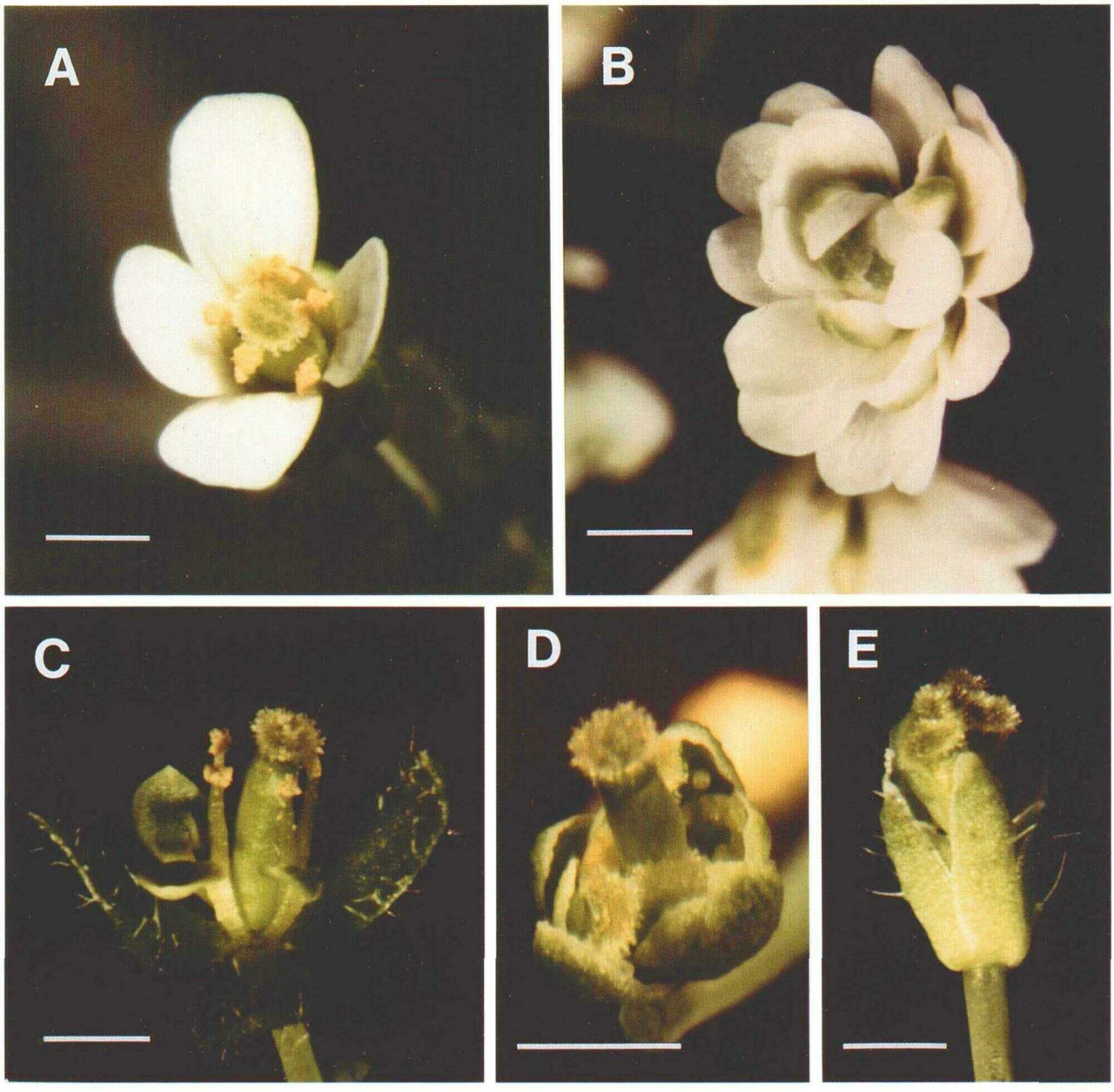

Figure 1. Phenotypes of Wild-Type and Mutant *A. thaliana* Flowers.

(A) Wild-type.
(B) *agamous*.
(C) *apetala2*.
(D) *apetala3*.
(E) *pistillata*.
The plants were grown at 25°C. Bar = 1 mm.

The original *ap2* mutant allele (Koornneef et al., 1980), which we designate *ap2-1*, is temperature sensitive, with different phenotypes at different temperatures (Tables 1 and 2). At all temperatures, the effects are on the outer two whorls. Plants grown at 25°C have an outer whorl consisting of four organs resembling cauline leaves, rather than four sepals (Figure 1C). That they are leaflike is shown by the presence of stellate trichomes, which are charac-

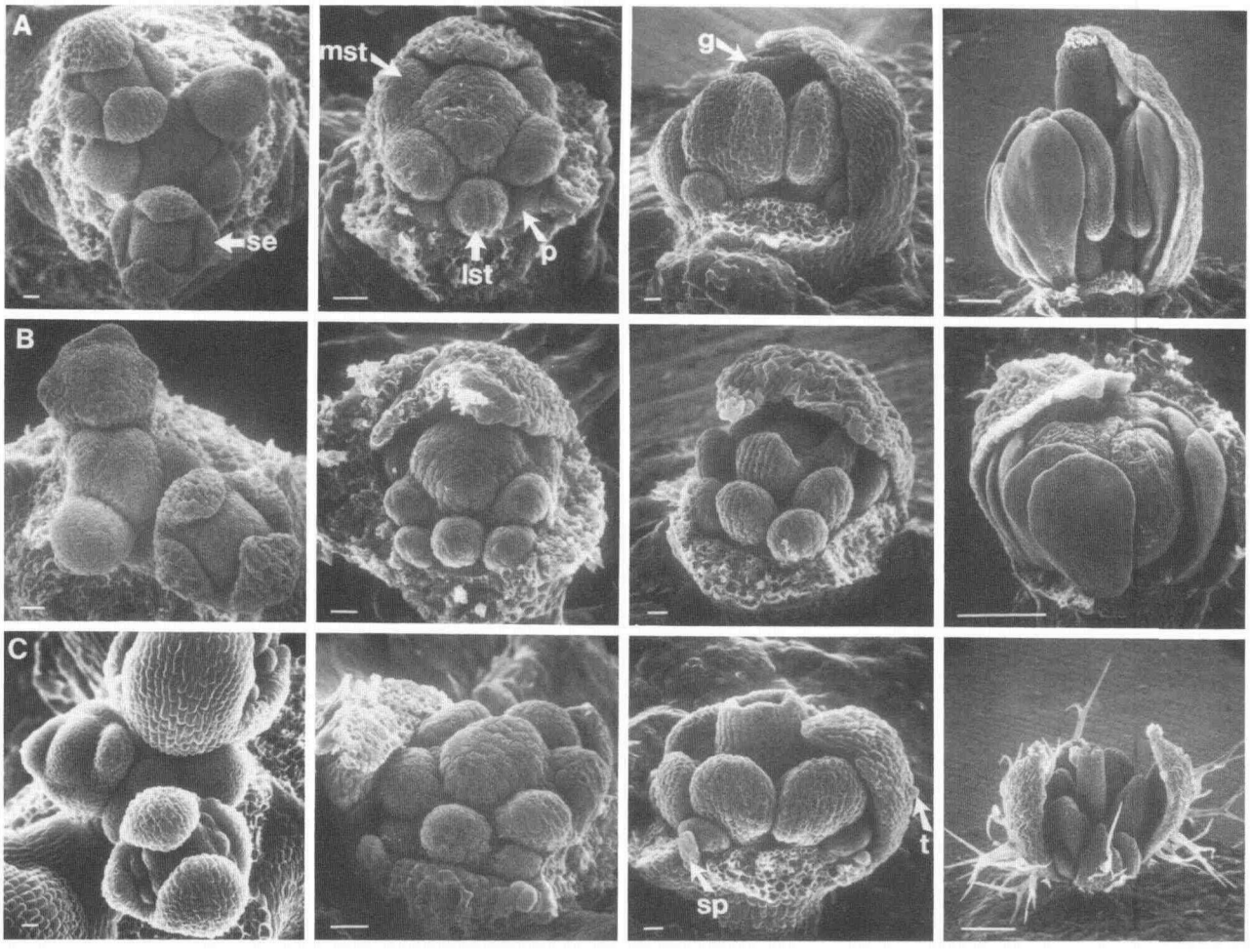

Figure 2. SEM Micrographs of Early Flower Development in Wild-Type and Mutant *Arabidopsis* Plants Grown at 25°C.

