MOLECULAR GENETICS OF FLOWER DEVELOPMENT IN *Arabidopsis thaliana*

Thesis by

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Dedicated to Bibi

who now soars with the cliff swallows.

A special friend who will always remain

a source of wonder and inspiration.

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John Lincoln Bowman

Abstract of Dissertation

Molecular Genetics of Flower Development in *Arabidopsis thaliana*

Flowers of *Arabidopsis thaliana* consist of a precise pattern of organs arranged in four concentric whorls, with each whorl containing a different type of floral organ in characteristic positions and numbers. *Arabidopsis* flowers begin their development as small outgrowths of cells on the flank of the inflorescence meristem. Cells within each flower primordium must somehow assess their position relative to others and subsequently differentiate accordingly. Homeotic mutations at four loci (*AGAMOUS*, *APETALA2*, *APETALA3*, *PISTILLATA*) identified in *Arabidopsis* appear to cause cells in two adjacent whorls of the developing flower primordium to misinterpret their position and differentiate inappropriately. The development of wild-type flowers and that of several alleles of each locus is analyzed as well as the development of double and triple mutant combinations between the homeotic mutations. Based on this genetic data, a model is proposed for how this limited number of floral homeotic genes, by being expressed in overlapping fields of cells in the meristem of the developing flower primordium, and acting alone and in combination, could specify the identity of each of the whorls. *AGAMOUS* and *APETALA2* are proposed to negatively regulate each other's activity with the result that they are expressed in mutually exclusive domains, *APETALA2* in the outer two whorls of the flower and *AGAMOUS* in the inner two whorls. The activities of *APETALA3* and *PISTILLATA* are proposed to be localized to the second and third whorls, with another gene, *SUPERMAN*, negatively regulating their activities in the fourth whorl. By protein sequence homology to known transcriptions factors, SRF of humans and MCM1 of yeast,

AGAMOUS encodes a putative transcription factor. In support of the proposed model, RNA tissue *in situ* hybridizations to developing flowers show that *AGAMOUS* RNA is spatially localized to the inner two whorls in developing floral buds. Furthermore, in *apetala2* mutant flowers, *AGAMOUS* RNA is detected in all floral whorls suggesting that *APETALA2* negatively regulates *AGAMOUS* expression in the outer two whorls at the transcriptional level. Expression patterns of *AGAMOUS* late during flower development suggest that *AGAMOUS* may also play a role in cell fate specification during cellular differentiation of stamens and carpels.

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Explanation of my contribution to the chapters of which I am not the primary author.

Chapters One, Two, and Three resulted from a collaboration with David Smyth, who was on sabbatical in Elliot Meyerowitz's lab in 1988. The experimental work in Chapter One was done jointly by David and myself. In addition, a significant contribution towards the completion of the manuscript was made by me.

Chapter Four was the culmination of a collaboration between several members of the Meyerowitz laboratory and Ken Feldmann, now at the University of Arizona. I was involved in every aspect of the experimental work and as well as being responsible for the initiation of the project.

Chapters Five and Six were both derived from the same experimental work, a collaboration with Gary Drews, a post doc in the lab. Equal contributions, both in experimental procedure and interpreting data, were made by both Gary and me to each of the resulting papers.

INTRODUCTION

GENETIC CONTROL OF PATTERN FORMATION DURING FLOWER DEVELOPMENT IN *ARABIDOPSIS*

John L. Bowman and Elliot M. Meyerowitz

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SUMMARY

Arabidopsis flowers develop from groups of undifferentiated cells on the flank of an inflorescence meristem. The cells in these flower primordia must somehow assess their position within the primordium and differentiate accordingly to produce a flower with a precisely defined pattern of organ types and positions. The molecular mechanisms by which this is accomplished are largely unknown. We are studying a set of genes whose mutations give homeotic phenotypes in *Arabidopsis* flowers. A genetic model to explain the specification of organ identity by combinatorial action of the products of these homeotic genes is presented, along with several aspects that are not readily addressed by the model. The recent cloning of one of the *Arabidopsis* homeotic genes, and an additional homeotic gene from *Antirrhinum*, has provided an opportunity for molecular tests of our genetic model. So far, the molecular data are in accord with the genetic model.

Introduction

Flowers begin their development as groups of undifferentiated cells, which comprise the floral meristem. The cells in these flower primordia then divide and differentiate to produce a flower with a precisely defined pattern of organ positions and types. During this process, cells within the primordium must somehow assess their relative position and differentiate accordingly. The mechanisms by which these cells sense and determine their position are largely unknown. It does appear that some processes utilized in animals are not involved. There is no cell migration in higher plants, and maternal deposition of positional information is not required, since most plant cells are totipotent. As a method of understanding the molecular mechanisms involved in pattern formation in the flower, our laboratory is studying genes whose wild-type products are required for proper pattern formation in *Arabidopsis thaliana* flowers (Pruitt et al., 1987; Bowman et al., 1988; Bowman et al., 1989; Yanofsky et al., 1990; Bowman et al., 1991).

Flower formation in *Arabidopsis* can be considered a series of developmental steps. The first of these, termed floral induction, is the decision to switch from vegetative growth to reproductive growth. This involves a reorganization of the apical meristem in response to both environmental and internal signals such as day length, temperature, and age (Vaughan, 1955; Miksche and Brown, 1965; Drews and Goldberg, 1989). Following floral induction, the reproductive (or inflorescence) meristem produces one to three cauline (stem) leaves, followed by an indeterminate number of individual flowers in a phyllotactic spiral (Smyth et al., 1990). Rapidly enlarging individual flower primordia (floral meristems), which exhibit determinate growth, are produced on the flank of the apical meristem. These cells divide to produce four whorls of organ primordia in a sequential manner, and in a well-defined whorled pattern (Smyth et al., 1990). Each of the organ primordia then differentiates into one of the four types of floral organs, depending upon its position within the flower. These events do not occur as discrete steps, but rather as a

continuous process, and it is possible that individual genes are involved in more than one of the steps. Mutations disrupting various stages in these processes have been isolated in *Arabidopsis*, providing genetic tools for elucidating the mechanisms of flower development (Koornneef et al., 1983; Pruitt et al., 1987; Bowman et al., 1988; Komaki et al., 1988; Haughn and Sommerville, 1988; Bowman et al., 1989; Meyerowitz et al., 1989; Hill and Lord, 1989; Okada et al., 1989; Kunst et al., 1989, Bowman et al., 1991). Our laboratory is primarily concerned with processes after floral induction, therefore those mutations affecting events following inflorescence meristem formation will be the focus of this review. Since apparently homologous mutations exist in *Antirrhinum majus* (Stubbe, 1966; Sommer et al., 1990; Carpenter et al., 1990; Schwarz-Sommer et al., 1990), similarities and differences in flower development between the two systems will be noted throughout.

Flower structure

The radially symmetric *Arabidopsis* flower, which is typical of the Brassicaceae, is composed of four concentric whorls of organs (Figure 1D): the first or outer whorl is occupied by four green sepals; four white petals are found in the second whorl positions, which are alternate and interior to the first whorl positions; four long medial (with respect to the inflorescence meristem) and two short lateral stamens, each composed of a filament capped with a pollen bearing anther, occupy the third whorl; and a gynoecium composed of a two-chambered ovary topped with a short style and capped with stigmatic papillae occupies the fourth whorl (Smyth et al., 1990). Each gynoecium contains approximately 30-50 ovules born in rows along the margins of fusion of the carpels. The flowers develop in a raceme so that a single plant has a series of flowers in different stages of development, the older flowers and mature fruits toward the base, and the younger flowers at the apex. The vegetative growth of *Arabidopsis* is characterized by the production of several rosette

leaves in a phyllotactic spiral, each with a secondary meristem in their axil (Smyth et al., 1990). After floral induction, one to three (average=2.05) cauline leaves are produced, each also associated with a secondary meristem in its axil, before the individual flowers are formed, again in a phyllotactic spiral (Smyth et al., 1990). Each of the secondary meristems is capable of producing its own cauline leaves and flowers.

The development of individual flowers, which is similar to that of *Cheiranthus cheiri* (Payer, 1857; Sattler, 1973) and *Brassica napus* (Polowick and Sawhney, 1986), has been described in detail and stages defined by Smyth et al. (1990) (Figure 1A-D). Briefly, flower development commences when a group of cells emerges to form a buttress on the flank of the inflorescence meristem (stage 1; Figure 1A). As these cells divide, an indentation separates the flower primordium from the inflorescence meristem (stage 2; Figure 1A). Sepal buttresses arise (stage 3; Figure 1A) on the flanks of the flower primordium. The abaxial sepal primordia arise first, followed by the adaxial and then the lateral sepal primordia. Shortly after these primordia grow to overlay the remaining flower meristem (stage 4), the second (petal) and third (stamen) whorl primordia become visible (stage 5). The sepals' continued growth causes them to enclose the developing bud entirely (stage 6; Figure 1B). The rapidly growing stamen (in the third whorl positions) primordia develop distinct filament and anther regions (stage 7; Figure 1C) and locules appear soon after in the anthers of the long (medial) stamens (stage 8). Petal elongation marks the beginning of stage 9 and they reach the height of the short stamens by stage 10. During these stages the gynoecium develops from those cells interior to the stamens. Development of the gynoecium begins as the remaining floral meristem, which is dome shaped at this time, grows into a cylinder due to cell division at the periphery of the dome. Stigmatic papillae appear on the rim of the cylinder at stage 11 (Figure 1D) about which time nectaries, which began their development during stage 9, mature at the base of the lateral stamens. All floral organs continue to grow, with the petals reaching the level of the long stamens by stage 12. Development through stage 12 takes approximately 13 days under

growth conditions of 25°C in 24 hour illumination. After stage 12, the bud opens and the petals and stamens continue to elongate until anthesis occurs. Later stages of development, through seed maturation have been described by Müller (1961).

Antirrhinum majus flowers also consist of four concentric whorls of organs and are produced in a raceme of spiral phyllotaxis, but are bilaterally symmetric (Stubbe, 1966). They are much larger than *Arabidopsis* flowers, and each is produced in the axil of a bract, an organ type not present in *Arabidopsis*. Those organs closest to the bract are referred to as lower while those on the opposite side are referred to as upper. The first whorl consists of five free green sepals, and the second whorl of five petals which are fused for part of their length forming a tube with five lobes. The lobes of the upper two petals are distinct in shape from the lower three. Five stamens comprise the third whorl, but in wild-type the uppermost is aborted in early development. The fourth whorl is occupied by an ovary composed of two carpels. The prominent zygomorphy of *Antirrhinum* flowers allows a class of mutations not identified in *Arabidopsis*, those in which the symmetry of the flower is no longer bilateral, but rather radial. The production and development of floral organ primordia is sequential; the outer whorl primordia form first and the central last, as is observed in *Arabidopsis* (Awasthi et al., 1984). Vegetative growth of *Antirrhinum* is characterized by pairs of leaves in decussate phyllotaxis. Following the transition from vegetative to inflorescence meristem, bracts are produced in a phyllotactic spiral with a flower in the axil of each.

Classes of mutations

One approach to elucidating the mechanisms by which pattern formation occurs is to identify mutations which specifically disrupt developmental processes, and to study the wild-type products of genes identified by such mutations. Mutations have been isolated which disrupt the major events of flower development after floral induction, and can be

divided into three classes: (1) mutations which disrupt the formation of individual flower primordia, (2) mutations which affect the generation of organ primordia within the flower and (3) mutations which alter the fate of organ primordia. Many of the mutations which affect the formation of floral organ primordia also alter their fate. These two processes are concurrent and will therefore be discussed together.

Mutations in genes involved in the production of flower primordia and the transition from the inflorescence meristem to the flower meristem

The production of individual flower primordia and the transition from inflorescence to floral meristem are defined by three types of mutations, many of which are not well-characterized. Thus, only brief descriptions are presented. *Arabidopsis* plants homozygous for the *pin-formed* mutation (Goto et al., 1987) fail to produce individual flower primordia even though the transition from vegetative to inflorescence meristem appears to have occurred. An inflorescence stem is produced but no flowers are formed, resulting in a pointed and flowerless stem emerging from the rosette leaves. The *sterilis* mutation of *Antirrhinum* is similar in phenotype, with an inflorescence stem producing floral bracts with no flowers in their axils (Stubbe, 1966).

Arabidopsis plants homozygous for a strong *leafy* mutation fail to make the transition from inflorescence meristem to floral meristem (Haughn and Somerville, 1988; Detlef Weigel, Ken Feldmann, David Smyth, and Elliot Meyerowitz, unpublished). Flower primordia appear to form properly in a phyllotactic spiral, but rather than differentiating into a flower, the primordium behaves as if it is an inflorescence meristem, remaining indeterminate and producing its own cauline leaves, and with each of these leaves, an associated secondary meristem. This process can be repeated in each of the secondary meristems resulting in an often highly branched structure consisting mostly of cauline leaves. Late in development, carpelloid organs are produced towards the terminal

portion of the branched structure. Mutations similar in phenotype in *Antirrhinum* include *floricaula*, *squamata*, and *squamosa* (Stubbe, 1966; Carpenter et al., 1990; Schwarz-Sommer et al., 1990).

The final type of mutation identified in this class is one in which the inflorescence meristem displays determinate growth and produces a terminal flower instead of continuing to produce the normal phyllotactic spiral of flowers. The *centroradialis* mutation in *Antirrhinum* displays this phenotype (Stubbe, 1966). Candidates for genes with similar functions in *Arabidopsis* include *triple flower* (Alvarez et al., 1990) and *premature termination of inflorescence-1* (Shannon et al., 1990). Homozygous plants of either of these *Arabidopsis* mutants produce a terminal structure composed of organs of more than one flower after a limited number of normal flowers are produced.

Mutations in genes involved in the generation of organ primordia and their subsequent specification

Three types of genes belong to this category: those that alter organ primordium formation, those that alter the specification of organ primordia (homeotic genes), and those that disrupt both processes. Mutations at two loci, *clavata1* and *clavata2* (*clv1* and *clv2*), appear to fall in the first category (Koornneef et al., 1983; Bowman et al., 1988). Homozygous, recessive mutations at either of the *CLAVATA* loci, result in the production of extra floral organs, although the organ identity in the individual whorls is not altered. The extra organs may occur in any whorl, but a gynoecium of four carpels is the most frequent floral phenotype. The number of extra organs in *clv1-1* flowers increases when the plants are grown at 16°C (Bowman et al., 1988). The phenotypic effects of these mutations are not restricted to the flower, however, since in addition to the floral organ phenotype, the spiral phyllotaxis of the rosette leaves and flower primordia is disrupted.

Thus, these loci appear to be involved in a fundamental process that controls the number and position of organ primordia throughout the plant.

The homeotic mutations which alter floral organ identity have been characterized in greater detail than the mutants in the other classes. Each of the floral homeotic mutations described in *Arabidopsis* alters the identity of two adjacent whorls of organs, and thus fall into three categories: (1) those that affect whorls one and two, (2) those that affect whorls two and three, and (3) those that affect whorls three and four. There is more than one locus that alters the fate of the second and third whorls. These types of mutants have also been identified in *Antirrhinum* (Stubbe, 1966; Carpenter et al., 1990; Sommer et al., 1990; Schwarz-Sommer et al., 1990) as well as in a number of other species (Masters, 1869; Penzig, 1890-4; Meyer, 1966; Meyerowitz et al., 1989). Mutations in which the identity of only a single whorl of organs is altered have not been documented in either *Arabidopsis* or *Antirrhinum*. Many of the homeotic loci of *Arabidopsis* seem to be involved in the production of organ primordia as well as specifying organ fate. The phenotypes of the homeotic mutants of *Arabidopsis* described below are for plants grown at 25°C in 24 hour illumination, unless otherwise noted, and are summarized in Table 1.

Mutations at the *APETALA2* (*AP2*) locus alter the identity of the outer two whorls of organs. A wide allelic series of recessive *ap2* mutations have been isolated in *Arabidopsis* (Koornneef et al., 1983; Komaki et al., 1988, Bowman et al., 1989; Meyerowitz et al., 1989; Kunst et al., 1989; Bowman et al., 1991), three examples of which are shown in Figure 2A-C. Each appears to be a loss of function mutation because they are all recessive, and trans-heterozygotes between alleles have a phenotype intermediate between the homozygous phenotypes.