The first (lefthand) panel in each series displays the apical meristem and stages 1 through 4 of flower development, with the exception of **(B)**, which shows through stage 6. The second panel shows stage 6, the third panel stage 8, and the fourth panel displays flowers near maturity. In the second and third panels, one to three outer whorl organs have been removed to reveal the inner whorls. In the fourth panel, outer and in some cases **(B,D,E)** second whorl organs have been removed.

(A) Wild-type. Sepal (se), petal (p), medial stamen (mst), and lateral stamen (lst) primordia, and the gynoecial cylinder (g) are indicated.

(B) *agamous*. Nested flowers are visible in the third and fourth panels.

(C) *apetala2*. A stipule (sp) is indicated in the third panel. A trichome (t) at an early stage of development is also noted. The appearance of stigmata on the outer whorl organs precedes their appearance on the gynoecium, as seen in the fourth panel.

teristic of leaves (sepals have few, simple trichomes), appearing as early as stage 7 on both sides of the organs. These trichomes are more dense on the abaxial surface; in genuine cauline leaves they are more dense on the adaxial. In addition, these organs senesce in a way uncharacteristic of sepals, in that they do not yellow and fall off shortly after anthesis. Furthermore, the early development of the organs of the *ap2* outer whorl is characterized by the presence of stipules, present in cauline leaves but not in genuine sepals (Figure 2C). In two respects these organs are not leaflike: they have the long (>100 μm) epidermal cells that are characteristic of sepals, but not leaves, on their abaxial surface. In addition, they often have stigmatic papillae at their tips, revealing a slight gynoecial transformation. The frequency with which stigma tissue is seen at the tips of these organs depends on the position of the flower in the inflorescence, with later flowers showing papillae more often. In addition, stigmatic papillae

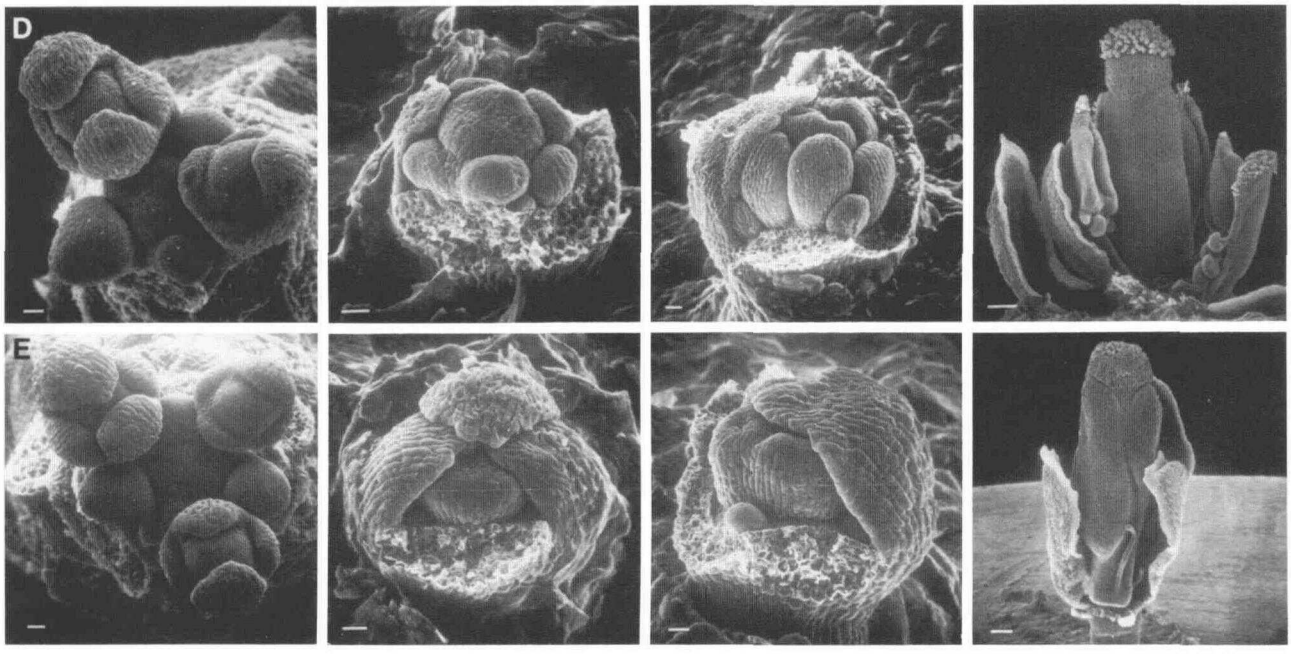

Figure 2 (continued).

(D) *apetala3*. Note the delayed development of the second whorl primordia in the third panel, even though they differentiate into sepals rather than petals (fourth panel).
(E) *pistillata*. The third whorl primordia fail to appear. Bar = 10 μm in the first three panels of each series; bar = 100 μm in the fourth panel.

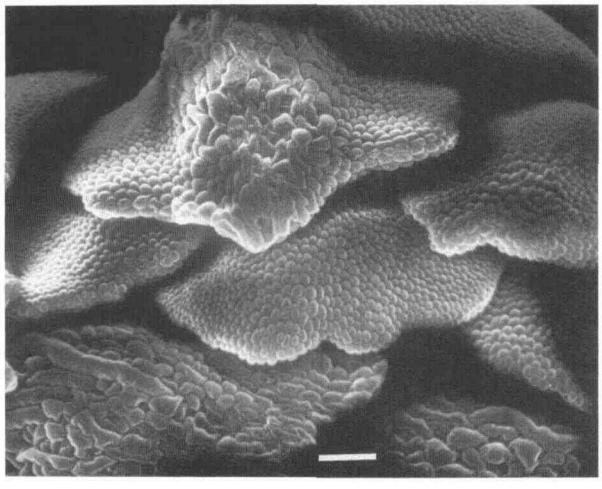

Figure 3. Distinct Petaloid and Sepaloid Regions Are Visible in a Mosaic Organ of an *agamous* Flower.

The transition between the two types of tissue is usually abrupt with a zone of one to three cells of intermediate phenotype. Bar = 30 μm.

occur more frequently on the medial than on the lateral organs. These papillae are first seen in stage 10 of developing *ap2* flowers, prior to the appearance of the stigma on the gynoecium.

The second whorl of *ap2* flowers grown at 25°C shows transformation of petals toward stamens. The transformation is seldom complete, with most organs having features of both petals and stamens; the degree of transformation increases with increasing age of the inflorescence (Figure 4). The intermediate and the most staminoid organs contain pollen grains in locules; only the most staminoid dehisce.