Plants homozygous for the mildest allele, *ap2-1* (Koornneef et al., 1983; Bowman et al., 1989), produce flowers in which the first whorl organs are leaves rather than as sepals (Figure 2A). These leaf-like organs have stellate trichomes on their adaxial surfaces and have stipules at their bases, both characteristics of leaf development. In addition, these

organs senesce on the time course of leaves, rather than sepals. Occasionally, a secondary flower develops in the axil of one of these outer whorl leaves. This can be interpreted as another leaf-like characteristic, since each leaf on a wildtype plant is associated with a secondary meristem in its axil. In *ap2-1* flowers, the second whorl organs are staminoid petals (Figure 2A) or morphologically normal stamens, which dehisce and sometimes have nectaries at their base (Figure 3). The third and fourth whorls of *ap2-1* flowers are normal.

ap2-2 homozygotes (Meyerowitz et al., 1989; Bowman et al., 1991) produce flowers in which the medial first whorl organs are solitary carpels and the lateral first whorl organs, when present (about 50% of the time), are leaf-like (Figure 2B). Both the lateral and medial first whorl organs may have sectors of stamen tissue at their outer margins. In *ap2-2* flowers, second whorl organs are completely missing, as are most of the third whorl organs (90% missing). When the third whorl organs are present, they develop into morphologically normal stamens. The central gynoecium often fails to fuse properly, and when it does fuse, its orientation is altered by 90° with respect to that seen in wildtype. The transheterozygote *ap2-1/ap2-2* displays a phenotype intermediate between these two extremes (Figure 2C; Bowman et al., 1991). The medial first whorl organs are phylloid carpels, while the lateral first whorl organs are leaf-like. The second whorl organs are always absent. The third whorl organs, which are normal stamens, are present in near-normal numbers. The central gynoecium is also phenotypically normal.

Two notable features of the *ap2* phenotype are (1) that only the outer two whorls of organs have their identity altered (the third and fourth whorl organs, when present, are phenotypically normal with respect to cell type) and (2) that the formation of the pattern of organ primordia is altered. When organs are absent, it is due to the failure to initiate an organ primordium rather than to the aborted development of an organ primordium (Figure 4A; Bowman et al., 1989). When several organ primordia are not formed, the positions of the remaining primordia are abnormal. For example, in *ap2-2* flowers the medial first whorl primordia are enlarged relative to wildtype, while the lateral first whorl primordia

develop in a position lower on the flower meristem than they do in wildtype (Figure 4B; Bowman et al., 1991). The positions of third whorl organ primordia may also be abnormal if fewer than six are formed (Figure 4A; Bowman et al., 1991).

All of the *ap2* alleles that we have tested (4 alleles) is temperature sensitive, suggesting that either the *AP2* gene product is inherently temperature sensitive or that the developmental process in which it is involved is temperature sensitive (Bowman et al., 1989; Bowman et al., 1991). The second whorl organs of *ap2-1* flowers are nearly phenotypically normal petals at 16°C while they are either stamens or fail to form at 29°C. To determine the temperature-sensitive developmental stage for the formation and specification of the second whorl organs, we performed temperature shift experiments (Bowman et al., 1989). The temperature sensitive period (tsp) for each of the processes was found to begin in stage two, when the flower primordium becomes distinct from the inflorescence meristem, but prior to the formation of any organ primordia, and continue into stage four, by which time the sepal primordia have formed, but no other primordia are yet visible (see Figure 1A). This suggests that the processes of organ primordium formation and organ specification are concurrent, and is consistent with AP2 having a role early in the formation and differentiation of the outer two whorls of organs. A generalized *ap2* phenotype of carpels, stamens, stamens, carpels may be derived from the allelic series.

A similar mutation, *ovulata*, has been described in *Antirrhinum* in which the identity of the outer two whorls has been altered, the outer whorl organs from sepals to carpels and the second whorl organs from petals to stamens (Carpenter et al., 1990). In contrast to the *ap2* alleles described, the *ovulata* mutation is dominant with the heterozygote having carpelloid outer whorl organs and staminoid petals in the second whorl. When homozygous, the outer whorl organs are very carpel-like, while the second whorl organs are morphologically normal stamens. In contrast to *ap2* mutations, organs are rarely missing in *ovulata*, even in homozygotes. The isolation of other alleles of *OVULATA* is necessary to determine if this is an allele- or locus-specific difference, since the nine

well-described *ap2* alleles in *Arabidopsis* vary in this respect. A semi-dominant mutation at the *AP2* locus in *Arabidopsis* has also been isolated; when heterozygous it displays a phenotype less severe than *ap2-1*, and when homozygous, flowers consist of little more than a two carpelled gynoecium (Uli Mayer, Gerd Jürgens, and Detlef Weigel, unpublished).

Two *Arabidopsis* mutations, *pistillata* (*pi*) and *apetala3* (*ap3*), cause the second whorl organ primordia to develop into sepals rather than petals, and the third whorl organ primordia to develop into carpels instead of the wildtype stamens (Koornneef et al., 1983; Bowman et al., 1989; Hill and Lord, 1989). In *ap3* flowers the organ primordia are all formed properly, but their subsequent differentiation is altered (Figure 2D). The *ap3-1* allele is temperature sensitive; the third whorl organs are phenotypically normal stamens at 16°C, and are solitary carpels at 29°C (Bowman et al., 1989). The second whorl organs are sepals at 29°C, but are slightly sepaloid petals at 16°C. The temperature sensitive period (tsp) of *ap3* for the specification of the third whorl organs begins in stage 5, at which time all organ primordia have formed, but have not yet begun to visibly differentiate, and ends early in stage 7, when the third whorl organ primordia begin to visibly differentiate into stamens in wildtype flowers (Bowman et al., 1989). The tsp of *ap3* for the second whorl organs also begins in stage 5, but extends into stage 7, and possibly later, (Bowman et al., 1989). *pi* alleles range in phenotype from ones indistinguishable from *ap3-1* (*pi-3*; Bowman et al., 1991), to ones in which third whorl organs fail to form (*pi-1*; Bowman et al., 1989; Hill and Lord, 1989). The development of *pi-1* flowers deviates from that of wildtype at the time during which the third whorl organ primordia are formed (stage 5; Bowman et al., 1989). In *pi-1* flowers the cells which would normally give rise to the third whorl organ primordia appear to be incorporated into the gynoecial cylinder causing the cylinder to be abnormal in size and shape, and thus producing a flower in which no third whorl organs are present, and in which the fourth whorl gynoecium is

abnormal (Figure 2E; Bowman et al., 1989; Hill and Lord, 1989). The *pi/ap3* phenotype can thus be summarized as : sepal, sepal, carpel, carpel.

Mutations in at least three loci (*deficiens*, *globosa*, and *sepaloidea*) in *Antirrhinum* give a phenotype similar to the *ap3* and *pi* mutations of *Arabidopsis* (Stubbe, 1966; Sommer et al., 1990; Carpenter et al., 1990). Some *deficiens-621* (*def-621*) flowers are similar in phenotype to *ap3* flowers but some display a more extreme phenotype, in which the third whorl develops into a ring of fused carpels, whereas the fourth whorl fails to develop (Carpenter et al., 1990). This extreme phenotype resembles *pi-1* flowers in the respect that only three whorls of organs develop. Since the *def-621* mutation is caused by the insertion of an active transposon, somatic reversions are often observed in flowers. Somatic reversion events in second whorl organs can produce small sectors of petal tissue in the sepals occupying second whorl positions suggesting that the fate of those cells is not irreversibly determined until late in development (Sommer et al., 1990; Carpenter et al., 1990). Small sectors from somatic excision events are not seen in third whorl organs. This is consistent with the temperature shift experiments in *Arabidopsis*, in which the tsp of *ap3* for the third whorl organs is a short well-defined developmental period, whereas the tsp of *ap3* for the second whorl organs extends until late in development (Bowman et al., 1989).

agamous-1 (as well as *ag-2* and *ag-3*) flowers of *Arabidopsis* consist of multiple whorls of sepals and petals, and display indeterminate growth (Figure 2F; Koornneef et al., 1983; Bowman et al., 1989; Yanofsky et al., 1990; Bowman et al., 1991). Observations on developing flowers elucidates the developmental basis of the phenotype (Bowman et al., 1989). The first two whorls of organ primordia are produced correctly and differentiate properly, but the third whorl organ primordia, while they are produced in the correct positions, differentiate into another whorl of petals rather than the wild-type stamens. The cells which would normally give rise to the gynoecial cylinder behave instead as if they constituted another flower primordium; this type of developmental defect

has been termed heterochronic. This internal flower primordium repeats the pattern and the cycle continues indeterminately, causing the phenotype commonly referred to as a "double flower." The organs of the internal flowers may be mosaics of petal and sepal tissue, with the petal tissue always at the margin, and the sepal tissue central. Thus the *ag* phenotype may be summarized as (sepal, petal, petal)$_n$ with respect to organ identity or (1, 2, 3)$_n$ in terms of organ position.

A mutation in *Antirrhinum*, *pleniflora*, is similar in phenotype to *agamous*, except that the organs produced interior to the third whorl organs of *pleniflora* flowers may be carpelloid in addition to being sepalloid and petaloid (Carpenter et al., 1990).

Another mutation of *Arabidopsis* which has phenotypic effects in the third and fourth whorls, is *superman-1* (Figure 2G; Uli Mayer, Gerd Jürgens, and Detlef Weigel, unpublished). In *superman-1* flowers, the first and second whorls develop normally into four sepals and four petals, respectively. Interior to these outer two whorls, 9-12 organ primordia are formed in a pattern that is not well-defined, either spatially or numerically (J.B. and Detlef Weigel, unpublished). Most of the primordia that form develop into stamens although the more peripheral may be petalloid and the more central may be carpelloid. The fate of the cells interior to the staminoid organs is variable. Nearly normal gynoecia, free carpelloid organs, thread-like filamentous structures, and no organ development at all have been observed. Further characterization of this allele as well as the generation of an allelic series for this locus should provide a clearer understanding of the role of *SUPERMAN* in organ primordium formation and specification of identity. A mutation with a similar phenotype has been described in an abstract by Schultz and Haughn (1990); allelism tests between this mutation and *superman-1* have yet to be performed.

Genetic interactions between the homeotic genes

Double and triple mutant combinations have been constructed between many of the homeotic mutations described for *Arabidopsis* (Bowman et al., 1989; Bowman et al., 1991), though, unfortunately, not for the similar *Antirrhinum* mutations. These genetic experiments have provided evidence for the interaction of the *AP2* and *AG* gene products in establishing organ identity and number as well as the *AP2* and *PI* gene products in the establishment of organ number.

ag-1 ap3-1 and *ag-1 pi-1* flowers consist entirely of sepals and display the indeterminate growth seen in *ag* flowers (Figure 5A; Bowman et al., 1989). The pattern of organ primordia observed in *ap3-1 ag-1* flowers is indistinguishable from that of *ag-1* flowers: $(1, 2, 3)_n$ (Bowman et al., 1989) In contrast, the pattern of organ primordia formed in *ag-1 pi-1* flowers is $(1, 2)_n$; the third whorl organ primordia are absent just as they are in *pi-1* flowers (Bowman et al., 1989).

ap2-2 pi-1 flowers are comprised of a central gynoecium usually comprised of four carpels and an occasional lateral first whorl leaf-like organ (Figure 5B; Bowman et al., 1991). Observations on developing flowers provide insight into the basis of the phenotype. Lateral first whorl organ primordia are initiated about half of the time, as seen in *ap2-2* flowers, and develop into cauline leaf-like organs. Medial first whorl organ primordia are initiated but are congenitally fused to each other, and grow to form a cylinder into which all of the remaining flower meristem is incorporated. The four carpels in the mature flower could arise from a fusion of the two first whorl primordia, which differentiate into carpels, and the cells which would ordinarily give rise to the two carpels of the central gynoecium. No second or third whorl organs are present, but nectaries may arise between the lateral first whorl positions and the central gynoecium. *ap2-2 ap3-1* flowers (Bowman et al., 1991) are similar to *ap2-2 pi-1* flowers except that there are occasional third whorl organ primordia which differentiate into carpels, and the medial first whorl carpels are often separate from the central carpels. Thus, the effects on the

specification of organ identity of the two mutations in each of the four double mutants described thus far are essentially additive in the double mutant flowers.

In contrast, non-additive effects in the specification of organ identity are seen in *ap2-2 ag-1* flowers (Figure 5C; Bowman et al., 1991). The outer whorl of *ap2-2 ag-1* flowers is composed of carpelloid leaves in the medial positions, while about half of the time cauline leaf-like organs develop in the lateral positions. The second and third whorl organs are petaloid stamens; hybrid organs with both an overall morphology as well as epidermal cellular morphology intermediate between that of wildtype petals and wildtype stamens. These organs have rudimentary locules containing pollen, but fail to dehisce. The number of second and third whorl organ primordia formed (7.6) is intermediate between that formed in wildtype flowers (10) and *ap2-2* flowers (.25). These organ primordia arise in a pattern resembling wildtype, with some of the primordia occupying positions occupied by organ primordia in wild-type flowers, while the remaining primordia are in abnormal positions. This pattern repeats indefinitely, producing a double flower with the following order of organs: (carpelloid leaves, petaloid stamens, petaloid stamens)$_n$ and (1, 2*, 3*)$_n$ in terms of organ primordia with the * denoting ectopic positions and incorrect numbers. Thus the *ag-1* mutation suppresses two aspects of the *ap2-2* phenotype: the carpellody of the medial first whorl organs and the failure of second and third whorl organ formation. That the *ag-1* mutation has first and second whorl effects in an *ap2-2* mutant background, while it has no such effects alone, strongly suggests that the *AG* and *AP2* gene products interact at some level.

Flowers of plants mutant for all three types of homeotic genes, *ap2-2 pi-1 ag-1* homozygotes, consist of many whorls of carpelloid leaves (Figure 5D; Bowman et al., 1991). Analysis of developing flowers shows that lateral first whorl organ primordia are formed about half of the time, and that they arise lower on the flower pedicel than the similar primordia in wildtype flowers. These develop into cauline leaf-like organs. The lower position and leaf-like differentiation of the lateral first whorl primordia are

characteristic of all flowers with the *ap2-2* genotype (*ap2-2*, *ap2-2 pi-1*, *ap2-2 ap3-1*, *ap2-2 ag-1*, and *ap2-2 pi-1 ag-1*; Bowman et al., 1991). The medial first whorl primordia develop in the correct positions, although they are enlarged relative to wildtype. Interior to the medial first whorl organ primordia, two more primordia are formed in lateral positions. This is followed by the production of two additional primordia in medial positions that are interior to all of the previously produced primordia. This process continues indefinitely, generating many whorls of alternating organs. Each of these primordia develops into a carpelloid leaf-like organ. These pairs of organs could be interpreted as a repeating pattern of (medial first whorl organs)$_n$ or (1)$_n$ if each successive internal flower is rotated 90° with respect to the next outer one. That the *pi-1* mutation prevents the production of second whorl organs in an *ap2-2 ag-1* background suggests that the products of these genes interact during the process of organ primordium formation, however, it is not possible to distinguish whether this interaction is between AP2 and PI or AG and PI or both.

ap2-1 ag-1 pi-1 and *ap2-1 ag-1 ap3-1* flowers consist entirely of cauline leaf-like organs with virtually no carpellody (Figure 6; Bowman et al., 1991). The *ap2-1* allele is the mildest mutant allele isolated thus far. Thus, an apparent low level of AP2 activity, in the absence of the other types of homeotic gene products, causes floral organs which would otherwise develop into carpelloid leaves, as in *ap2-2 ag-1 pi-1* flowers, to develop into cauline leaf-like organs, complete with stipules, stellate trichomes, and an occasional floral meristem in the axil. Explanation for this are that the ground state of organs produced by the floral meristem is carpelloid leaves or that there is another as yet unidentified gene involved in carpel formation that the *AP2* gene product represses.

Several of the genes involved in floral morphogenesis encode transcription factors

During floral morphogenesis, cells in the developing flower primordia must assess their position relative to nearby cells, or assess it globally, and differentiate accordingly. Two types of genes are likely to play a role in these processes: those that encode transcription factors and those that encode components of signal transduction pathways. Indeed, many genes identified with the morphogenesis of other systems such as *Drosophila melanogaster* and *Caenorhabditis elegans* encode these types of proteins. The recent cloning of several of these genes in *Arabidopsis* and *Antirrhinum* and their identification as putative transcription factors suggests that this is also the case for the genes controlling morphogenesis in plants.

The AGAMOUS *and* DEFICIENS *genes encode putative DNA-binding proteins*

The recent molecular cloning of the *AGAMOUS* gene (Yanofsky et al., 1990) from *Arabidopsis* and *DEFICIENS* gene (Sommer et al., 1990) from *Antirrhinum* have been described. In each the predicted protein sequence contains a region with remarkable similarity to the DNA-binding region of transcription factors from yeast and humans, MCM1 (Passmore et al., 1988) and SRF (Norman et al., 1988), respectively. The region of homology is limited the DNA-binding and dimerization domains in SRF, and does not extend to the rest of the predicted protein sequence. This region of homology has been designated as the MADS-box, referring to four of the proteins known to contain it: MCM1, *AGAMOUS*, *DEFICIENS*, and SRF (Schwarz-Sommer et al., 1990). A family of genes homologous to the *AGAMOUS* gene has been isolated in *Arabidopsis*, some of which appear to be preferentially expressed in flowers (Yanofsky et al., 1990). Likewise, a family of genes homologous to the *DEFICIENS* gene has been identified in *Antirrhinum*, two of which appear to correspond to the *GLOBOSA* and *SQUAMOSA* genes (Schwarz-Sommer et al., 1990). It will be of interest to see if there is a family of genes related to *DEFICIENS* in *Arabidopsis* and one related to *AGAMOUS* in *Antirrhinum*. The roles of

each of the MADS-box genes during floral morphogenesis are very different: the *AGAMOUS* gene is involved in specifying the identity of the third and fourth whorl organs as well as the determinate growth of the flower, the *DEFICIENS* and *GLOBOSA* genes are required for the proper specification of organ identity in the second and third whorls, and the *SQUAMOSA* gene is involved in the switch from vegetative meristem to inflorescence meristem. Thus, there appears to be a family of related genes that act to direct many steps of floral morphogenesis. A similar situation has been observed in animals where a single DNA-binding motif has been found to be used in different regulatory pathways.

The AGAMOUS *and* DEFICIENS *genes are expressed in complex spatial and temporal patterns*

The localization of *AG* mRNA in developing *Arabidopsis* flowers has been analyzed by tissue *in situ* hybridization experiments (Yanofsky et al., 1990; Gary Drews, J.B., and E.M., submitted). *AG* mRNA is detectable as early as stage three, at which time the outer whorl organ primordia have formed, but no other organ primordia are visible (Gary Drews, J.B., and E.M., submitted). Expression is localized to those cells which will give rise to the third and fourth whorl organs, and continues until late in development, past stage 12, in both the third and fourth whorl organs (Yanofsky et al., 1990; Gary Drews, J.B., and E.M., submitted). RNA blot analysis with dissected floral organs performed with the *DEFICIENS* gene in *Antirrhinum* shows that *DEFICIENS* expression is concentrated in those whorls that are affected in *deficiens* mutants (Schwarz-Sommer et al., 1990). A low level of expression was also detected in first and fourth whorl organs, where the *deficiens* mutation does not have an obvious phenotypic effect (Schwarz-Sommer et al., 1990). That the mRNA of each is preferentially expressed in those floral organs which are altered when the genes are mutated, implies that the genes are spatially as well as temporally regulated.

The processes of specification of organ identity and organ primordia formation are concurrent

Each of the homeotic flower genes of *Arabidopsis* appears to have a role early in the development of the flower, when the organ primordia are being generated. Evidence for this comes from phenotypic effects, temperature sensitive periods (tsps), and mRNA expression data. The tsp for the second whorl organs of *ap2-1* flowers begins in stage two (when no organ primordia are visible yet; Bowman et al., 1989), *AG* mRNA is detectable at stage three (when the first whorl organ primordia become visible; Gary Drews, J.B., and E.M., in preparation), the *pi-1* mutation has phenotypic effects at stage five (when the second and third whorl organ primordia are forming; Bowman et al., 1989), and the tsp for the third whorl organs of *ap3-1* flowers begins in stage five (Bowman et al., 1989). The fates of the cells which will give rise to the organs of the second whorl are at least in part determined by the *AP2*, *PI*, and *AP3* gene products. In *ap2-1* flowers the second whorl organs can be stamens, while in *ap3* and *pi* flowers the second whorl organs are sepals rather than the wildtype petals. In the temperature sensitive alleles, *ap2-1* and *ap3-1*, the fates of the cells which will give rise to the second whorl organs can be manipulated by altering the growth temperature. The tsp for the specification of identity of the second whorl organs in *ap2-1* flowers begins in stage two and extends into stage four, during which time the first whorl organ primordia are formed. In contrast, the tsp for the specification of identity of the second whorl organs in *ap3-1* flowers begins in stage five, at which time all the organ primordia have formed, and extends at least into stage seven, when the third whorl primordia have begun to differentiate, but the second whorl organ primordia have not yet begun their visible differentiation. This suggests that the specification of organ identity is initiated before the visible formation of the organ primordia and the process is not complete until after the formation of the organ primordia.

Mutations at the *AP2* locus can disrupt both of these processes in the second whorl and the tsps for each process encompass approximately the same developmental time period (Bowman et al., 1989).

Although the identity of the second whorl primordia is not irreversibly determined at stage five, its possible fates may be restricted (Bowman et al., submitted). For example, the cells which give rise to the second whorl organs choose between sepal/petal and stamen/carpel fates during stages 2-4, depending upon the action of the *AP2* gene product. Those same cells make the decision between petal and sepal fates slightly later in development, depending upon the action of the *AP3* gene product. After stage four the option to differentiate into a stamen may no longer be open, but the cells are not irreversibly committed to differentiate into those of a petal.

That most of the homeotic flower mutations of *Arabidopsis* disrupt organ primordium formation, while most of those isolated thus far in *Antirrhinum* do not, is the most obvious phenotypic difference between the two systems. The more severe phenotypes of *deficiens* alleles do resemble those of *pi-1* flowers, in that they consist of only three whorls of organs: sepals, sepals, carpels (Carpenter et al, 1990). The number of carpels composing the *pi-1* gynoecium is variable, ranging from 2 to 5 with an average of 2.7 (Bowman et al., 1989). The carpels of *deficiens* flowers are also variable in number (Sommer et al., 1990; Carpenter et al., 1990). Klemm (1927) described a *globifera* mutant (which is the same allele as *deficiens* described above) in which the gynoecium appeared to be a fusion of both the third and fourth whorls, and could be comprised of up to seven carpels. Thus, the *deficiens* mutation in *Antirrhinum* is similar in phenotype to that of *pi-1* flowers in *Arabidopsis* in terms of organ loss as well as altered organ identity. The extra organs of *pleniflora* mutants may be carpelloid, and do not form an inner flower with the normal *Antirrhinum* floral structure (Carpenter et al., 1990). In comparison, the extra organs of *ag* flowers of *Arabidopsis*, are flowers (Bowman et al., 1989). Finally, leaf-like organs or loss of organ primordia, both prominent features of the *ap2* phenotype

in *Arabidopsis*, have not been observed in *ovulata* flowers (Carpenter et al., 1990; Schwarz-Sommer et al., 1990), the *Antirrhinum* equivalent. Since a limited number of alleles of these loci have been analyzed, a wider allelic series of the *Antirrhinum* mutations might resolve these differences. Alternatively, this might be a fundamental difference between the two plant species in the mechanisms of floral morphogenesis.

Models of floral morphogenesis

Many early models of flower development rely on communication between developing organs in adjacent whorls to sequentially specify the identity of the organs in each whorl (Heslop-Harrison, 1963; McHughen, 1980; Green, 1988). This is clearly not the case in *Arabidopsis* since the proper specification of any whorl of organs is not dependent on the correct differentiation of either the next outer or the next inner whorl of organs (Bowman et al., 1989). For example, normal petals can form in *ap2-1* flowers in the second whorl when grown at 16°C despite the fact that the outer whorl organs develop as leaves rather than the wildtype sepals. Conversely, wildtype petals form in the second whorl of *ag* flowers despite the third whorl organ primordia differentiating incorrectly into petals rather than stamens. Examples exist for each of the other floral organ types as well. It is clear that there are few constraints on the identity of the organs in any particular whorl. For example, all four floral organ types in addition to leaf-like organs can be observed in second and third whorl positions in various single, double, and triple mutant flowers of *Arabidopsis*. This also seems to be the case for *Antirrhinum* although the lack of any reported double and triple mutant phenotypes leaves the possibility of some constraints in this species open.

The major hormones known to act in plants do not seem to be involved in specifying organ identity in any simple way in *Arabidopsis*. Application of exogenous gibberellic acid, indole acetic acid, or kinetin has no effect on phenotype of wildtype or

homeotic mutant flowers (Bowman et al., 1989; Bowman, unpublished). Additionally, mutants which fail to produce or respond properly to ethylene, auxins, gibberellins, or abscisic acid develop phenotypically normal flowers (Koornneef et al., 1985; Bleecker et al., 1988; King, 1988).

Models, in which the flower primordium is thought of as concentric fields of cells with each field being specified by a combination of gene products, have been proposed (Brieger, 1935; Holder, 1979; Bowman et al., 1989; Kunst et al., 1989; Carpenter et al., 1990; Schwarz-Sommer et al., 1990; Bowman et al., 1991). In this type of model, the flower primordium is divided into radially concentric fields, each of which is comprised of two adjacent whorls. Our model (Bowman et al., 1991) presented for *Arabidopsis* has three fields (Figure 7): the first and second whorls comprise field A and is identified by AP2; whorls two and three comprise field B, which is specified by AP3 and PI; field C is made of whorls three and four and is identified by AG. The combination of homeotic gene products present in each whorl unambiguously identifies each whorl, and the combination of gene products is responsible for specifying the fate of the cells within the whorl. It follows that in wild-type whorl one (occupied by sepals in wild-type) is specified by AP2, whorl two (occupied by petals in wild-type) by the combination of AP2 and AP3/PI, whorl three (occupied by stamens in wild-type) by the combination of AP3/PI and AG, and whorl four (occupied by carpels in wild-type) by AG. For example, if AP2 is the only product present in a particular whorl, then the organ primordia of that whorl develop into sepals, and if AP3/PI is present in addition to AP2 then petals would develop, and so on. We also proposed that the *AG* and *AP2* gene products act antagonistically, based on the genetic data that the *ag-1* mutation has phenotypic effects in the first and second whorls in an *ap2-2* background, but not in the *ag* single mutant (Bowman et al., 1991). Thus, in an *ap2* background the phenotypic effects of the wild-type *AG* gene product expand to encompass both fields A and C, all four whorls. And conversely, in an *ag* background the phenotypic effects of the wild-type *AP2* gene product expand into field C, encompassing all whorls.

If one or more of these gene products are missing, the distribution of the remaining gene products determines organ identity. For example, if the *AP3* and/or *PI* gene products are missing, leaving only the *AP2* and *AG* gene products in their wildtype domains, the outer two whorls develop into sepals, while the inner two develop into carpels. If both the *AG* and *AP3* gene products are missing, the phenotypic effects of the *AP2* wild-type product are observed in all four whorls. Since AP2 is the only functional homeotic gene product, a flower in which all organs are sepals develops. Similar arguments can be made for *ag*, *ap2*, *ag pi*, *ap2 ap3*, and *ap2 pi* flowers. In *ap2 ag* flowers both the *AP2* and *AG* gene products are not functional, leaving only the *AP3/PI* products in field B. Thus the distribution of gene products in each whorl is different from any distribution in wildtype. The production of both petals and stamens is dependent on the *AP3/PI* gene products, the decision to produce a petal or a stamen however, depends on the combinatorial action of *AP2* and *AG* gene products, respectively. Those organs produced in field B (whorls two and three) of *ag ap2* flowers are neither wildtype petals nor wildtype stamens, but intermediate in phenotype between petals and stamens (Bowman et al., 1989; Bowman et al., 1991). In those whorls in which none of the three types of homeotic gene products are functional, whorls one and four of *ap2 ag* flowers as well as all positions in *ap2 ag pi* flowers, carpelloid leaves are produced (Bowman et al., 1989; Bowman et al., 1991). The models proposed on the basis of single mutant phenotypes in *Antirrhinum* (Carpenter et al., 1990; Schwarz-Sommer et al., 1990) are consistent with, and are subsets of, this model.

The model successfully predicts the basic phenotypes of the singly, doubly, and triply mutant flowers. There are, however, a few details in *Arabidopsis* are not explained by this simplistic model (see Bowman et al., 1991 for a comprehensive discussion). For example, in *ag-1* homozygotes (in the double and triple mutants) both carpelloid and staminoid organs develop. If the *AG* gene product is responsible for directing the development of carpels, then the stigmatic tissue and the ovules observed in *ap2-2 ag-1* and *ap2-2 ag-1 pi-1* flowers pose a quandary. If there is residual *AG* activity in *ag-1* flowers,

then some carpelloid tissue would be expected. Therefore one possible solution would be that the *ag-1* allele is hypomorphic but not null. The molecular data do not support this hypothesis, however. *ag-1* and *ag-2* flowers are very similar in phenotype. The *ag-2* mutation is caused by a 35 kilobase insertion of T-DNA into an intron towards the 5' end of the gene while the *ag-1* mutation is caused by a single base change at a splice site acceptor site also towards the 5' end of the gene (Yanofsky et al., 1990). An alternative hypothesis is that the *AG* product merely sets up a prepattern on which other genes, whose expression may not be strictly dependent on *AG*, act to direct carpel differentiation in wildtype flowers. These other genes could be regulated by general factors, but in the absence of the *AG* and *AP2* gene products, could be expressed ectopically. The second and third whorl organs of *ap2-2 ag-1* flowers are actually much more stamen-like than petal-like, with locules full of pollen and a general morphology of wildtype stamens. It may thus be that the proper differentiation of stamens is also not directly dependent on the *AG* gene product. The slight petallody of the organs could be due to residual *AP2* activity, since the *ap2-2* mutation may not be null. Another complication of the *ag* phenotype is the occurrence of the internal flowers. One interpretation is that there are not any fourth whorl organs in *ag* flowers; those cells which would ordinarily develop into the gynoecium instead develop into another flower primordium. This primordium repeats the process producing organ primordia in the pattern seen in the first three whorls and another flower primordium internal to the three whorls of organ primordia. This continues indeterminately such that no fourth whorl primordia are ever produced. Therefore, the process of specification of organ identity starts over after the third whorl and the specification of the fourth whorl becomes irrelevant.

A second complication is the presence of mosaic organs. Stamen-carpel mosaics are observed in the first whorl of *ap-2* flowers (Bowman et al., 1991) and sepal-petal mosaics are seen in the internal flowers in *ag* (Bowman et al., 1989). The stamen-carpel mosaic organs consist of sectors of wild-type stamen and wild-type carpel tissue with the

stamen tissue always at the margins of the organs and the carpel sectors occupying the central region. The outer organ primordia of *ap2-2* flowers encompass a larger proportion of the flower primordium than do the first whorl organ primordia in wild-type flowers. It might be the case that the enlarged primordia of *ap2-2* flowers contain cells from more than a single whorl or field. The shape of the primordium is such that the cells at the margins could come from cells that would ordinarily form second or third whorl organs while those in the central portion of the primordia arise from the region that usually gives rise to the outer whorl (Figure 4B). This would explain the consistent spatial relationship of the stamen and carpel sectors. Since the processes of specification of organ identity and organ primordium formation are concurrent (see above) and the *AP2* gene product has a role in each of these, it is conceivable that the two processes might not be precisely coordinated in *ap2* mutants. A similar argument could be made for the mosaic organs in *ag* flowers. In summary, the model presented for the specification of organ identity based on simple combinatorial distributions of homeotic gene products, successfully predicts the specification of organ identity except in cases when organ number or position is drastically altered and does not address the issues of organ primordia formation.

How the products and phenotypic effects of the homeotic genes are localized to specific whorls is also unknown. It is true that the mRNA of both *AG* and *DEFICIENS* is preferentially present in those whorls which are altered in mutants, implying that transcriptional (or RNA stability) control is involved (Yanofsky et al., 1990; Schwarz-Sommer et al., 1990; Gary Drews, J.B., and E.M., submitted). Additionally, the spatial pattern of the *AG* mRNA is altered in an *ap2* background (Gary Drews, J.B., and E.M., submitted), as predicted by our model. This not only implies that there must be an initial prepattern which regulates the expression of the homeotic genes, but also that their final pattern of expression depends on interactions between the homeotic gene products. Thus, the mechanism of establishment of initial homeotic gene expression is different from the maintenance of their expression. One can envision a variety of types of interactions

between prepatterns and between the homeotic genes. For *AG*, one might imagine the initial expression pattern to be uniform, with subsequent modification by *AP2*. There is, however, no evidence of *AG* being expressed in all four whorls initially (Gary Drews, J.B., and E.M., submitted) although the method of detection used, *in situ* hybridization to tissue sections, might be unable to detect very low levels of expression. Since the whorls of organ primordia are generated sequentially, the outer whorl first and the fourth whorl last, it is possible that this developmental feature is utilized in the pattern formation process. *AP2* expression probably precedes that of *AG*, since the tsp determined for the *ap2-1* allele begins in stage two (Bowman et al., 1989), whereas *AG* mRNA is not detectable until stage three (Gary Drews, J.B., and E.M., submitted). The *AP2* gene product could become established in those cells destined to give rise to the outer whorls before *AG* expression commences. This might preclude the expression of *AG* in these whorls, since in an *ap2* mutant background, *AG* is expressed in the outer whorls. *AG* could then be expressed in those cells destined to become the third and fourth whorl primordia, consequently either reducing the expression of *AP2*, or suppressing the activity of the *AP2* gene product in some manner in the inner whorls. The spatial regulation of the *AP3* and *PI* gene products could also be dictated by temporal aspects in the first, second, and third whorls. The phenotypic effects of their wild-type products could be masked in the fourth whorl by an as yet unidentified gene. The phenotype of *superman-1* flowers (Uli Mayer, Gerd Jürgens, J.B., and Detlef Weigel, unpublished) suggests that the *SUPERMAN* gene product has a role in defining the patterns of activity of the other homeotic genes. One interpretation of *SUPERMAN* is that both the second and third whorl regions are expanded at the expense of the fourth whorl. A candidate for an even earlier regulator of the homeotic genes is the *LEAFY* gene (Haughn and Sommerville, 1988; David Smyth, Detlef Weigel, J.B., and E.M., unpublished). *leafy* mutants exhibit a phenotype similar to but not identical to that of *ap2-2 ag pi-1* triple mutants (see above; Bowman et al., 1991) suggesting that it might be involved in the initial appearance of the homeotic gene products.