At 16°C, the outer whorl of *ap2-1* homozygous flowers is the same as at 25°C: conversion of sepals to leaves with stigmatic papillae at their tips. At the lower temperature, however, there is very little stigmatic tissue evident. The second whorl is quite different than at the higher temperature, exhibiting in the first flowers on a stem an outward rather than an inward homeosis: the organs range from petals to leaflike structures (Figure 4, Table 2). Organs intermediate between petals and leaves may contain a distinct, longitudinal boundary between green and white regions, but the white regions have epidermal cells that

Table 1. Summary of Phenotypes

Landsberg erecta Wild-Type (wt)		First Whorl Sepals	Second Whorl Petals	Third Whorl Stamens	Fourth Whorl Carpels
agamous	16–25°C	wt	wt	Petals	Flower development repeats
apetala2	16°C	Leaves	wt or slightly phylloid petals	wt	wt
	25°C	Stigmoid leaves	Staminoid petals	wt	wt
	29°C	Carpelloid leaves	Absent	wt	wt
apetala3	16°C	wt	Sepaloid petals	wt	wt
	25°C	wt	Sepals	Carpelloid stamens	wt
	29°C	wt	Sepals	Carpels	wt
pistillata	16–25°C	wt	Sepals	Absent	Extra carpels

show characteristics of both petal and leaf cells (Figure 5). Even the organs most resembling petals are seen to have stomata, which are not ordinarily found on petals. This outward transformation decreases in later flowers, with those flowers after the first 10 showing a slight inward transformation, as at higher temperatures.

At 29°C, the outer whorl consists of leaves with a greater transformation toward gynoecial tissue than at lower temperatures. Stigmatic tissue occurs at the tip of almost every organ and may extend down the lateral margins. Naked ovules develop on one (13 out of 136 organs counted) or both (2 out of 136 organs) margins; this occurs primarily on the medial organs. The most carpelloid organs resemble solitary unfused carpels, but with the stellate trichomes characteristic of leaves on their abaxial surface. The second whorl organs of flowers grown at 29°C either fail to develop at all or are transformed more toward stamens than at 25°C. As at 25°C, the extent of staminody of these organs increases with increasing inflorescence age. The effects of temperature on the development of the organs of the second whorl are detailed in Table 2.

Observations of developing flowers indicate that the failure of an organ to appear in the second whorl is a result of a failure of the organ primordium to initiate development. At no temperature does there appear to be a correlation between the phenotype of a second whorl organ and its position within the whorl. Nearly wild-type and almost completely transformed organs may develop in adjacent positions. The organs of the second whorl, regardless of their eventual developmental fate, develop on a time course characteristic of wild-type petals: they develop after the organs of the other whorls of the flower.

The fact that the *ap2-1* allele is temperature sensitive allows temperature shift experiments to reveal the time at which the wild-type *AP2* gene product is active in flower development. Two types of experiment were performed. First, simple temperature shifts were done. Plants were taken from an incubator at 16°C and transferred to one maintained at 29°C, or vice versa. Since each plant had many flowers at many different stages of development, the effect of a shift on the organs of the second whorl was recorded at all stages of floral development. As seen in the data in Figure 6, A and B, temperature shifts in either direction indicate that the function of the *AP2* product is no later than the developmental period from stage 2 to stage 4. Later than this, temperature shifts have no effect. This developmental time extends from just prior to the appearance of the outer whorl primordia to the time just before the appearance of the second whorl primordia.

The second type of temperature shift experiment done was a temperature pulse, in which plants at 16°C were shifted to 29°C for 48 hr, and then shifted back to the lower temperature, or, conversely, plants at 29°C were

Table 2. Phenotypes of Second Whorl Organs in *ap2* Flowers[a]

	16°C	25°C	29°C
	%	%	%
Absent	<1	29	73
Stamen	0	0	3
Deformed Stamen	0	2	5
Filament	0	0	3
Petaloid Stamen	<1	24	7
Staminoid Petal	6	37	10
Petal	82	9	0
Phylloid Petal	6	0	0
Petaloid Leaf	3	0	0
Cauline Leaflike	2	0	0

[a] The second whorl organs of the first 10 to 14 flowers produced on at least four plants were scored and classified according to the phenotypes described in Figure 4.

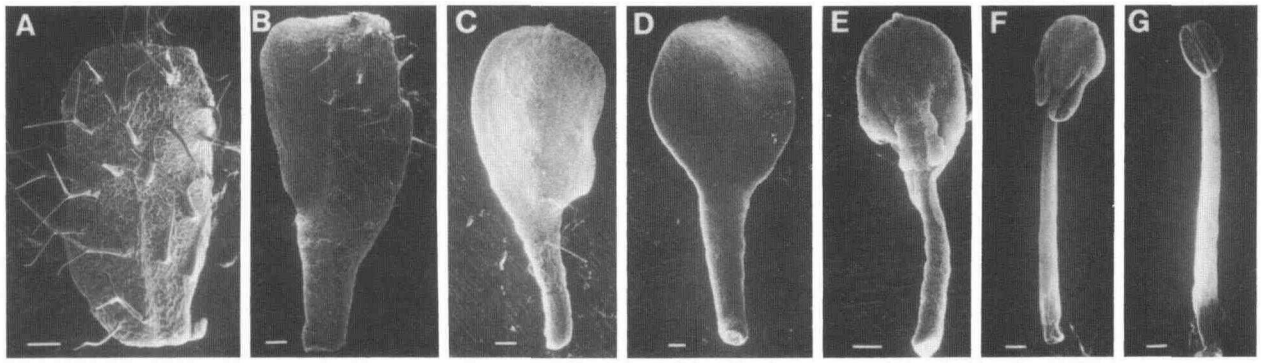

Figure 4. SEM Micrographs of Organs Observed in the Second Whorl of *apetala2* Flowers.

Petals and leaflike organs are common at 16°C, stamen-like petals typical at 25°C, and petal-like stamens or no organ occurring at 29°C (Table 1). To allow comparison, the intermediate forms were categorized.

(A) Organs with no trace of white tissue were classified as cauline leaflike, whereas those with a small amount **(B)** were termed petaloid leaves.

(C) Mostly white organs or those all-white organs possessing trichomes were classified as phylloid petals.

(D) Morphologically wild-type petals were typical at 16°C.

(E) White, petal-shaped organs possessing rudimentary locules were termed staminoid petals.

(F) Those organs classified as petaloid stamens were shaped like stamens but with some white petal tissue, usually near the top of the organ. Misshapen stamens and filaments lacking anthers were observed at a low frequency.

(G) Morphologically wild-type stamens occur at a low frequency at 29°C.