Other genes must be involved as well, since the phyllotaxy of the triple mutant flowers and *leafy* mutants differs. It should be noted that the mRNA and protein expression of the homeotic genes does not have to be an all-or-nothing phenomenon in those whorls in which they have phenotypic effects. That the *DEFICIENS* gene may be expressed in all four whorls, although at a low level in those whorls in which no phenotypic effects are observed, suggests a pattern in which there are threshold levels of expression required to produce phenotypic effects is possible, as suggested by Schwarz-Sommer et al (1990). A model similarly relying on threshold levels of gene activity to produce phenotypic effects has been proposed for specification of the *hairy achenes* character in *Microseris* inflorescences (Bachmann, 1983). Thus, a characteristic aspect of plant development, that of the sequential production of organs from a group of meristematic cells, could play a pivotal role in the generation of patterns in flower development.

Evolutionary considerations

In addition to the apparently homologous mutations in *Antirrhinum*, mutations described in hundreds of other species of plants (including *Matthiola*, *Cheiranthus*, *Petunia*, and *Primula*; see Masters, 1869; Penzig, 1890-4; Meyer, 1966; Meyerowitz et al., 1989) resemble the homeotic flower mutations of *Arabidopsis*. Thus, the mechanisms used in flower development of *Arabidopsis* and *Antirrhinum* are likely to be utilized in other flowering plants as well. Flowering plants appeared recently in the fossil record (120-140 million years ago; lower cretaceous) and rapidly became the dominant class of plants (80-90 million years ago; upper cretaceous). Due to the sparse nature of the early fossil record it is difficult to ascertain the relative order of appearance of the floral organ types, but it is apparent that their evolution was rapid since the four basic types were present even in early flowers (120-140 million years ago; Friis and Crepet, 1987). It has been suggested that the floral organs are modified leaves and that carpels evolved from

sporophylls of primitive vascular plants (Stebbins, 1976). When all three types of flower homeotic genes are mutant in *Arabidopsis*, a flower in which all organs are leaves or carpelloid leaves, which could be interpreted as sporophylls, is produced. This suggests that the three classes of genes represented by the known *Arabidopsis* homeotic genes may be responsible for the majority of fundamental features associated with the flower.

The two cloned homeotic genes (*AGAMOUS* in *Arabidopsis* and *DEFICIENS* in *Antirrhinum*; Yanofsky et al., 1990; Sommer et al., 1990), both putative transcription factors, are involved in distinct developmental processes in pattern formation in the flower, and share homology in their DNA-binding motif. It will be of interest to see if any of the other floral homeotic genes are also members of the same family of proteins. If so, it is possible that much of the genetic machinery required for the development of flowers may have evolved from a small number of progenitor genes. Early duplication and divergence could produce a small number of gene families whose members direct the development of the basic floral structure.

Future prospects

To understand the mechanisms by which flower development occurs, it will be necessary to analyze each of these genes, and probably many others, at the molecular level. Both *Antirrhinum* and *Arabidopsis* are amenable to approaches to clone genes of which nothing more than their phenotype is known. Several endogenous transposons in *Antirrhinum* make gene tagging by transposon mutagenesis an attractive method in this system (Coen and Carpenter, 1986). The large floral organs also make feasible the construction of organ-specific cDNA libraries. Differential screening or subtractive hybridizations can be used to identify organ specific clones or enrich libraries for genes of interest (Sommer et al., 1990). In contrast, the genome of *Arabidopsis* contains no known active transposons. Insertional mutagenesis can, however, be performed using the T-DNA

of *Agrobacterium* (Feldmann and Marks, 1987; Feldmann et al., 1989; Yanofsky et al., 1990; Feldmann and Meyerowitz, 1991) In addition, the introduction of heterologous transposons may allow gene tagging by transposon mutagenesis (Van Sluys et al., 1987; Masterson et al., 1989; Schmidt and Willmitzer, 1989). Although the genome of *Arabidopsis* has no known active transposons, others of its characteristics, such as its small size (70,000-100,000 kilobases; Leutwiler et al., 1984) and the near-absence of dispersed repetitive elements (Pruitt and Meyerowitz, 1986), can be exploited to clone genes by chromosome walking. RFLP maps with over 250 total markers have been constructed to provide starting points (Chang et al., 1988; Nam et al., 1989); walks of several hundred kilobases are already feasible if genomic libraries based on phage P1 or yeast artificial chromosome vectors are used. In addition, the possibility of physically mapping the entire *Arabidopsis* genome is being pursued. This would provide immediate access to any locus that can be mapped, consequently expanding the realm of plant molecular biology.

The small flowers of *Arabidopsis* preclude experiments relying on the isolation of large amounts of floral organ specific mRNA. Other attributes of *Arabidopsis*, however, such as its rapid generation time and the ability to be transformed by *Agrobacterium* (Lloyd et al., 1986; Masterson et al., 1989; Schmidt and Willmitzer, 1989), allow other types of analyses, such as the production of double and triple mutants, and the misexpression of cloned homeotic genes, which are at present impracticable in *Antirrhinum*. Thus, both *Arabidopsis* and *Antirrhinum* have distinct advantages that compliment each other. Since the flowers of each system are similar in architecture, it is likely that the general processes of development will also be similar. The advantages of each system can then be applied to the solution of a common problem: the mechanism of flower development.

Acknowledgments

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Table 1. Summary of Phenotypes of *Arabidopsis* Flowers

whorl	first (medial/ lateral)	second	third	fourth
wild type	sepals	petals	stamens	carpels
apetala2-1	leaves	staminoid petals	stamens	carpels
apetala2-2	carpels/ absent	absent	absent/ stamens	carpels
apetala2-1/2	carpels/ leaves	absent	stamens	carpels
apetala3-1	sepals	sepals	carpels	carpels
pistillata-1	sepals	sepals	absent	extra carpels
pistillata-3	sepals	sepals	carpels	carpels
agamous-1,2,3	sepals	petals	petals	another flower
superman-1	sepals	petals	stamens (stamens)	carpels

Figure 1. SEM micrographs depicting the early development of wildtype *Arabidopsis* flowers grown at 25°C.

(A) Apical meristem and flower development through stage 3. Stages of the buds are indicated. Bar = 10 μm.

(B) Stage 6 bud. Three sepals, two medial and one lateral have been removed. Bar = 10 μm.

(C) Stage 7 bud. All four sepals have been removed to expose the petal (p), medial stamen (ms),and lateral stamen (ls) primordia and the gynoecial cylinder (g). Bar = 10 μm.

(D) Stage 11 bud. Three sepals have been removed revealing the inner three whorls of organs.

Bar = 100 μm.

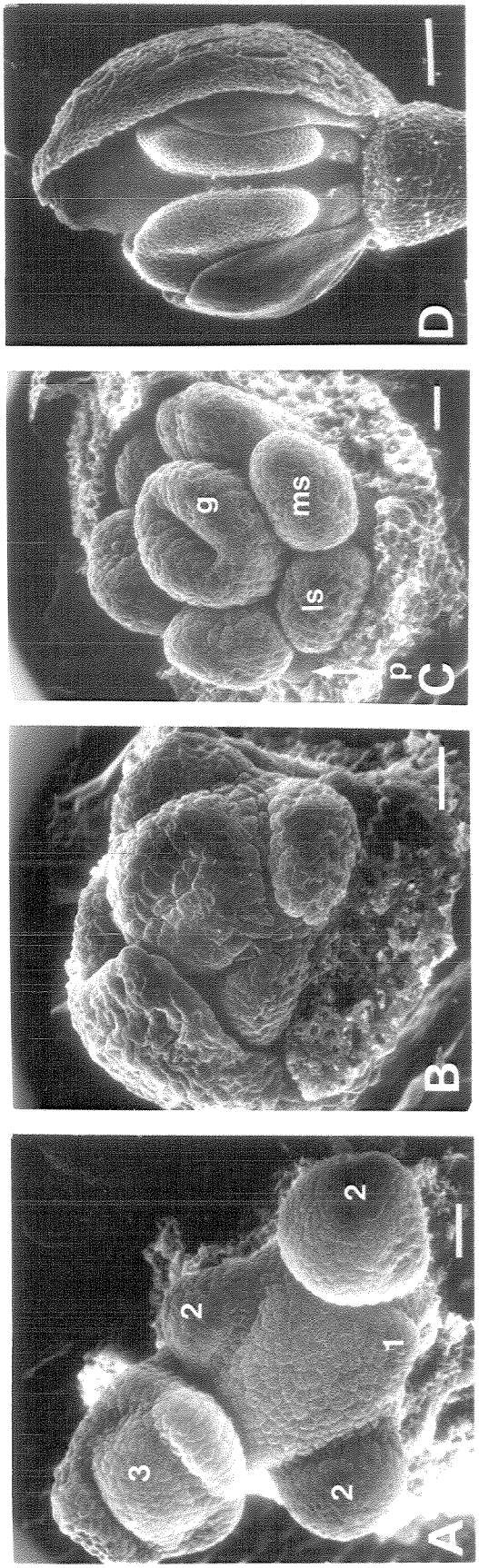

A
1
2
2
2
3
B
C
g
ms
ls
p
D

Figure 2. SEM micrographs of *Arabidopsis* homeotic flower mutants. All plants were grown at 25°C.

(A) *apetala2-1*. Cauline leaf-like organs are present in the outer whorl while staminoid petals are visible in the second whorl.

(B) *apetala2-2*. A stamen-carpel mosaic organ is indicated (arrow) in a medial first whorl position and the central gynoecium is not fused properly.

(C) *apetala2-1/2*. Note the difference in phenotype between the medial (carpel-like) and lateral (leaf-like) first whorl organs.

(D) *apetala3-1*. Note the second whorl sepal and the carpelloid third whorl organs. Some first and second whorl organs have been removed.

(E) *pistillata-1*. A second whorl sepal, which is smaller than the first whorl sepal, and the abnormal gynoecium are visible. The small dome of cells at the base of the flower (arrow) is a nectary. Several first and second whorl organs have been removed.

(F) *agamous-1*. cross section through a flower showing the indeterminate growth. The apical meristem is visible in the lower left (arrow).

(G) *superman-1*. Some first whorl sepals and second whorl petals have been dissected off to reveal what appears to be two whorls of stamens and some carpelloid tissue interior to the stamens.

Bar = 100 μm.

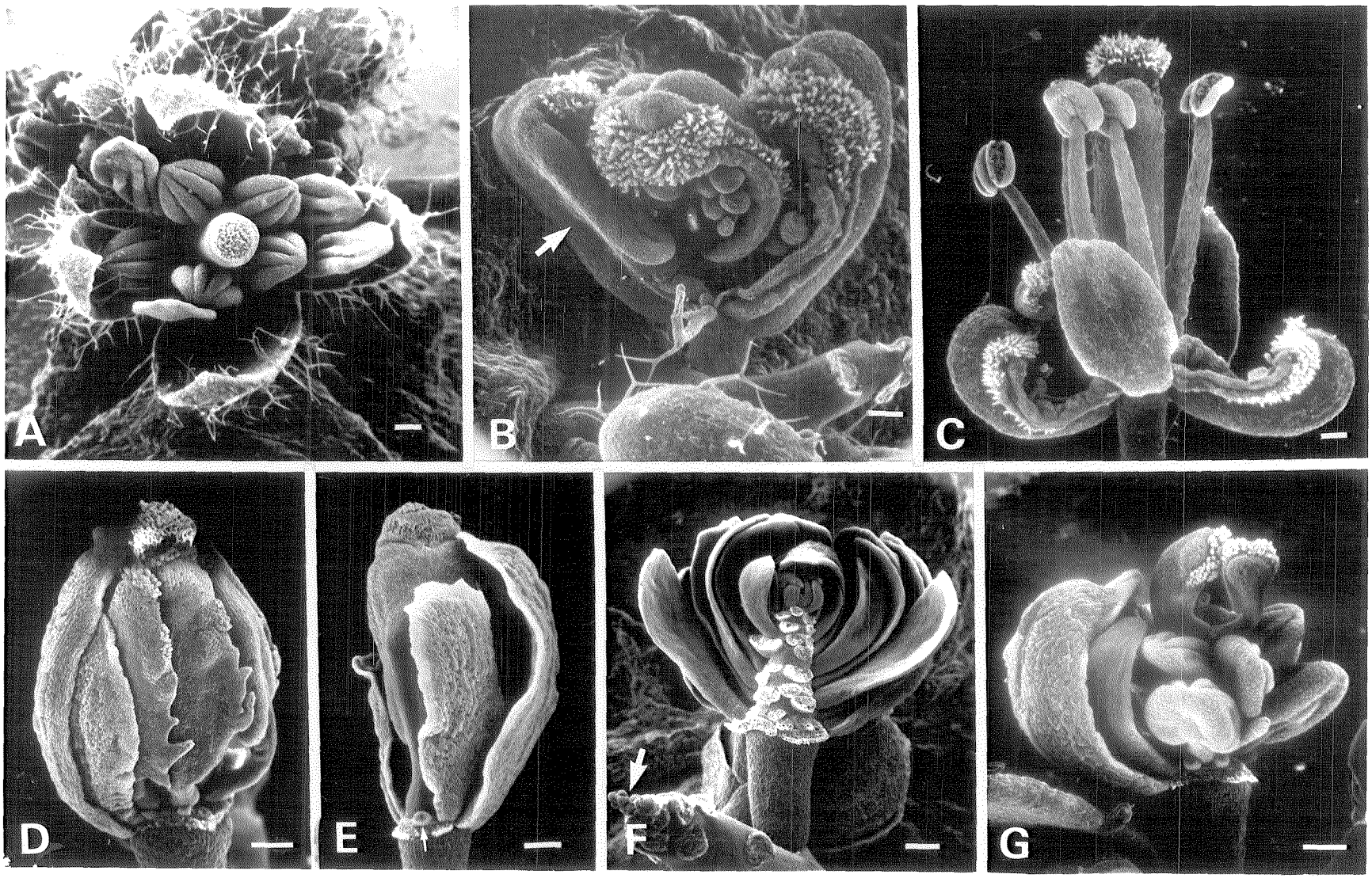

A
B
C
D
E
F
G

Figure 3.

(A) An *ap2-1* flower at an early stage of development (stage 7). When grown at 29°C, *ap2-1* flowers often fail to form second whorl organs (Bowman et al., 1989); this is due to a failure to form second whorl primordia. The remaining third whorl organs are also in ectopic positions (compare with Figure 1C). The small group of cells indicated (arrow) arises from the base of an outer whorl organ which would develop into a cauline leaf-like organ and therefore is a stipule. Bar = 10 μm.

(B) Apex of an *ap2-8* plant. Flowers of *ap2-8* homozygotes are similar in phenotype to those of *ap2-2* homozygotes. The medial first whorl primordia are enlarged and the lateral first whorl primordia arise lower on the flower primordium compared to those of wildtype (Figure 1A). Bar = 10 μm.

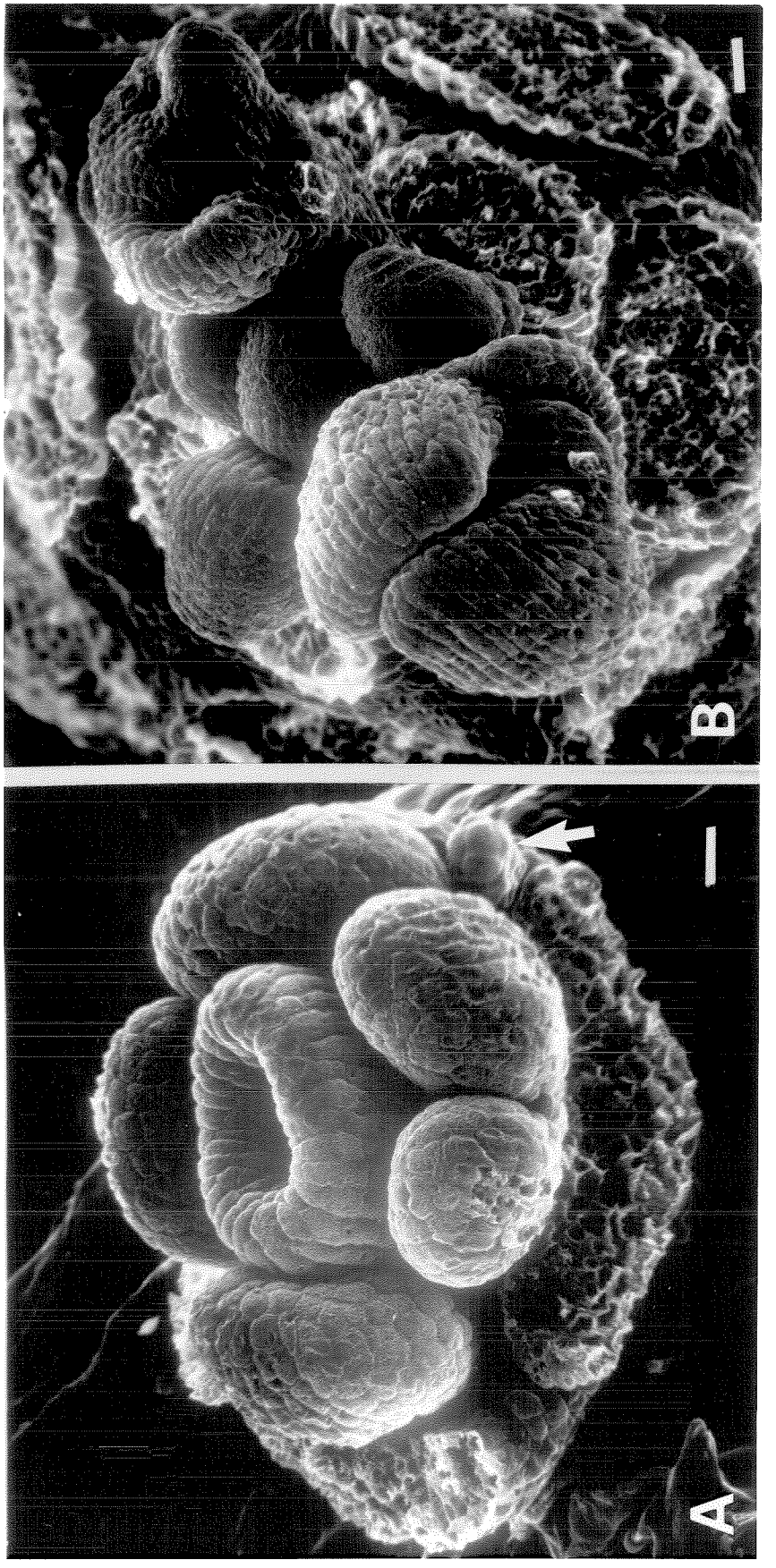

A
B

Figure 4. Medial view of the base of an *ap2-1* flower grown at 29°C; the first whorl organs have been removed. Second whorl organs when present (they are missing about 3/4 of the time, see Figure 3A) often develop into stamens. All second and third whorl positions visible are occupied by stamens and are indicated as: second (2), medial third (3), and lateral third (3L) whorls stamens. The domes and other outgrowths of cells therefore do not represent aborted organ primordia. The domes at the base of the stamens (large arrows), both second and third whorl, are nectaries and have stomata on their apices. The other filamentous outgrowths (small double arrows) arise from the base of the first whorl organs which were cauline leaf-like and therefore these outgrowths are stipules. Bar = 100 μm.

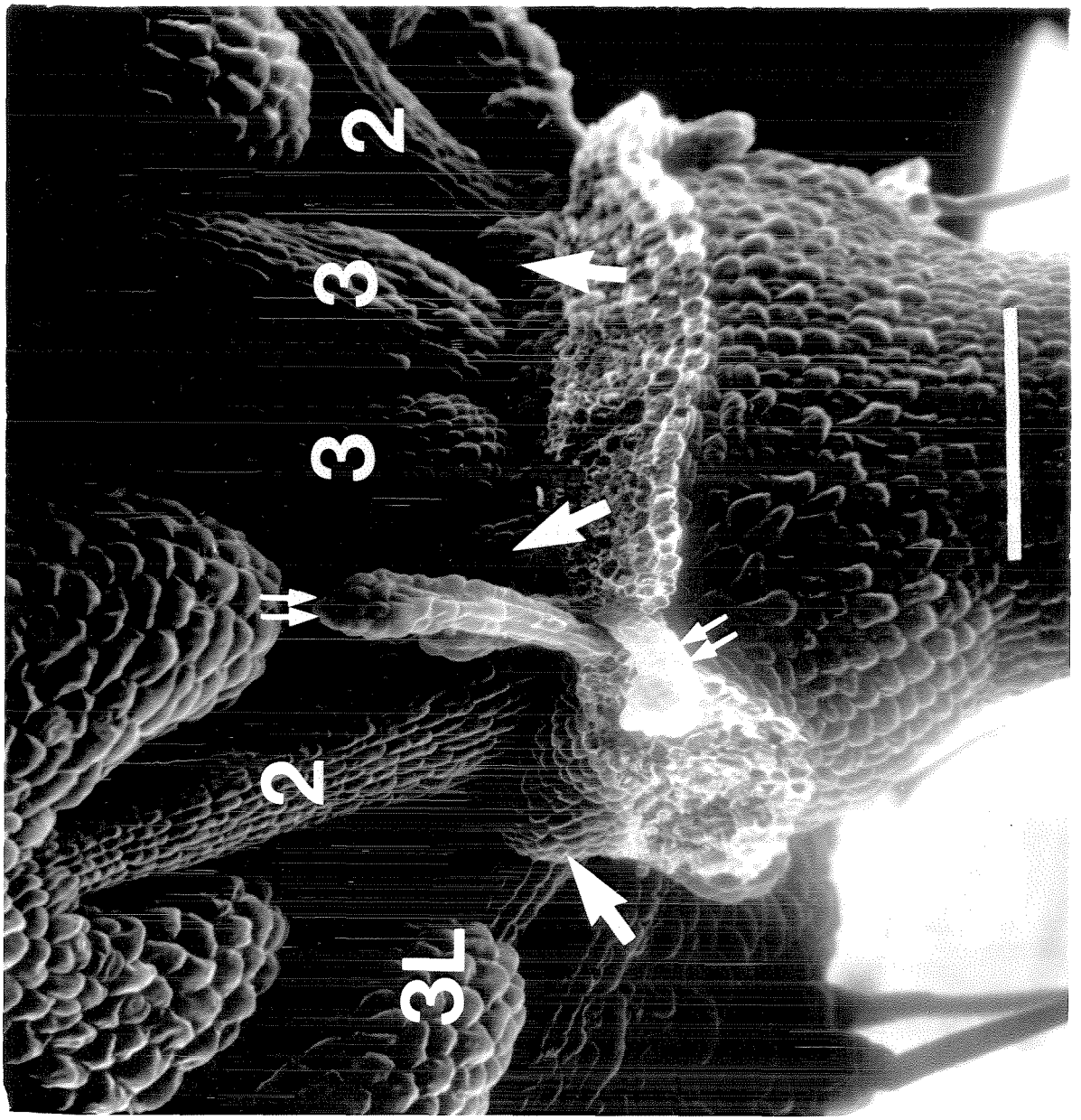

2
3
3
2
2
3L

Figure 5. SEM micrographs of double and triple mutant flowers

(A) *ap3-1 ag-1*. All organs are sepals.

(B) *ap2-2 pi-1*. Flowers consist of only a central gynoecium usually composed of four carpels.

(C) *ap2-2 ag-1*. Medial first whorl organs are carpelloid leaves (c/l) with stigmatic tissue visible along the margin and the second and third whorl organs are petaloid stamens (p/s).

(D) *ap2-2 pi-1 ag-1*. All organs are carpelloid leaves. Both trichomes and stigmatic tissue are evident.

Bars = 100 μm.

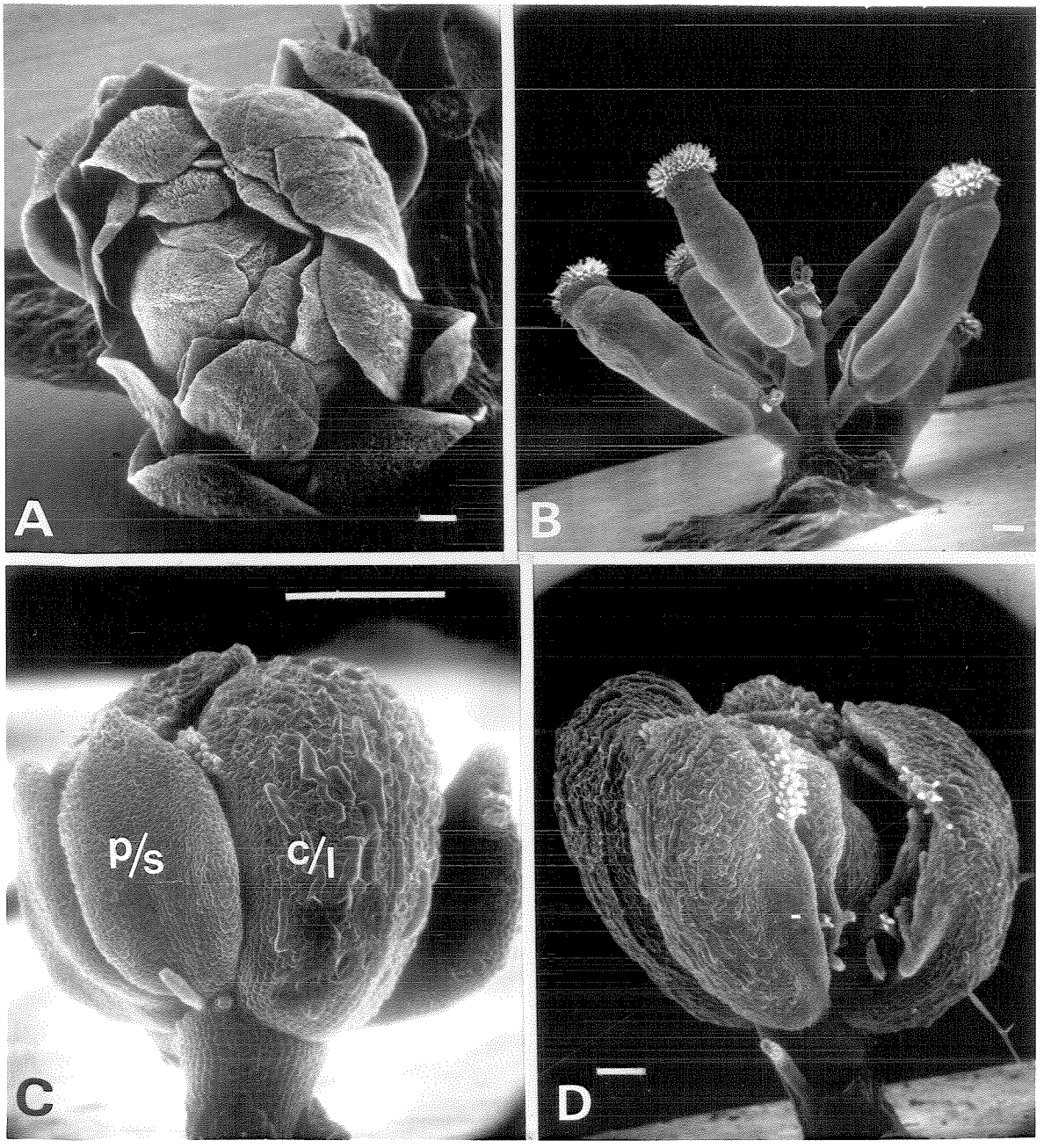

A
B
C
p/s
c/l
D

Figure 6. *ap2-1 pi-1 ag-1* flower. All organs are cauline leaf-like with prominent stellate trichomes on their abaxial surfaces and stipules at their bases. Bar = 100 μm.

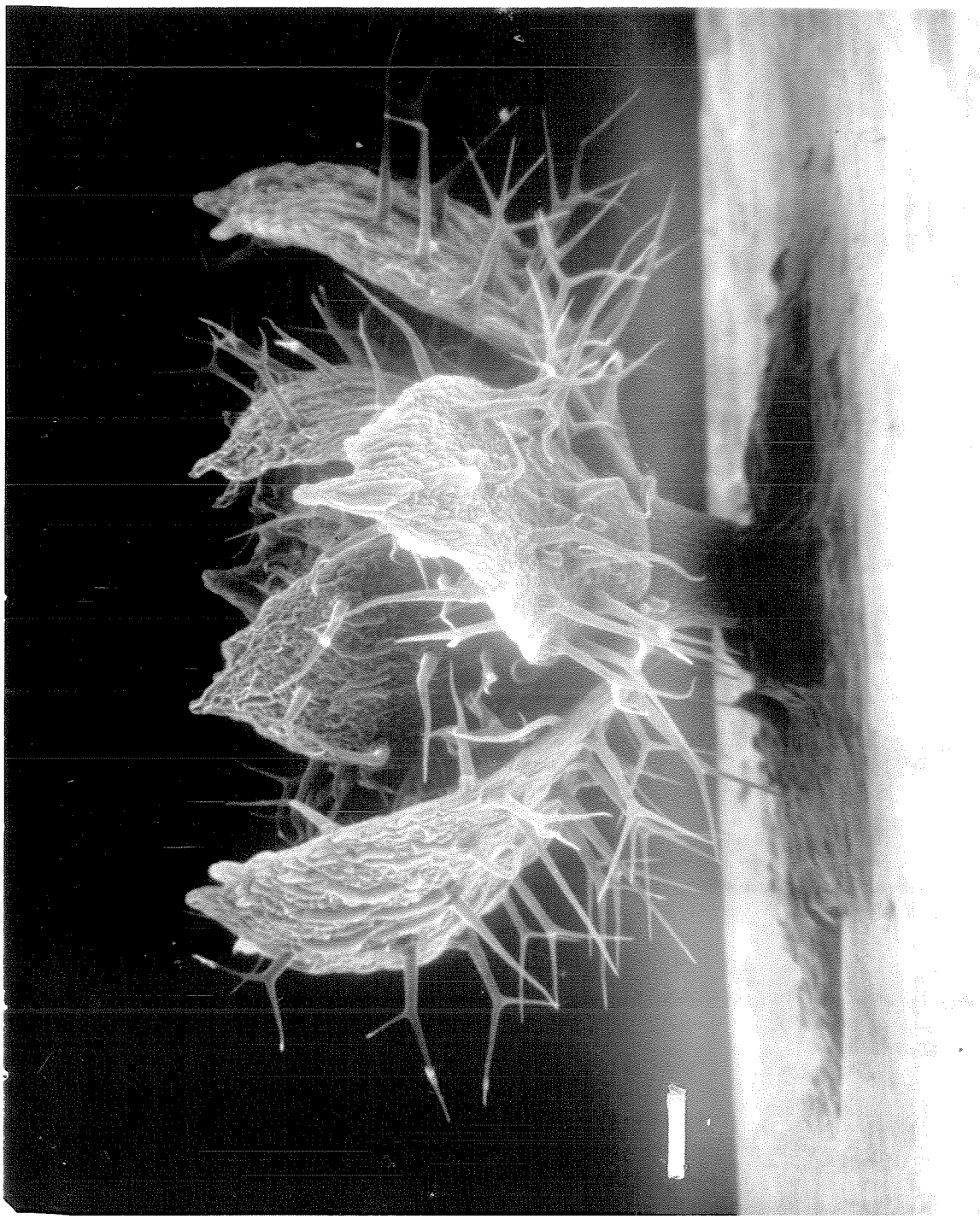
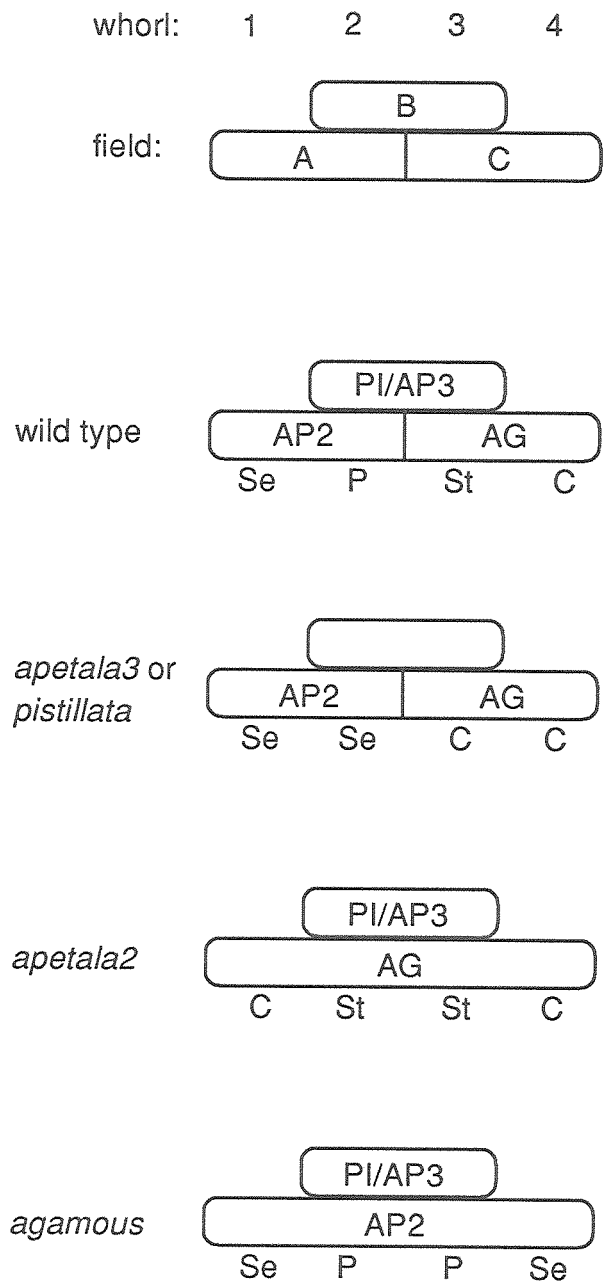


Figure 7. A simple model depicting how the three classes of floral homeotic genes in *Arabidopsis* could specify the identity of each of the four whorls of floral organs. Details of the model are in the text. Se = sepals, P = petals, St = stamens, and C = carpels.


whorl: 1 2 3 4
field:
B
A
C
wild type
PI/AP3
AP2
AG
Se P St C
apetala3 or pistillata
AP2
AG
Se Se C C
apetala2
PI/AP3
AG
C St St C
agamous
PI/AP3
AP2
Se P P Se

CHAPTER ONE

EARLY FLOWER DEVELOPMENT IN *ARABIDOPSIS*

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Early Flower Development in *Arabidopsis*

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The early development of the flower of *Arabidopsis thaliana* is described from initiation until the opening of the bud. The morphogenesis, growth rate, and surface structure of floral organs were recorded in detail using scanning electron microscopy. Flower development has been divided into 12 stages using a series of landmark events. Stage 1 begins with the initiation of a floral buttress on the flank of the apical meristem. Stage 2 commences when the flower primordium becomes separate from the meristem. Sepal primordia then arise (stage 3) and grow to overlie the primordium (stage 4). Petal and stamen primordia appear next (stage 5) and are soon enclosed by the sepals (stage 6). During stage 6, petal primordia grow slowly, whereas stamen primordia enlarge more rapidly. Stage 7 begins when the medial stamens become stalked. These soon develop locules (stage 8). A long stage 9 then commences with the petal primordia becoming stalked. During this stage all organs lengthen rapidly. This includes the gynoecium, which commences growth as an open-ended tube during stage 6. When the petals reach the length of the lateral stamens, stage 10 begins. Stigmatic papillae appear soon after (stage 11), and the petals rapidly reach the height of the medial stamens (stage 12). This final stage ends when the 1-millimeter-long bud opens. Under our growing conditions 1.9 buds were initiated per day on average, and they took 13.25 days to progress through the 12 stages from initiation until opening.