Outer surfaces are shown in **(A)**, **(B)**, and **(C)**; inner surfaces in **(D)**, **(E)**, **(F)**, and **(G)**. Bar = 100 μm.

shifted to 16°C for 90 hr (a developmental time at 16°C equivalent to 48 hr at 29°C), and then returned to 29°C. Flowers developed to show the phenotype corresponding to the temperature they experienced at stage 2 to 4, indicating that the *AP2* product acts no earlier and no later than this period of development. That *ap2* flowers held at 16°C for a brief period in their development can form petals shows that the *AP2* product need only be active for this brief period to specify the initiation and differentiation of the organs of the second whorl. The temperature-sensitive period, from stages 2 to 4, lasts approximately 75 to 90 hr at 16°C, and only 30 to 50 hr at 29°C.

apetala3 (*ap3*)

The *ap3-1* allele of the *AP3* gene (Bowman et al., 1988), like *ap2-1*, is a recessive temperature-sensitive mutation. This third chromosome mutation, like *ap2-1*, also causes transformations in two adjacent whorls, but they are the second and third, rather than the first and the second, ones (Table 1). At 25°C or 29°C, *ap3-1* homozygotes develop flowers in which the organs of the second whorl are sepals (Figure 1D), indistinguishable from wild-type sepals except by their slightly smaller size. Despite their transformation, these organs develop in the positions, and on a time course, characteristic of petals. The organs of the third whorl of *ap3* flowers grown at or above 25°C range from apparently normal stamens to carpelloid stamens to normal-appearing, but unfused, carpels, as shown in Figure 7. The degree of carpellody increases with increasing temperature and increasing age of the inflorescence, and the organs replacing the two lateral stamens are less carpelloid than those replacing the four medial stamens (Table 3). These organs, when fully carpelloid, have up to five well-developed ovules along each margin and are capped with stigmatic papillae. The appearance of their epidermal cells in the scanning electron microscope is identical with the appearance of the epidermal cells of genuine ovaries. In some flowers two or three of the six carpelloid organs may fuse to form a hemispherical structure. Intermediate organs are mosaics, consisting of a patchwork of sectors with epidermal features of either stamens or carpels. A single organ may possess both ovules and pollen.

The development of *ap3* flowers at 25°C is morphologically identical to wild-type until stages 7 to 8, when the filament and anther would normally become distinct. At this time it becomes clear that the organs of the third whorl are not producing these structures, but are elongating vertically and forming the epidermal cell files seen on normal gynoecia (Figure 2D). The stigmas of the isolated carpels appear slightly earlier than those of the the central gynoecium, with ovule formation beginning before this stage.

At 16°C, plants homozygous for *ap3-1* have a second

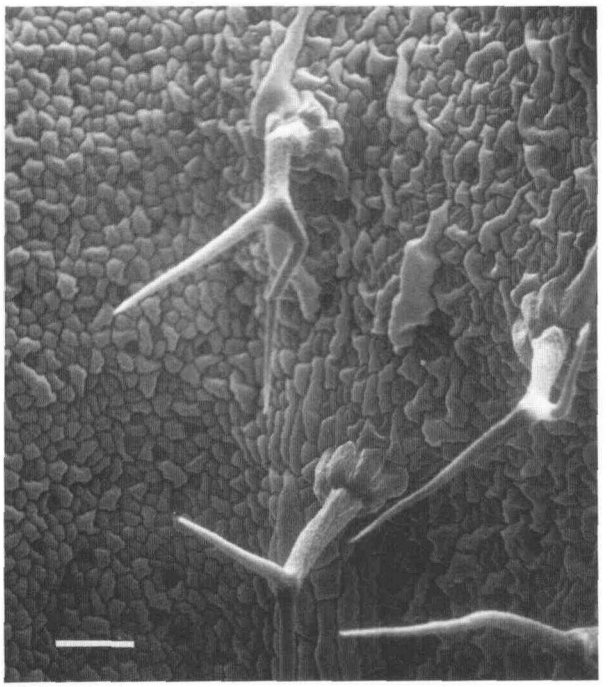

Figure 5. Higher Magnification (bar = 30 μm) of the Petaloid Leaf Shown in Figure 4B Showing the Patches of Green Leaflike Tissue with Wild-Type Leaf Epidermal Morphology and the White Tissue with an Epidermal Morphology Intermediate between that of Petals and Leaves.

A similar situation is observed in staminoid petals: patches of stamen tissue with nearly wild-type stamen epidermal morphology are adjacent to white petal-like tissue with an epidermal morphology intermediate between that of petals and stamens.

whorl that is nearly wild-type, with organs that are partly or completely white and with the smooth margins of petals, rather than the uneven edges of a sepal. The organs are not completely normal petals: they fail to reach normal petal size, and their epidermis is like that of sepals, consisting of stomata and irregularly shaped cells without the radial cuticular thickenings of petal epidermal cells. The organs in the third whorl of *ap3* plants grown at 16°C all develop as stamens; at this temperature the flowers can self-fertilize and produce homozygous seed.

Figure 8, A and B, summarizes temperature shift experiments with *ap3-1* homozygotes, performed to determine the temperature-sensitive period of the third whorl in these flowers. This period is later than with *ap2-1*. A shift up as late as stage 5 can cause complete conversion of third whorl organs to carpels, and there is a partial conversion after a temperature shift up as late as stage 7 and perhaps even 8. A shift down, from 29°C to 16°C, has a clear effect until after stage 6. Thus, the *AP3* gene product

(A) *apetala2*	16°C to 29°C					
absent	65	13	48			1
stamen		1				
deformed stamen	1	1	6	2		
petaloid stamen		1	6	3		
staminoid petal	1		1	10		2
petal	1			22	30	39
phylloid petal				1	1	18
petaloid leaf				4	3	4
leaf-like	1			2	4	1
stage of flower at time of shift	m	b (1)	p (2)	se (3,4)	se+ (4,5)	bud (6+)

(B) *apetala2*	29°C to 16°C					
absent		1	9	11	9	8
stamen						
deformed stamen			1			6
petaloid stamen				1	2	5
staminoid petal	2	1	4	3	4	4
petal	43	14	15	1	1	5
phylloid petal	1					
petaloid leaf	1	1	2			
leaf-like			1			
stage of flower at time of shift	m	b (1)	p (2)	se (3,4)	se+ (4,5)	bud (6+)

Figure 6. Temperature-Sensitive Period (TSP) of the Phenotype of the Second Whorl Organs of *apetala2-1* Flowers.

Since each inflorescence consists of flowers at many stages of development, shifting a small number of plants provides data on all stages of flower development. Plants were either germinated and grown at the permissive (16°C) temperature and shifted to the restrictive (29°C), or the converse shift was performed. The developmental stage of all flowers at the time of the shift was noted. Because all stages defined using SEM are not distinguishable in the dissecting microscope, the following stages were used: those flowers that were not initiated at the time of the shift were placed in the meristem (m) stage. Stages 1 and 2 were defined as buttress (b) and primordia (p), respectively. Stages 3, 4, and 5 were combined into two stages: sepals (se) and sepals-plus (se+). The sepals stage has only sepals initiated, whereas the sepals-plus stage has third whorl primordia initiated but the sepals have not yet enclosed the bud. The remaining flowers (stage 6 on) were simply classified as buds. When the flowers had matured, the second whorl organs of each were scored as outlined in Figure 4. The numbers are the number of organs of each type.

(A) Four *ap2-1* plants shifted from 16°C to 29°C.

(B) Four *ap2-1* plants shifted from 29°C to 16°C. The TSP of the second whorl appears to encompass stages 2, 3, and part of 4.