INTRODUCTION

The genetic control of flower development in *Arabidopsis* has been the focus of several recent studies including our own (Pruitt et al., 1987; Komaki et al., 1988; Bowman et al., 1989; Hill and Lord, 1989; Kunst et al., 1989; Okada et al., 1989). In each case the effects of mutations that specifically disrupt flower morphogenesis have been characterized in detail. It is argued that such mutations are likely to occur in genes whose products control the regulatory decisions that underlie flower development. Ultimately, the cloning and molecular characterization of such genes are likely to allow their mechanism of action to be deduced (Meyerowitz et al., 1989). To understand better the aberrant development in mutant plants, we have characterized wild-type development in detail.

The mature flower of *Arabidopsis thaliana* has a simple structure typical of the Brassicaceae. It has a calyx of four free sepals and a corolla of four petals, whose positions alternate with those of the sepals. There are four medial and two lateral stamens, the former longer than the latter. The superior gynoecium has two carpels whose locules are separated by a false septum. Ovules arise on the parietal placentae on each side of the septum.

The structure and development of the *Arabidopsis* flower from the time of its opening to the release of mature seed from the fruit have already been characterized in detail (Müller, 1961). Changes in the apical meristem as it moves from vegetative to floral growth also have been described by several authors (Vaughan, 1955; Miksche and Brown, 1965). However, a detailed study of the intervening early stages of flower development in *A. thaliana* has not been reported.

In this paper we describe the structure of *Arabidopsis* buds by scanning electron microscopy from their first appearance as a buttress on the apical meristem until they open as fully developed flowers. By examining the shape, size, and surface features of developing floral organs, we have divided the continuous process into 12 stages defined by landmark events. The duration of each stage has also been estimated. This analysis provides a series of reference observations of importance for interpreting the mechanisms of action of genes that control flower development in this model species.

RESULTS

Description of Developmental Events

Flower initiation in *Arabidopsis* occurs continuously in an indeterminate spiral at each floral apex. Thus, flowers can be placed in order of age and developmental stage by their

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position on an inflorescence. In this study all flowers on the main apex of three plants in which the oldest flowers had just opened were analyzed in detail. One plant was 22 days old, the other two were 24 days old. Observations were made directly on primordia and buds at the earliest stages of development. At later stages the buds are enclosed by sepals. To reveal the organs developing underneath, one medial and the two lateral sepals were dissected from the bud. After detailed examination they were photographed from the side and the lengths of the remaining lateral sepal; one or two of the petals, one or two long stamens, a short stamen, and the gynoecium were recorded as the linear distance between the base and the tip of the organ in each case. (In some buds further organs had to be removed in turn to reveal previously hidden organs.) Detailed measurements of buds from one of the three inflorescences are shown graphically in Figure 1. The overall appearance of another inflorescence before dissection is shown in Figure 2A.

Based on observations and measurements from these inflorescences, we have divided the continuous process of flower development from initiation until the bud opens into 12 stages, as shown in Table 1. A summary of information on later stages has been added from the study of Müller (1961).

Stages 1 to 5

Flowers arise on the flank of the apical meristem, which is a dome of cells about 45 μm in diameter at this time (Figures 2B to 2D). Stage 1 begins when a floral buttress appears. This increases in size by lateral outgrowth from the apex. At the time it reaches a breadth of about 22 μm to 25 μm, a groove separates it from the main apex. This defines the beginning of stage 2. Growth of the hemispherical primordium continues almost at a right angle to the main apex, which is itself lengthening and widening at the point of attachment of the bud. Stage 3 begins when sepal primordia appear. By now the flower primordium is 30 μm to 35 μm in diameter and becoming stalked with an incipient pedicel. Its growth is also becoming more vertical. The abaxial sepal primordium arises first, followed soon after by the adaxial and the two laterals. The sepal primordia arise initially as ridges that lengthen and curve inward until they begin to overlie the dome-shaped flower primordium. Again, the abaxial sepal is the first to do this, at which time stage 4 begins. Pedicel elongation continues concurrent with an increase in the diameter of the developing bud to about 65 μm to 70 μm.

Stage 5 begins with the appearance of stamen and petal precursors (Figure 2C). Primordia of the four medial (long) stamens are first seen as wide outgrowths on the central dome of cells. Barely visible are the four petal primordia that arise between the sepals and close to their base. The two lateral (short) stamens develop from primordia that appear a little later in stage 5 (Figure 3A). [This definition of the beginning of stage 5 is slightly earlier than that in our earlier paper (Bowman et al., 1989), where it was defined as occurring when the lengthening medial sepals overlapped.] Stage 5 ends and stage 6 begins when the bud is fully enclosed by both medial and lateral sepals. The tip of the abaxial sepal overlies that of the adaxial, whereas the two shorter lateral sepals meet or overlap underneath (Figure 2A).

Stages 6 to 12

From the start of stage 6, sepals completely cover the bud. During stage 6, primordia of the four long stamens bulge out and become distinct from the central dome of cells (Figure 3B). The two lateral stamen primordia arise slightly lower on the dome and develop slightly later. The petal primordia increase in size somewhat, but are still relatively small. A rim around the central dome of the flower primordium now begins to grow upward to produce an oval, hollow tube that will be the gynoecium.

Stage 7 begins when the growing primordia of the long stamens become stalked toward their base (Figure 3C). This region will give rise to the filament, the wider upper region to the anther. This occurs when the long stamen primordia are around 25 μm to 30 μm long overall. Vertical growth of the slotted gynoecial tube keeps pace with that

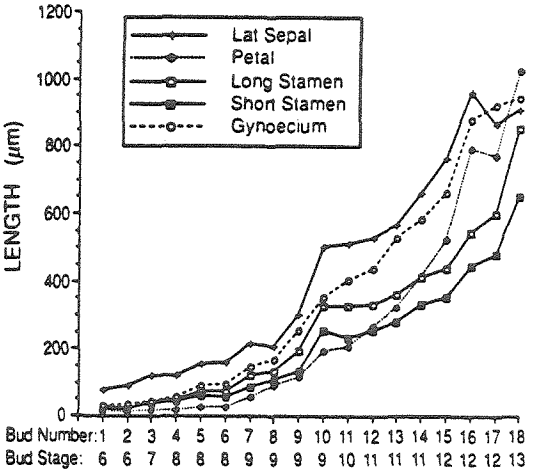

Figure 1. Length of Flower Organs in Buds on the Main Inflorescence Apex of a 22-Day-Old Plant.

The youngest bud scored (bud 1) was at stage 6 and the oldest (bud 18) had reached stage 13. The stage reached by each of the other buds is indicated.

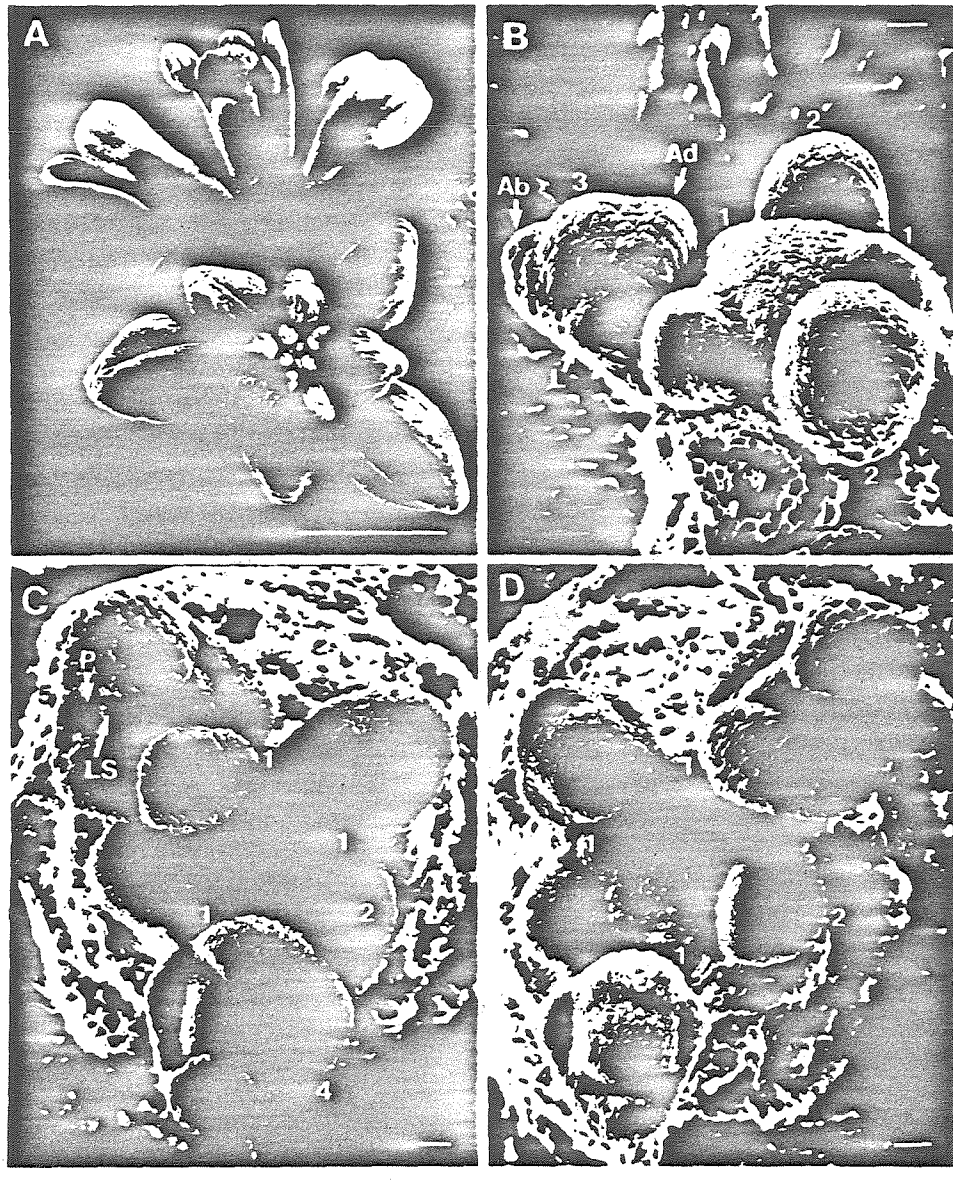

Figure 2. Scanning Electron Micrographs of the Main Flowering Apex of *Arabidopsis*.

(A) Vertical view of the apex of a 24-day-old plant in which the oldest flower has reached stage 14. (The sepals and petals of this flower have spread during preparation.) Bar = 1000 μm.

(B) Lateral view of the youngest buds on an inflorescence after the older buds have been removed. The stage reached by each bud is shown. The abaxial (Ab), adaxial (Ad), and lateral (L) sepal on the stage 3 bud are also indicated. Bar = 10 μm.

(C) and (D) Vertical views of two inflorescences in which buds are arising in the opposite spiral sense. In (C) younger buds appear in a counter-clockwise direction from the next older bud; in (D) the direction is clockwise. The stage of each bud is indicated. Petal (P) and long stamen (LS) primordia are just visible on the oldest, stage 5 buds. Bars = 10 μm.

Table 1. Summary of Stages of Flower Development in *A. thaliana* Listing the Landmark Events that Define the Beginning of Each Stage and Its Approximate Duration

Stage	Landmark Event at Beginning of Stage	Duration[a] (hr)	Age of Flower at End of Stage[a] (days)
1	Flower buttress arises	24	1
2	Flower primordium forms	30	2.25
3	Sepal primordia arise	18	3
4	Sepals overlie flower meristem	18	3.75
5	Petal and stamen primordia arise	6	4
6	Sepals enclose bud	30	5.25
7	Long stamen primordia stalked at base	24	6.25
8	Locules appear in long stamens	24	7.25
9	Petal primordia stalked at base	60	9.75
10	Petals level with short stamens	12	10.25
11	Stigmatic papillae appear	30	11.5
12	Petals level with long stamens	42	13.25
13[b]	Bud opens, petals visible, anthesis	6	0.5
14	Long anthers extend above stigma	18	1
15	Stigma extends above long anthers	24	2
16	Petals and sepals withering	12	2.5
17	All organs fall from green siliques	192	10.5
18	Siliques turn yellow	36	12
19	Valves separate from dry siliques	up to 24	13
20	Seeds fall		

[a] Estimated to nearest 6 hr.

[b] Results for stages 13 to 20 (after the flower opens) are summarized from Müller (1961) where they were named B3 to B10. Their timings are given separately because a different strain was grown under different conditions from those used in the present study.

of the long stamens (Figure 3D). Petal primordia are now hemispherical, although still relatively small (about 25 μm in diameter) (Figure 3C). The beginning of stage 8 is defined by another landmark of stamen development, when anther locules are visible as convex protrusions on the inner (adaxial) surface of the long stamens (Figures 3E and 3F). These stamens have a total length of 55 μm to 60 μm at this time, most of it made up by the developing anther. Locules also appear soon after in the short stamens.

Petal growth now accelerates, and when the petal primordia become stalked, stage 9 begins (Figure 4A). This stage involves a rapid lengthening of all organs, especially the tongue-shaped petals (Figure 1). These increase in length fourfold to fivefold, from about 45 μm up to 200 μm. The stamens also grow rapidly. By the end of stage 9, the medial stamens are around 300 μm long. Most of this growth occurs in the anther region, which still accounts for over 80% of their total length. The gynoecium continues to elongate as an open, oval tube with a somewhat tapered apex. The growth of sepals keeps pace so that the bud remains completely closed.

Stage 10 begins when the fast-growing petals reach the top of the short stamens (Figure 4B). Soon afterward, stage 11 begins when the upper surface of the gynoecium develops stigmatic papillae, although their outward growth is limited at first to regions not in contact with the overlapping sepals (Figure 4C). Once the petals reach the top of the long stamens, flowers move into the final bud stage, stage 12 (Figure 4D). [This corresponds to stage B2 of Müller (1961).] During stage 12 petals continue to lengthen relatively rapidly. The growth rate of lateral sepals continues to slacken while stamens and the gynoecium lengthen in concert. The anthers have almost reached their mature length of 350 μm to 400 μm for both the long and short stamens. Lengthening of the filaments now accelerates rapidly. The upper part of the gynoecium becomes differentiated into a short, 100-μm- to 120-μm-long style, which is slightly constricted toward its base. A sharp boundary separates it from a cap of stigmatic papillae (Figure 4D).

Bud stage 12 ends when the sepals open and stage B3 (Müller, 1961) (here renamed 13) begins (Table 1). The petals can be seen between the sepals and continue to elongate rapidly. The stigma is already receptive at this stage and anthesis occurs. Stamen filaments extend even faster, and the length of the long stamens outstrips that of the gynoecium at the beginning of stage 14 (Figure 2A).

Mature Flower

The mature floral organs show a range of different surface morphologies. The outer sepal surface has many stomata interspersed among irregularly shaped epidermal cells (Figure 5A). In addition, there are always several characteris-

tically elongated cells (up to about 300 μm long) extending longitudinally. Probable progenitors of these cells can be first recognized at stage 8 when the lateral sepals are only about 150 μm long (Figure 3E). The distal edges of the mature sepals are bordered by smaller epidermal cells that are pale green in the live plant (Figure 5A). The outer surface of the sepal may also have an occasional unbranched trichome (Figures 2A and 6C). These are lacking from the inner surface, as are stomata and the elongated cells. Both surfaces of the petal blade are covered with specialized, dome-shaped cells whose surface is finely ridged in a radial pattern (Figure 5B). Petals lack stomata.