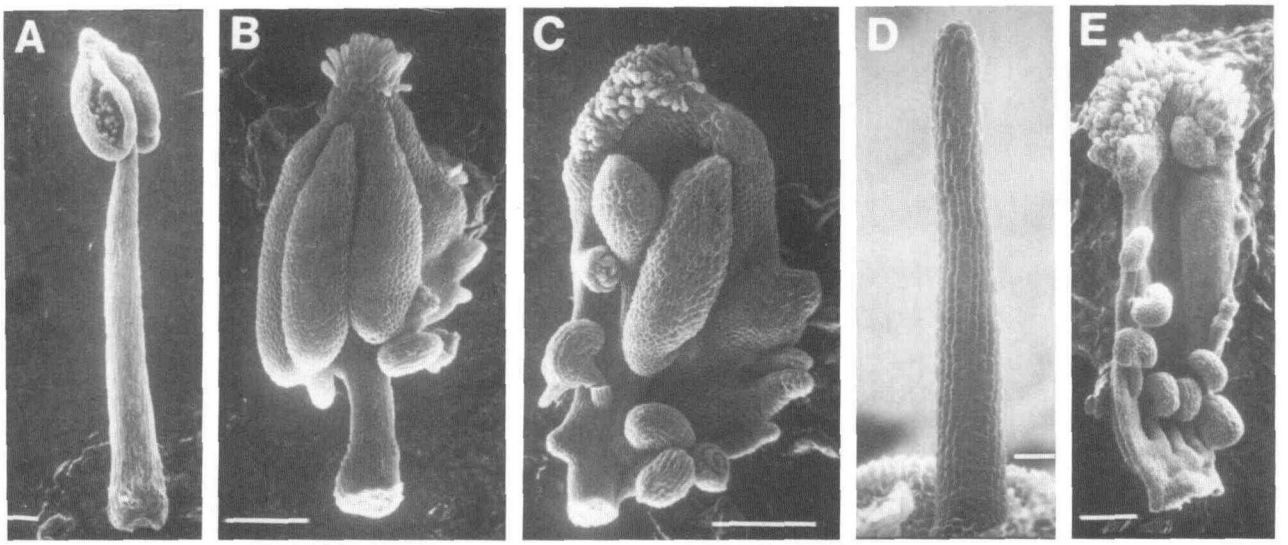

Figure 7. SEM Micrographs of the Inner Surface of Organs Observed in the Third Whorl of *apetala3* Flowers.

(A) Morphologically wild-type stamens are observed at 16°C; carpel-like organs **(E)** are typical at 29°C. Mosaic organs possessing characteristics of both occur at intermediate temperatures (Table 3). The mosaic organs were categorized to permit comparisons of phenotypes under different growth conditions. If stamens were capped with stigma but no other deformities of the anther were present, or if the anther was misshapen but not carpelloid, the organs were classified as deformed stamens (not shown).

(B) Those with the shape of stamens, with prominent locules, but capped with stigma and showing developing ovules at the base of the outer locules were termed carpelloid stamens. The most developmentally advanced ovules occurred at the base of the anthers, whereas less well developed ovules occurred farther up.

(C) Organs shaped like carpels, capped with stigmatic papillae, and possessing ovules, but with remnants of locules and a filamentous base were termed staminoid carpels.

(D) Filaments with no anther but sometimes topped with stigma may form at higher temperatures.

Bar = 100 μm in **(A)**, **(B)**, **(C)**, and **(E)**, and 30 μm in **(D)**.

appears to be effective in specifying the fate of third whorl organ primordia up to the time when they begin their differentiation. The results of temperature pulse experiments are in accord with those of the shift experiments. If plants are grown at 16°C, shifted to 29°C for 54 hr, and then returned to 16°C, flowers that were in stages 5 up to and past bud closure (stage 6 and perhaps beyond) are affected. The converse experiment, in which plants are changed from 29°C to 16°C for 123 hr (a developmental time at 16°C roughly equivalent to 54 hr at 29°C), and then returned to 29°C, flowers in stages 5 and 6 are affected. The *AP3* gene product thus acts much later than that of *AP2*. The *AP3* temperature-sensitive period lasts approximately 40 to 50 hr at 29°C, and 80 to 100 hr at 16°C.

The temperature-sensitive period of the second whorl in *ap3* flowers is more difficult to specify since the organs of this whorl are not completely converted to wild-type at 16°C. Nonetheless, in a shift-down experiment, the 16°C phenotype of the organs of the second whorl was observed in flowers that were at the same or even slightly more advanced developmental stages at the time of the shift than those in which third whorl effects are seen. Thus again, the *AP3* product acts in flowers up to the time when differentiation of affected organs begins.

pistillata (*pi*)

PI is a gene on chromosome 5; the recessive mutant allele *pi-1* (Koornneef et al., 1983) affects the development of all floral organs except the sepals. The organs of the second whorl develop as small sepals rather than petals, the organs of the third whorl do not develop at all, and the central gynoecium develops abnormally (Table 1). The mature flowers thus consist of two outer, alternate whorls of sepals surrounding a large club-shaped gynoecium, usually composed of more than two carpels (Figure 1E). The ovary may exhibit unfused carpel margins and vertical filamentous appendages fused to its outer surface.

The development of *pi* flowers is indistinguishable from that of wild-type until the time of the appearance of the

Table 3. Phenotypes of Third Whorl Organs in *ap3* Flowers[a]

	16°C, All Positions	25°C, Medial Only		29°C,	
		1–7[b]	8–15[b]	Lateral	Medial
			%		
Carpel	0	0	29	6	73
Filament	0	0	0	8	8
Staminoid Carpel	0	0	40	9	4
Carpelloid Stamen	0	9	28	25	7
Deformed Stamen	0	37	3	38	5
Stamen	100	53	0	0	0
Absent	0	1	1	15	4

[a] Third whorl organs of the first 15 flowers produced on at least four plants were scored and classified according to the outline in Figure 7.

[b] Numbers refer to position of flowers within the inflorescence, with 1 being the first flower produced.

primordia of the second and third whorls (stage 5). In *pi* mutants the second whorl primordia appear in the appropriate place at the correct time, but the third whorl primordia are not seen (Figure 2E). The gynoecium forms from the cells encircled by the second whorl primordia, so that it appears that the cells that would ordinarily be in the third whorl are instead incorporated into the developing ovary. Gynoecium development proceeds with characteristically rapid vertical growth of the periphery of the central dome of the flower primordium, but the diameter of the cylinder that is formed is much greater than in wild-type. Growth of the cylinder can soon be seen to be irregular, with extra carpels often forming and regions sometimes lagging behind in vertical growth. One to four filamentous appendages emerged from the surface of the gynoecium in 56% of 75 flowers examined; these appear to arise at the margin between carpels and can be fused to the ovary at their base or along their entire length. These develop late, from the wall of a developmentally advanced gynoecium. The mature gynoecium has two to five apparent carpels, with an average of 2.7 (206 carpels counted in 75 flowers). The style and stigma are expanded in correlation with the increase in carpel number.

While the abnormal ovary is forming, the primordia of the second whorl differentiate just as in flowers of *ap3-1* homozygotes at restrictive temperatures: they differentiate into sepals, but following a developmental time course characteristic of petals (Figure 2E).

Double Mutants

Plant lines homozygous for pairs of the four mutations described were constructed to examine the epistatic relations of these genes and their phenotypic interactions. Two general classes of interaction were observed: combinations in which the effects of the two mutations appeared purely additive (*ag ap3*; *ag pi*) or close to additive (*ag ap2*), and combinations in which phenotypes were observed that are not seen in plants homozygous for only one of the mutations (*ap2 ap3*; *ap2 pi*). *ap3 pi* mutant plants have not been studied: the crosses to produce them gave no plants that looked different from single homozygotes, indicating the possibility that the double mutant phenotype is identical to that of one of the single mutants.