The surface of the anthers consists of epidermal cells of uniform size (Figure 5C) that have already been formed by bud stage 10 (Figure 4B). The surface of the ovary in the mature flower is made up of vertical files of epidermal cells (Figure 5D). On the short style the cells are larger and are interspersed with stomata. The densely packed stigmatic papillae are 20 μm to 35 μm long when the bud opens at the end of stage 12. The development of the stigma is shown in Figures 5E to 5G. By the end of stage 9 there is not yet any differentiation at the tip of the gynoecium, which still has a slotted opening to the interior (Figure 5E). This opening is maintained through stage 10, although it becomes smaller. In addition, the eventual disc shape of the stigma surface is becoming apparent (Figure 5F). During stage 11, stigmatic papillae come to cover the entire stigmatic surface. The gynoecium closes over, and the short style begins to develop (Figure 5G).

Nectaries are present in mature flowers at the base of the stamens (Figures 5H to 5J). Lateral nectaries arise at the base of the short stamens toward the end of stage 9 (Figure 5H). They first appear as an outgrowth several cells thick. By stage 11 they have formed either a large ridge (Figure 5I), a single dome, or two domes of cells, usually with several stomata at the apex in each case (Figure 5J). One or the other of the lateral stamens was absent in one-quarter of the flowers we analyzed in detail (12 out of 47). In these the lateral nectary occupying the empty space was usually larger and more dome shaped. The occurrence of medial nectaries at the base of the long stamens was sporadic. If present, they were narrower than the lateral nectaries and arose later.

Commencement of Flower Development

Before flower development starts, the main shoot apex produces a limited number of rosette leaves. In a sample of 117 plants grown under our conditions, the mean number of rosette leaves was 7.25 (SE = 0.07, range = 5 to 9).

To trace the change from vegetative to floral development, a sample of plants was fixed every 2 days beginning 12 days after incubation at 25°C. Buds were first visible on 14-day plants, by which time some of the first-formed buds had reached stage 3 (Figure 6A). At 16 days several of the oldest buds on the main axis of some plants had developed beyond stage 5 (Figure 6B). Buds also arise on secondary floral apices; these occur in the axils of all cauline leaves on the main stem. In our 117 plants there were usually two such cauline leaves per main stem (mean = 2.05 ± 0.04, range 1 to 3). These leaves have clearly developed stipules at each side of their point of attachment to the main stem (Figures 6B and 6C). At 16 days these secondary apices were much less advanced than the main apex. The oldest buds, flanked in turn by secondary cauline leaves, had only reached stage 2 or 3.

At around 18 days, the main inflorescence begins to elongate rapidly as the plant starts to bolt (Figure 6C). At this time between four and seven buds per plant were observed to be at stage 6 or beyond on the main apex. The secondary branches of the main inflorescence continue to develop, with buds now at stage 5. The cauline leaves that overlie these buds develop trichomes. Further inflorescences arise in the axils of the rosette leaves. At 18 days these have only just begun to develop, visible under a cover of their own cauline leaf primordia.

Duration of Stages 1 to 5

To estimate the relative numbers of each of the five early stages on the main axis, counts were made by light and scanning electron microscopy on 20 plants, four sampled on each of days 18, 20, 22, 24, and 26, as shown in Table 2. An average of 7.8 floral primordia were present per plant. There was no apparent change in the proportions of flower primordia in each of the five stages during this period. Overall, stages 1 and 2 were present most often with an average of more than two primordia per plant. Stages 3 and 4 were less frequent (1.40 and 1.35 per inflorescence), whereas there was only a 0.7 chance of seeing a stage-5 bud.

The absolute times spent in these stages can be assessed if an estimate of the rate of bud production is available. This was obtained by recording the numbers of flowers on 20 plants on seven consecutive days beginning on day 25, as shown in Table 3. Because of the difficulty in accurately scoring primordia at stages 1 to 5 on live plants, only buds with closed sepals (stage 6 onward) were recorded. Nevertheless, this allowed the number of buds per day that passed the stage 5/6 boundary to be deduced (Table 3). This was relatively constant from days 26 to 29, averaging 1.9 buds per day. [It fell significantly over the last 2 days (days 30 and 31), and these results have not been used in the following calculations.]

Because an average of 1.9 buds per day move *out of* stage 5 over days 25 to 29, it can be argued that this is also an estimate of the rate of movement of buds *into* stage 1 over the earlier period of days 18 to 26 because the number of buds in stages 1 to 5 is constant over this

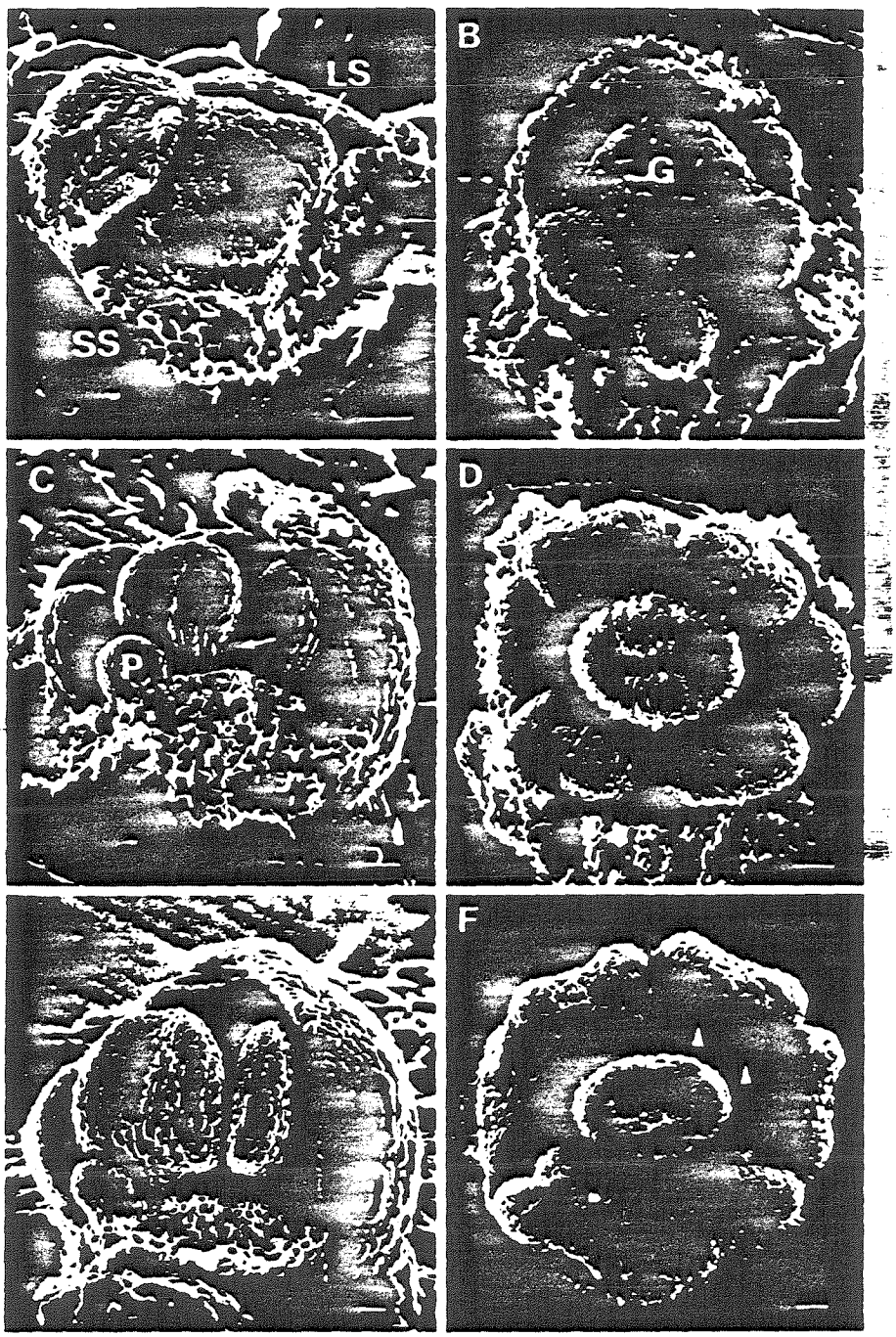

Figure 3. Individual Buds at Stages 5 to 8 with Sepals Dissected Away To Reveal Inner Organs.

period (Table 2). Under such circumstances, stages 1 to 5 occupy a total of 4.1 days on average (i.e., 7.8 buds per plant arising at the rate of 1.9 buds per day). Rounded off to the nearest 6 hr, stage 1 lasts for 24 hr, stage 2 for 30 hr, stages 3 and 4 are 18 hr each, and stage 5 occupies only 6 hr (Table 1).

Duration of Stages 6 to 12

The data in Table 3 also allow the total time in stages 6 to 12 to be estimated. The number of buds moving into stage 6 was in overall balance with the number moving out of stage 12 (into 13 and beyond). For days 25 to 29, the average number moving out was 1.7 buds per day, close to the 1.9 buds per day estimated earlier as moving into stage 6. Thus, the average flux of buds moving through this period is 1.8 buds per day. Given that there is an average of 16.4 buds per inflorescence in these stages (Table 3), a bud takes 9.1 days on average to pass through stages 6 to 12.

Finally, an estimate of the times spent in each of stages 6 to 12 depends on counts of the numbers of bud per plant at each stage. This requires the removal of sepals and was done only for the three inflorescences used to define stages. The number of buds at each stage for one of these is given in Figure 1. (Note that there was an additional bud at stage 6 on this inflorescence that was too small to measure accurately.) In the second inflorescence there were two buds at stage 6, two at 7, two at 8, five at 9, one at 10, two at 11, four at 12, and one at stage 14. In the third inflorescence there were two buds at stage 6, three at 7, one at 8, four at 9, one at 10, two at 11, three at 12, and one at stage 14. Because of these limited data, stages have been estimated only to the nearest 6 hr (Table 1). The longest stage is stage 9 which, with 4.3 buds per inflorescence on average, lasts 2.5 days. Stage 10 is the shortest—about 12 hr.

Direction of Inflorescence Spiral

Buds arise acropetally on an apex, with each new bud arising around the apex at an angle of between 130° to 150° to the earlier bud (Figures 2C and 2D). The direction a new bud may take can be counter-clockwise (Figure 2C) or clockwise (Figure 2D) from the previous bud, as viewed from the top. This direction is maintained by later buds on an axis giving, respectively, a righthanded or a lefthanded helix.

One hundred seventeen mature plants were scored for the direction of spiralling of their main axis. The numbers were close to equality—60 spiralled clockwise, 57 counter-clockwise. Other axes arise on each plant as secondary and tertiary branches of the main stem and as rosette inflorescences. These do not necessarily adopt the same helical sense as the primary apex. Indeed, there is some tendency for them to spiral the other way. On the 60 plants whose main apex spiralled clockwise, 136 further apices spiralled clockwise but there were 183 spiralling counter-clockwise. On the 57 counter-clockwise plants, equivalent figures were 120 counter-clockwise but 135 clockwise. Overall, 256 apices adopted the same spiral sense as their main apex, whereas 318 were different, a significant departure from equality ($\chi^2 = 6.70$ with 1 *df*, $P < 0.01$).

DISCUSSION

Overall, the pattern of development of the *Arabidopsis* flower is similar to that described for other species in the family Brassicaceae, such as the wallflower *Cheiranthus cheiri* (Payer, 1857; Sattler, 1973) and oil seed rape *Brassica napus* (Polowick and Sawhney, 1986), in spite of the fact that *Arabidopsis* flowers are much smaller throughout all stages of development. For example, in *B. napus*, the early flower primordium is about 170 μm in diameter when sepals first appear (30 μm to 35 μm in *Arabidopsis*), and the mature bud is 5 mm long (1 mm in *Arabidopsis*). It will be of interest to determine whether this is a consequence of differences in the number of cells, their size, or both.

Organogenesis

There has been controversy about the relative time of appearance of floral organs and their location in whorls in

Figure 3. Individual Buds at Stages 5 to 8 with Sepals Dissected Away To Reveal Inner Organs.

(A) A bud at stage 5 in which a medial and lateral sepal have been removed. One of the small petal primordia (P) is indicated. The primordia of the long stamens (LS) are larger than that of the short stamen (SS).
(B) Lateral view of a bud at stage 6 in which the sepals had fully enclosed the bud. The stamen primordia are now dome shaped, whereas the petal primordia are still relatively small. The gynoecium (G) will arise from the central dome of cells.
(C) Medial view of a bud at stage 7 showing that the long stamen primordia are now constricted toward their base (arrow). The petal primordia (P) have become dome shaped.
(D) Vertical view of a stage-7 bud showing that the stamens do not yet show locule ridges on their adaxial surface. The gynoecium (G) is growing vertically as a slotted tube.
(E) A bud at stage 8 in which the stamen primordia have increased markedly in size, especially in relation to the petal primordia.
(F) Vertical view of a stage-8 bud in which locules (arrows) are now clearly visible in the stamens.
Bars = 10 μm.

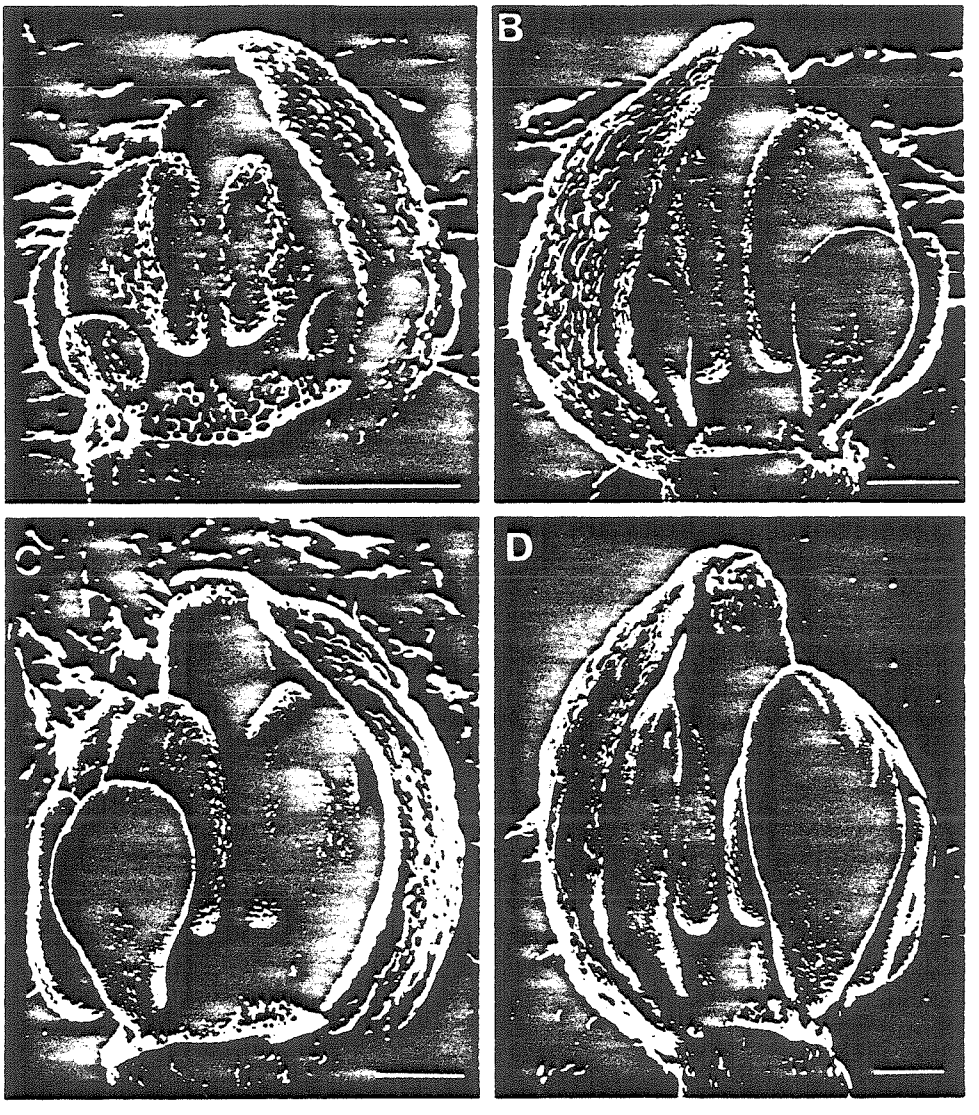

Figure 4. Lateral Views of Buds at Stages 9 to 12.

(A) A bud early in stage 9 showing that the petal primordia have become wider toward the top as they start growing rapidly.
(B) A bud in which the petals have just reached the height of the lateral (short) stamens, marking the beginning of stage 10. Buds more than double in size during the lengthy stage 9 [c.f. (A)].
(C) Stigmatic papillae (arrow) appear on the top of the gynoecium at the start of stage 11.
(D) Stage 12 is the final stage before the bud opens. It commences when the petals reach the height of the long stamens.
Bars = 100 μm.

the Brassicaceae flower. Whether all four sepals occupy one whorl or the two laterals occur in a separate whorl outside that of the abaxial and adaxial sepals has been in question. The order in which sepals arise does not help settle the matter. It is clear in all species examined that the abaxial sepal is the first to appear. In *Arabidopsis* we agree with Hill and Lord (1989) that the lateral sepals arise at around the same time as the adaxial sepal, although they are reported to arise earlier in *Cheiranthus* (Payer, 1857; Sattler, 1973) and *B. napus* (Polowick and Sawhney, 1986). What is established is that the laterals arise lower on the floral primordium (e.g., Figure 2B), and that their vasculature in the mature flower detaches below that of the medial sepals (Arber, 1931a).