The additive combinations all included *agamous*. *ag ap3* flowers grown at 25°C have the multiplication of organs

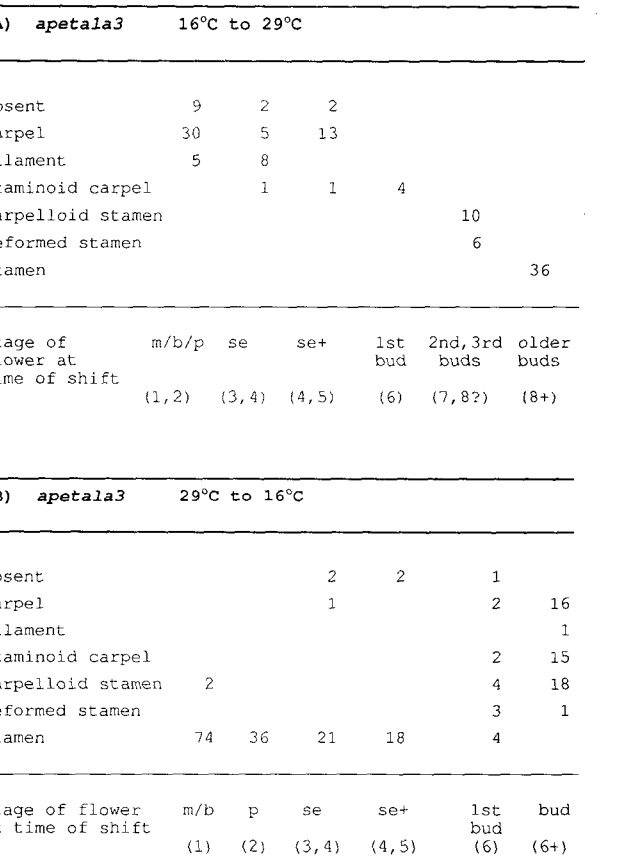

Figure 8. The TSP of the Third Whorl of *apetala3* Flowers.

The procedures outlined in Figure 6 were followed with the exception that the youngest enclosed bud (1st bud), as well as the next two youngest buds in the 16°C to 29°C shift (2nd, 3rd buds), were tallied separately from the rest of the older enclosed buds. The stages of these buds were inferred from SEM of dissected buds of other inflorescences. Medial third whorl organs were classified as described in Figure 7.

(A) Two *ap3-1* plants shifted from 16°C to 29°C.

(B) Four *ap3-1* plants shifted from 29°C to 16°C. The TSP of the third whorl in *ap3* flowers extends from stage 5 up to and possibly including stage 8.

and indeterminate growth of *ag* flowers, but instead of whorls of sepals and petals, as in *ag* homozygotes, they have whorls of sepals only (Figure 9A). *ag pi* flowers (Figure 9B) also consist of many whorls of sepals. The outer two whorls are initiated correctly, but the third whorl primordia fail to appear, as in *pi* flowers. The remaining tissue follows the pattern of indeterminate growth characteristic of *ag*, with nested internal flowers.

ag ap2 double mutants grown at 25°C (Figure 9C) have an overall morphology characterized by indeterminate growth and mosaic organs, as do *ag* single mutants. The identity of the organs is altered from that in *ag*, however. All organs and sectors of organs that would be sepaloid in *ag* flowers are leaflike (with no stigmatic tissue) in *ag ap2* double mutant flowers. The leaflike structures are dense with trichomes but lack any sign of the stigmatic papillae found on the leaflike organs of *ap2* flowers. The remaining organs and sectors, which would be petals in an *ag* single mutant, are short, fleshy structures similar in shape to rudimentary petals, but occasionally possessing the external ridges that cover the locules of wild-type anthers, and showing the yellow-green color of developing anthers. At 16°C the *ag ap2* double mutants still have leaves in place of sepals, but the remaining organs are petals. One feature of the double mutant not usually seen in *ag* alone is a greater degree of pedicel elongation between the nested flowers.

Turning to the nonadditive interactions, double mutant flowers homozygous for both *ap2* and *pi* (Figure 9D), grown at 25°C, have an outer whorl of four cauline leaflike organs topped with stigmatic papillae, as in the *ap2* single mutant, but with an increased tendency toward carpellody. The second whorl is variable, in both organ number and organ identity. There are one to four organs, with an average of 2.5 (54 organs in 22 flowers scored). When there are four organs, they occupy the positions normally occupied by petals in wild-type flowers. When there are fewer organs, their positions are irregular. The identity of these organs varies from cauline leaf to solitary carpel, with most being intermediate and having characteristics of both leaf and carpel. These organs show no staminody, in contrast to the *ap2* phenotype. Frequently these second whorl organs are fused along one margin with the gynoecium, as shown in Figure 10. Mixed second whorl organs are mosaics of leaf and carpel, with a transition zone one to five cells wide between the typical epidermal cell types of these organs visible at the boundaries between different types of tissue. There are no third whorl organs, as in *pi* single mutants, and the gynoecium is similar to that in *pi* homozygotes.

At 16°C, *ap2 pi* double mutant flowers consist of leaflike organs, sometimes topped by stigmatic tissue, in the positions normally occupied by sepals and petals. These surround a gynoecium like that of *pi* single mutants. Second whorl organs are only occasionally missing (3 missing out of 28 flowers), and can possess ovules (20 organs out of 109 counted) or be fused to the gynoecium (2 out of 109).

The phenotype of *ap2 ap3* double mutants (Figure 9E) is also nonadditive and dependent on temperature. At 25°C, the four organs of the outer whorl are similar to those in *ap2* flowers, but with a greater degree of carpellody, in that nearly all have stigmatic papillae, and many of those in medial positions have ovules on their margins. Early flowers have second whorl organs resembling leaves, but most also have rudimentary locules, and are thus staminoid. This is unlike the second whorl organs in the *ap2 pi* double mutants, which show no staminody. In later flowers the second whorl organs show increased carpellody, so that they can consist of a mosaic mixture of leaf, stamen, and carpel. After the tenth flower on a stem, subsequent flowers usually lack all second whorl organs. The positions usually occupied by the four long medial stamens are either filled by solitary carpels (51% in 15 scored flowers), by anthers (9%), or by filaments without anthers (10%); the remaining 30% of the positions had no organs. Twenty-eight percent of the positions usually occupied by the two short lateral stamens had carpels; 19%, mixed stamen/carpels; and 53%, no organ. Examination of developing flowers shows that missing organs result from failure of formation of an organ primordium.

At 16°C, the first whorl of *ap2 ap3* double mutants is made of four leaves, and the second whorl is made of green organs that appear to be leaves, but with far fewer trichomes than the organs of the outer whorl. Four of 104 of these organs scored had ridges of the type that cover anther locules, three of 104 had stigmatic tissue at their apex. These organs develop on the time course of petals. The third whorl primordia develop into stamens, although frequently capped by a stigma. The lateral stamens are often missing. These flowers are self-fertile, indicating normal pollen development and dehiscence of at least some of the anthers.

DISCUSSION

Our reason for analyzing these homeotic mutations is to understand the processes that allow cells in flowers to recognize their appropriate developmental fate. Similar studies of developmental mutations in *Drosophila* have revealed many of the strategies by which cellular identity is established in early insect embryos (Lewis, 1978; Akam, 1987; Scott and Carroll, 1987).

It seems that all of the genes described here act in allowing cells to recognize their position in the developing flower. *AP2* and *PI* may also be required for the appearance of organ primordia in some whorls. None of the mutations has any regular effect other than elimination of organs, or converting their fate. Beyond this general appraisal, we can only describe the functions of the products of the

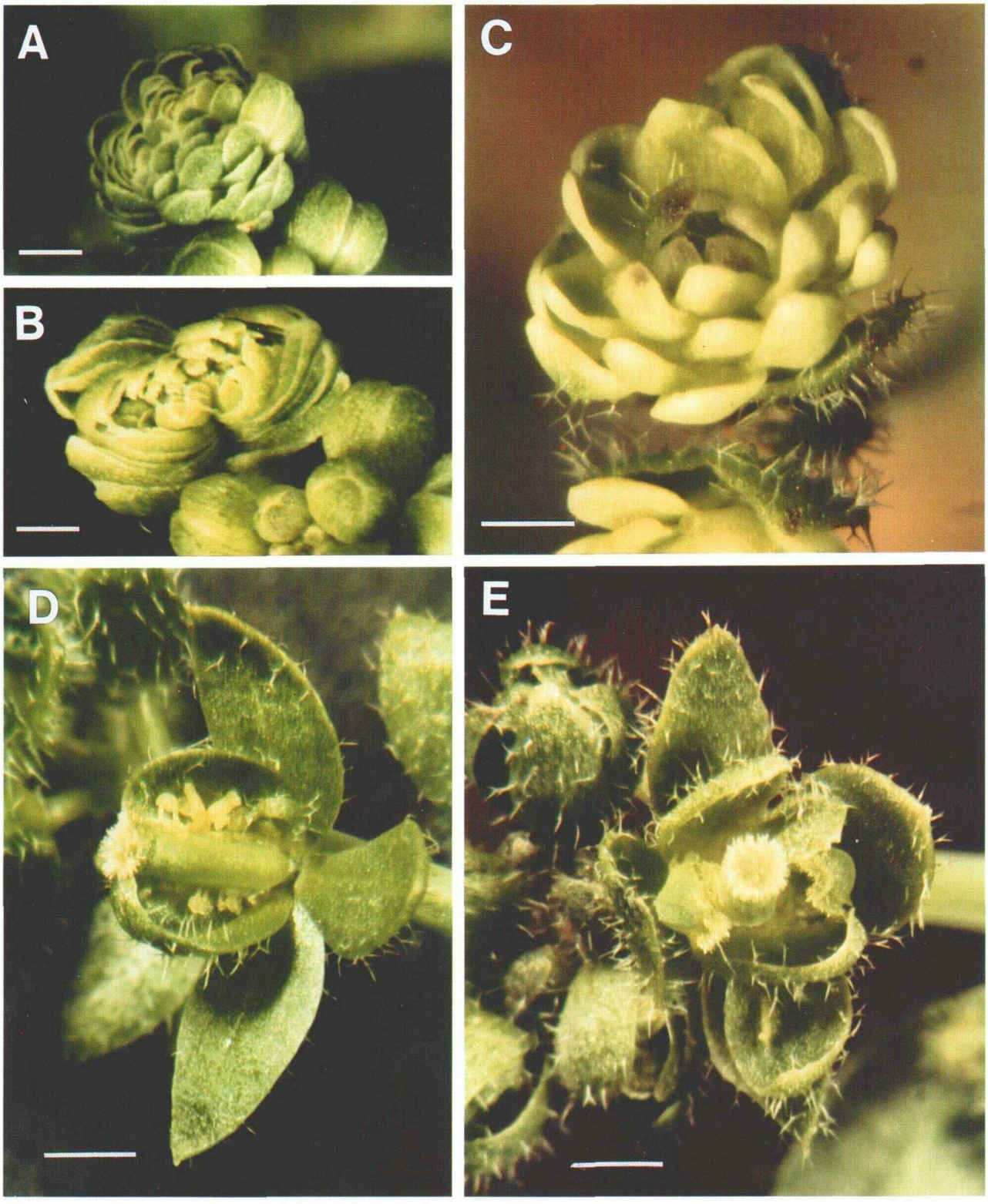

Figure 9. Phenotypes of Double Mutant Combinations Grown at 25°C.

(A) *ag ap3*.
(B) *ag pi*.
(C) *ag ap2*.
(D) *ap2 pi*.
(E) *ap2 ap3*.
Bar = 1 mm.

Figure 10. Fusion of Second Whorl Organs to the Central Gynoecium in an *ap2 pi* Flower.

Note the row of ovules present where the two organs are fused. Also note the carpel-like tissue and leaflike tissue sectors in the second whorl organ on the left.
Bar = 100 μm.

flower development genes in the most general terms. The *AP2* product, for example, must be involved in the process by which the organs of the first and second whorls interpret their position, and it acts at the time when the primordia of these organs are first forming. This product could, therefore, be a part of a signal from some region of the plant or flower to these whorls, part of the receptor for such a signal, or part of the machinery of the cell that acts subsequently to stimulation of the receptor. In the absence of knowledge of the cell types in which the *AP2* product acts, we cannot differentiate between these general hypotheses. Identification of the cell type in which the gene product acts could be obtained either by mosaic analysis or by using molecular cloning of the gene to identify and locate the gene product.

One principle suggested by the phenotypes of the plants described is that carpel fate may be a ground state, and that the wild-type products of these genes act to alter this ground state to allow other organs to differentiate. Carpellody is the most prevalent phenotype among these mutations: *ap3* makes the third whorl carpelloid, *ap2* causes carpellody of the first whorl, and in the *ap2 pi* and *ap2 ap3* double mutants, every organ exhibits carpellody. Other examples of *Arabidopsis* mutations whose phenotypes include free carpels are known (Röbbelen, 1965; Haughn and Somerville, 1988). Even in wild-type plants, the final flowers to develop can exhibit extreme carpellody. *agamous* is an exception to this: either singly or in a double mutant combination, *ag* has not been observed to have any organ with carpelloid characteristics. Perhaps the wild-type product of this gene is required for any cell to differentiate to a type specific to carpels.

A comparable conclusion might be drawn regarding *PI*, since flowers homozygous for *pi-1* alone or with other mutations never have cells differentiating in a manner characteristic of staminal cells. The wild-type *PI* product may be required for any cell to differentiate to a stamen-specific fate.

The nearly additive interactions observed between *ag* and each of the other mutations suggests an absence of interaction of the *AG* gene product and the products of the other genes. In contrast, double mutant combinations involving *ap2* and either *ap3* or *pi* display phenotypes that are not observed in the single mutants, suggesting direct or indirect interaction at some level. The nature of these interactions precludes establishment of epistatic relationships between the genes: for example, the second whorl organs of *ap2 ap3* can be leaflike or carpelloid, indicating that neither gene is epistatic to the other.

It must be pointed out that only one allele of each of these mutations has been described here, and the different phenotypes found at different temperatures for the temperature-sensitive alleles *ap2-1* and *ap3-1* indicate that many of the phenotypes seen in these mutants are due to partial loss of function of the wild-type product. Consistent with this, the phenotype of a newly isolated mutant allele of *ap2* (designated *ap2-2*; D.R. Smyth, J.L. Bowman, and E.M. Meyerowitz, work in progress) is much more abnormal than *ap2-1*. At 25°C, its flowers usually have only two outer whorl organs that are carpelloid, no second or third whorl organs, and a relatively normal gynoecium. When in heterozygous state with *ap2-1*, an intermediate phenotype results. Another three mutations with phenotypes between these extremes, *flo2*, *flo3*, and *flo4* (Haughn and Somerville, 1988), have recently been shown to be allelic with *ap2-1* (L. Kunst, J. Martinez-Zapater, and G.W. Haughn, personal communication). One important task for the future is to obtain a wider allelic series for each of these genes.

It has been suggested that communication between developing organs of adjacent whorls leads to sequential specification of the fate of the primordia in each whorl (Heslop-Harrison, 1963; McHughen, 1980; Green, 1988). However, it cannot be that each whorl depends on the proper differentiation of the adjacent and outer one, since there are examples in the results reported here of incorrect specification of each whorl, with correct specification of the adjacent inner whorl. For example, *ap2-1* homozygotes

at 16°C have leaves instead of sepals, but nearly normal petals; *ap2-1* plants that at 29°C have staminoid organs instead of petals have a normal third whorl of stamens; *ap3-1* plants at 25°C or 29°C have carpels in the third whorl, but a normal gynoecium. That inner organs specify the adjacent outer whorl cannot be simply true, either.

Another class of model that is better supported by the evidence is that the flower primordium is divided into fields or compartments, each consisting of adjacent whorls. The early-acting gene *AP2* may specify a developmental state for the cells that will later give rise to whorls 1 and 2, whereas the wild-type *AG* gene may specify a different state for those that will become whorls 3 and 4. Similarly, the *PI* product may set aside a separate fate for those cells that will give rise to whorls 2 and 3. Thus, the combined action of all of these genes is the delineation of concentric ring-shaped compartments, each with a different fate. Even if something like this does occur, the present information is insufficient to exclude other classes of models or to allow any speculation on biochemical mechanisms. One thing is clear: there are few, if any, restrictions on the ultimate fate of the cells in any whorl. For example, the organs of the second whorl can be leaves, sepals, petals, stamens, or carpels, and those of the third whorl can be sepals, petals, stamens, or carpels, all as a result of the manipulation of only a small number of the many genes that must be involved in specifying these organs.

One question raised by any model requiring communication between adjacent developing regions is whether the hormones known to act in plants are involved. The fact that a carpelloid stamen mutation in tomato can be reverted to wild-type by application of gibberellic acid (GA_3, Sawhney and Greyson, 1973) emphasizes the importance of this question. Two lines of evidence indicate that the known hormones are not involved in the phenotypes of the mutations described here. The first is that application of exogenous gibberellic acid (GA_{4+7} or GA_3), indole acetic acid, and kinetin had no effect on any of the mutants described (J.L. Bowman and E.M. Meyerowitz, unpublished data). The second is that there are *Arabidopsis* mutants known that either fail to produce, or fail to respond properly to gibberellins, auxins, abscisic acid, and ethylene; none of these mutations give phenotypes involving homeotic conversions (Koornneef et al., 1985; Bleecker et al., 1988; King, 1988).

The fact that mosaic organs are composed of distinct regions, with the epidermal cells in each region resembling those normally found in a single organ, may indicate that individual cells in organ primordia make autonomous and heritable decisions as to their fate at a time when the primordium consists of only a few cells, and then multiply to form a clone of cells whose differentiation reflects the choice made by their common ancestor. Also, most individual epidermal cells in the mutants differentiate into cell types normally found in wild-type flowers, thus showing normal cellular differentiation but in inappropriate places. Exceptions to this are those cells on the borders between mosaic patches, which may be intermediate in morphology, and organs in the second whorl of *ap2-1* flowers.

A notable feature of the development of most of the abnormal organs is that they develop on a time course characteristic of their whorl and not of their organ identity. With one exception, the various organs that develop in whorl 2 develop later than the adjacent whorl 3 organs; this is true even when all of the organs of both whorls are of the same type (as, for example, in *ap2-1* at 29°C). The identity of the organ to which a primordium develops, and the time course of its development, are thus separable. The only exceptions to this are the petals that form in whorl 3 of *agamous* flowers, which develop in parallel with the second whorl petals.

Finally, it should be noted that the mutations described here resemble similar, perhaps homologous, mutations in other species of plants. *agamous* is one typical sort of double flower (Masters, 1869; Reynolds and Tampion, 1983); similar phenotypes were described in *Matthiola* more than 400 years ago (Dodoens, 1568: see Saunders, 1921). Other genera in which mutants giving this phenotype are known include *Cheiranthus* (Masters, 1869), *Arabis* (Bateson, 1913), *Petunia* (Sink, 1973), and many others. A similar, perhaps allelic *Arabidopsis* mutation, *multipetala*, has been described as well (Conrad, 1971). A *Capsella* mutant with a phenotype quite similar to *ap2-1* was described as long ago as 1821. More recent descriptions of this mutant are given by Dahlgren (1919) and Shull (1929). *ap3* analogs have been reported in *Cheiranthus* (Nelson, 1929) and in *Primula* (Brieger, 1935); many other carpelloid stamen strains have been described (Meyer, 1966), as have strains like *ap3* or *pi* with conversion of petals to sepals (see Renner, 1959). The numerous reports of mutants resembling those described in this paper indicate that the processes of floral development in *Arabidopsis* are unlikely to be fundamentally different from those in any other plants.

METHODS

The alleles studied, *agamous-1*, *apetala2-1*, *apetala3-1*, and *pistillata-1* are in the Landsberg ecotype and homozygous for the *erecta* mutation. They were obtained from Maarten Koornneef (Department of Genetics, Wageningen Agricultural University, The Netherlands). Genetic nomenclature used here is based on recommendations of the Third International Arabidopsis Meeting (East Lansing, Michigan, 1987). Wild-type alleles are symbolized in block capitals and italics; mutant alleles in lower case italics. Individual mutant alleles are designated by a number that follows the mutant symbol and a hyphen (e.g., *ap2-1*, *ap2-2*). If not specified, it is assumed that the mutant allele is number 1. Doubly mutant stains were constructed by manual cross-pollination, using as parents strains homozygous for individual mutations. The resulting F_1 plants were allowed to self-pollinate, and double

mutants were selected from the F_2 plants. To establish strains involving *agamous-1*, which is sterile when homozygous, heterozygotes were used as initial parents. Seeds were planted on a peat moss/potting soil/sand (3:3:1, v:v:v) mixture in 55-mm pots. The plants were grown in incubators under constant cool-white fluorescent light at 16°C, 25°C, or 29°C, and 70% relative humidity.

For scanning electron microscopy (SEM), young, primary inflorescences were fixed in 3% glutaraldehyde in 0.025 M sodium phosphate (pH 7.0) at 4°C overnight, and then transferred to 1% osmium tetroxide in 0.05 M sodium cacodylate buffer (pH 7.0) at 4°C for 12 to 24 hr. They were then rinsed in 0.025 M sodium phosphate (pH 7.0) and dehydrated in a graded ethanol series at 4°C. This material was critical point dried in liquid carbon dioxide. Individual flowers were removed from inflorescences and mounted on SEM stubs. Organs were dissected from individual flowers by applying pressure with glass needles. The mounted specimens were coated with gold and palladium (4:1) in a Technics Hummer V sputter coater after each dissection. SEM was performed on an ETEC Autoscan scanning electron microscope at an accelerating voltage of 20 kV, and the images were photographed on Kodak 4127 film.

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NOTE ADDED IN PROOF

Recent observations have shown that stigmatic tissue, which was not previously seen in *ag* homozygotes, may develop on the leaflike organs of *ap2 ag* double mutant flowers grown at 29°C.

The isolation and characterization of two new *ap2* alleles with phenotypes intermediate between those of *ap2-1* and *ap2-2* have been reported recently (Komaki, M. K., Okada, K., Nishino, E., and Shimura, Y. [1988]. Isolation and characterization of novel mutants of *Arabidopsis thaliana* defective in flower development. Development **104,** 195–203).