Petals arise in one whorl in the Brassicaceae flower. Because their early development is relatively slow, there has been some doubt about whether they arise before or after the stamens. The petals have been reported to arise before the stamens in *C. cheiri* (Payer, 1857; Sattler, 1973) and after the stamens in *B. napus* (Polowick and Sawhney, 1986). In *Arabidopsis* we could not confidently separate their origin in time from that of the long stamens (see also Hill and Lord, 1989).

In *A. thaliana* and *B. napus* (Polowick and Sawhney, 1986) the four inner stamens arise a little earlier than the two outer ones, whereas in *Cheiranthus* the order reportedly is reversed (Payer, 1857; Sattler, 1973). In *Arabidopsis* we found that one of the outer stamens was absent in about one-quarter of the flowers examined. This has also been reported in three other races in which many flowers had only four stamens (Müller, 1961). The pattern of gynoecium development seems to be similar in *Arabidopsis* (the present study; Hill and Lord, 1989; Okada et al., 1989), *Cheiranthus* (Payer, 1857; Sattler, 1973), and *B. napus* (Polowick and Sawhney, 1986). The major difference lies in the stigma, which is markedly bilobed only in the latter two species.

Finally, our observations on nectaries, which concur with those of Müller (1961), show that they are variable in presence, size, and shape. They arise late in flower development (stage 9), and it may be that their growth is limited by available space and nutrients.

Origin of Flowers

The apical meristem of *Arabidopsis* changes from slightly convex to distinctly dome shaped during the transition from vegetative to floral growth (Vaughan, 1955; Miksche and Brown, 1965). Flower primordia then arise on the flank of the apical meristem by periclinal divisions in cells beneath the two-layered tunica. Under continuous light at 25°C, stage-3 buds were observed here after 14 days' incubation. Thus, the first bud is likely to have arisen at around 12 days in our plants, several days later than that observed by Miksche and Brown (1965) on Estland plants in soil-less culture. We estimated that an average of 1.9 buds arose per day on the main apex of 20 plants in this study. The only other report from *Arabidopsis* is for three plants of race Dijon grown at 20°C that produced buds at a slightly faster rate—2.3 buds per day (Müller, 1961). It is likely that these rates are influenced to a large degree by environmental factors.

The presence of stipules at the base of young cauline leaves in *Arabidopsis* (Figures 6B and 6C) is of interest. Payer (1857) was unable to see stipules in any species of the Brassicaceae, but Arber (1931b) reported well-developed stipules on young leaves of *Nasturtium officinale*. We have reported earlier that the presence of stipules on outer-whorl organs of *apetala2-1* flowers was evidence of their leaf-like structure (Bowman et al., 1989). Arber (1931a, 1931b) also commented on the presence of filamentous outgrowths at the base of some leaves and pedicels in a range of Brassicaceae species. She called these "squamules," and distinguished them from stipules by their smaller diameter and their presence at the base of pedicels as well as leaves. We were unable to see such organs on any leaves or buds of wild-type *Arabidopsis*, although we have seen them in various mutants, including *apetala1*. They may well be the "filamentous sepals" that Kunst et al. (1989) record between the outer floral organs of *apetala2-7* mutants and that Komaki et al. (1988) report in mutant FI-54.

The direction of flower production on any one main apex of *Arabidopsis* is apparently decided at random and is maintained unchanged. In this regard it resembles the pattern of phyllotaxis in many species including, for example, *Pharbitis nil* (Imai, 1927), *Nicotiana tabacum*, and *N. rustica* (Allard, 1946). It does seem in *A. thaliana* that the spiral direction taken by secondary and tertiary branches is somewhat influenced by the spiral pattern of the primary stem. We do not know whether this is the case in other plant species.

Conclusion

Our definition of 12 stages in early flower development in *Arabidopsis* will be useful in interpreting the action of genes that control this process. Already we have indirect evidence that the product of the *APETALA2* gene is active during stages 2 to 4 (Bowman et al., 1989). Sepals, which are affected by the *apetala2-1* mutation, arise during this time. In the case of *APETALA3*, the gene product is apparently active later when petals and stamens, the organs affected by mutations in this gene, are differentiating (Bowman et al., 1989). The *pistillata* mutation also affects petals and stamens, and it is clear that in this case "petal" primordia arise as normal, but they differentiate with sepal-like properties under a petal timetable (Bowman et al.,

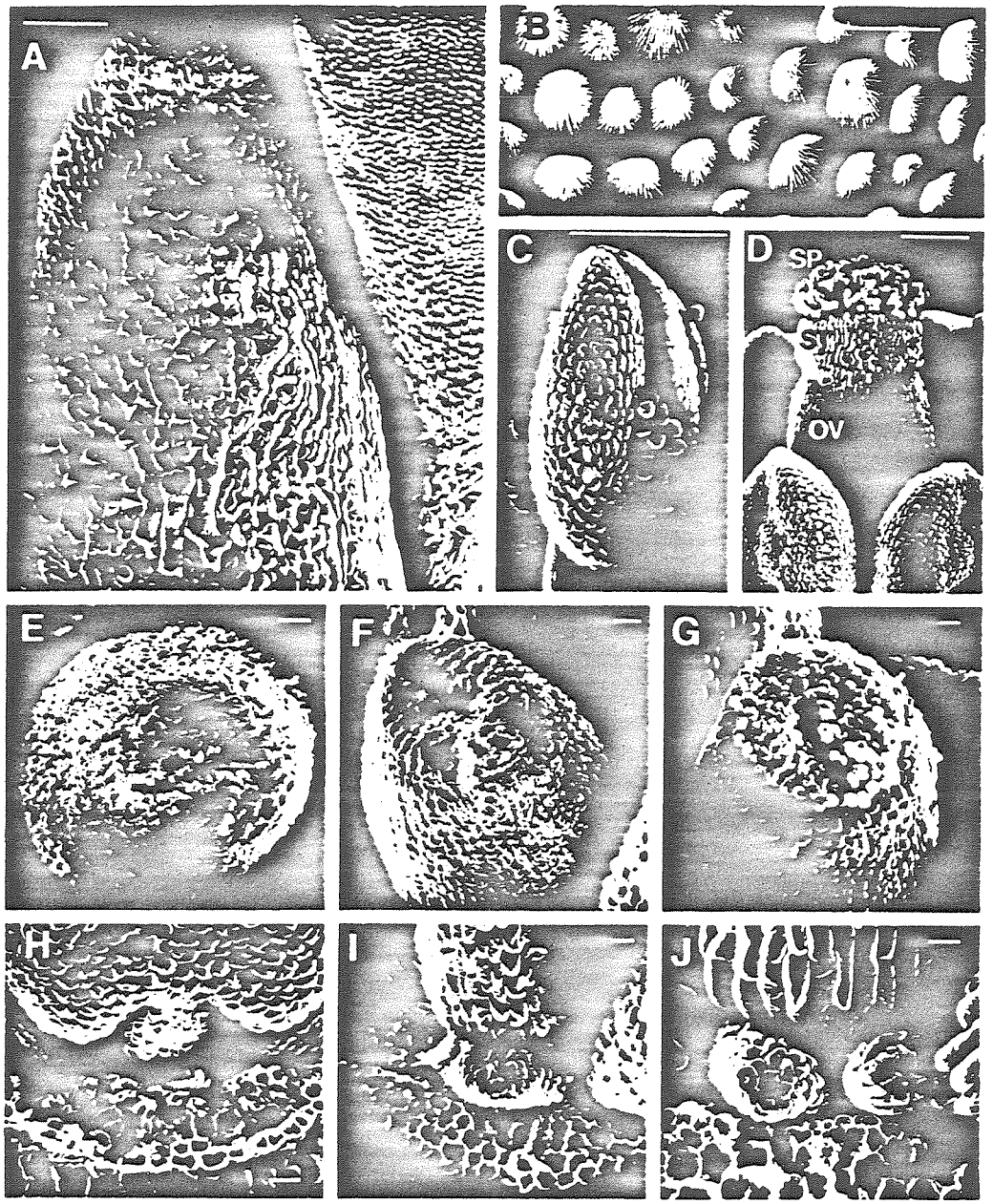

Figure 5. Surface Morphology of Mature and Developing Floral Organs.

(A) Outer surface of a mature sepal (left) showing several long epidermal cells (arrow), stomata, and a fringe of smaller cells. The lower part of the adjacent petal (right) shows a regular transition from longer cells in the claw to more ovoid cells in the blade. Bar = 100 μm.
(B) Higher magnification of the ridged cells that cover the surface of the blade of mature petals. Bar = 10 μm.
(C) A mature stamen after dehiscence showing epidermal cells of uniform size and pollen grains. Bar = 100 μm.

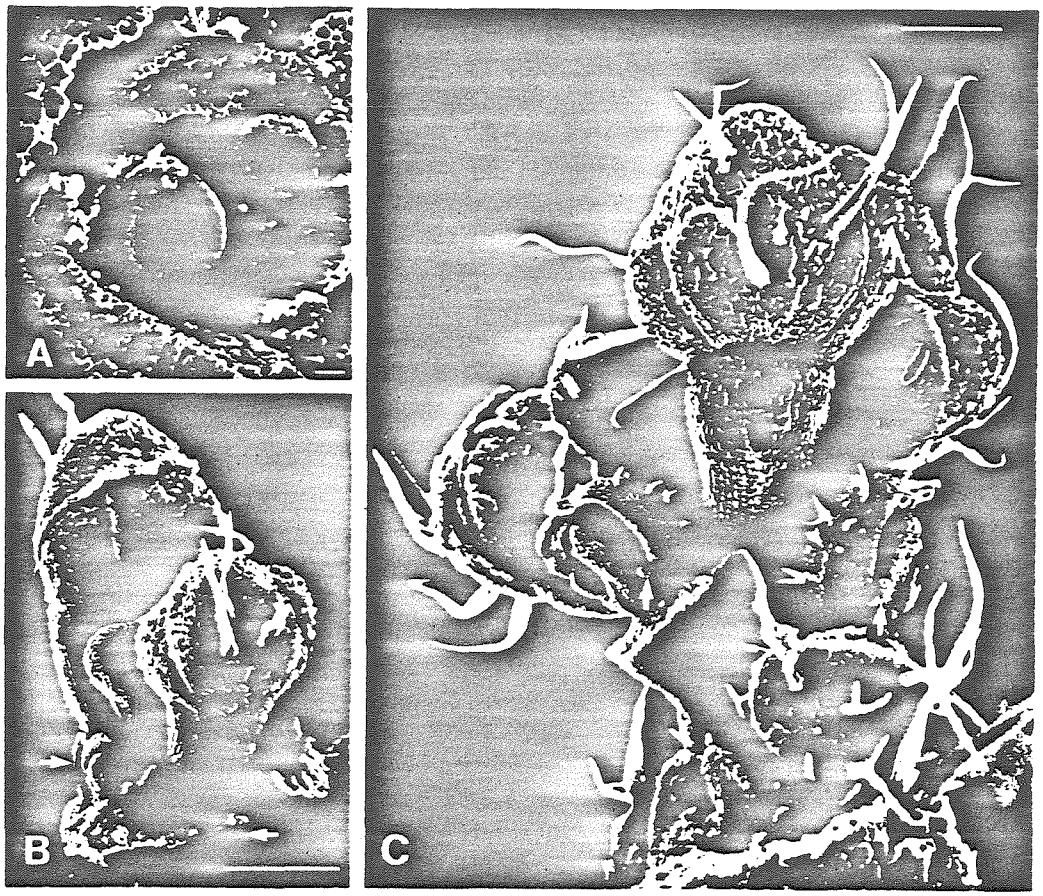

Figure 6. Appearance of Immature Inflorescences on Plants at the Commencement of Flowering.

Rosette and cauline leaves have been removed.

(A) Vertical view of the main apex of a 14-day-old plant showing that the oldest bud has advanced to stage 3. Bar = 10 μm.

(B) A 16-day-old-plant with four buds beyond stage 5 (sepals closed) on the main apex. Two cauline leaves have been removed from the main stem although their stipules remain (arrows). Secondary apices within these cauline leaves have also initiated flower development. These apices are flanked in turn by younger cauline leaves, and their oldest flower primordium is only at stage 2. Bar = 100 μm.

(C) Lateral view of an 18-day-old plant just beginning to bolt. The two secondary apices visible have advanced in concert, with buds reaching stage 5 on each. Their cauline leaves show developing trichomes and stipules (arrows). A new apex is just visible in the axis of one of the dissected rosette leaves (lower left) although flower development is not yet detectable under its cauline leaf primordia. Bar = 100 μm.

Figure 5. (continued).

(D) The upper parts of a gynoecium in a stage-12 bud showing stigmatic papillae (SP), large cells and stomata on the short style (ST), and smaller epidermal cells on the surface of the ovaries (OV). Bar = 100 μm.

(E) to (G) Structure of the developing stigma. The surface of the gynoecium is smooth and slotted at the end of stage 9 (E). A cap becomes apparent at stage 10 when the surface is still not closed (F). The appearance of papillae covering the stigmatic surface defines the start of stage 11 (G). Bars = 10 μm.

(H) to (J) Structure of developing nectaries (lateral sepal removed). These first appear at the base of the lateral stamens in stage 9 (H) and grow outward during stages 10 and 11 (I). They reach maturity when the flower opens and reaches stage 14 (J). Stomata often occur at their apex. The righthand nectary in (J) has shrunken, probably because it has already released its nectar. Bars = 10 μm.

1989; Hill and Lord, 1989). The description of the surface characteristics of normal organs (the present study; Bowman et al., 1989; Hill and Lord, 1989; Kunst et al., 1989) also allows the effects of mutational changes, including the nature of mosaic structures, to be inferred.

When these and other genes are cloned, the site and time of their expression can be determined directly. In this way we may ultimately understand how gene activity directs flower development.

METHODS

All plants were of the Landsberg ecotype and were homozygous for the *erecta* mutation, which reduces plant height. Seeds were planted on the surface of a mixture of 3 volumes of commercial potting mix, 3 volumes of peat, and one of sand, spaced at about 4 cm^2 per plant. They were treated at 4°C for 2 days and then transferred to a growth chamber. The age of a plant was recorded from the time of this transfer. Conditions were held at 25°C and 70% relative humidity, and plants were grown under continuous light provided by cool-white fluorescent tubes.

For scanning electron microscopy, whole inflorescences were fixed in 3% glutaraldehyde in 0.025 M sodium phosphate buffer (pH 6.8) at 4°C for at least 12 hr. They were then rinsed in the buffer and further fixed in 1% OsO_4 in 0.05 M sodium cacodylate buffer (pH 7.0) for several hours before dehydration through a graded ethanol series. Inflorescences were then critical point dried using CO_2. Whole inflorescences were mounted on stubs and shadowed with gold and palladium (4:1) before viewing with an ETEC Autoscan scanning electron microscope. In many cases individual buds were then dissected from the inflorescence, outer organs removed using glass needles, and the buds examined again after further shadowing. Photographs were taken on Kodak type 4127 film.

ACKNOWLEDGMENTS

We thank our fellow workers for discussions and interest and Pat Koen of the Electron Microscopy Facility at Caltech for technical advice. This study was assisted by National Science Foundation grant PCM-8703439 to E.M.M. J.L.B. is supported by training grant 5T32-GM07616 of the National Institutes of Health.

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Table 2. Numbers of Flower Primordia at Stages 1 to 5 on the Main Apex of Four Inflorescences. Four Plants Were Sampled Every Other Day from 18 to 26 Days after Incubation at 25°C

	No. of Flower Primordia at Stages Shown							
Age (days)	1	2	3	4	5	Total (1–5)	6–12	13–15
18	9	9	5	5	3	31	24	0
20	8	10	6	6	1	31	42	0
22	8	11	6	5	4	34	67	1
24	9	8	5	5	3	30	68	2
26	7	8	6	6	3	30	61	10
Total	41	46	28	27	14	156		
Mean/apex	2.05	2.30	1.40	1.35	0.70	7.80		

Table 3. Cumulative Totals of Flowers per Plant on 20 Plants Scored Each Day from 25 to 31 Days after Incubation at 25°C

	Mean No. of Flowers per Plant at Stages Shown			Mean No. of Flowers Advancing	
Age (days)	6–12	13–17	Total	Into Stage 6	Out of Stage 12
25	15.9	0.5	16.4	–	–
26	16.0	2.2	18.1	1.7	1.7
27	16.7	3.8	20.5	2.4	1.6
28	16.9	5.3	22.2	1.7	1.5
29	16.7	7.4	24.1	1.9	2.1
30	16.2	8.9	25.1	1.0	1.5
31	15.6	10.6	26.2	1.1	1.7

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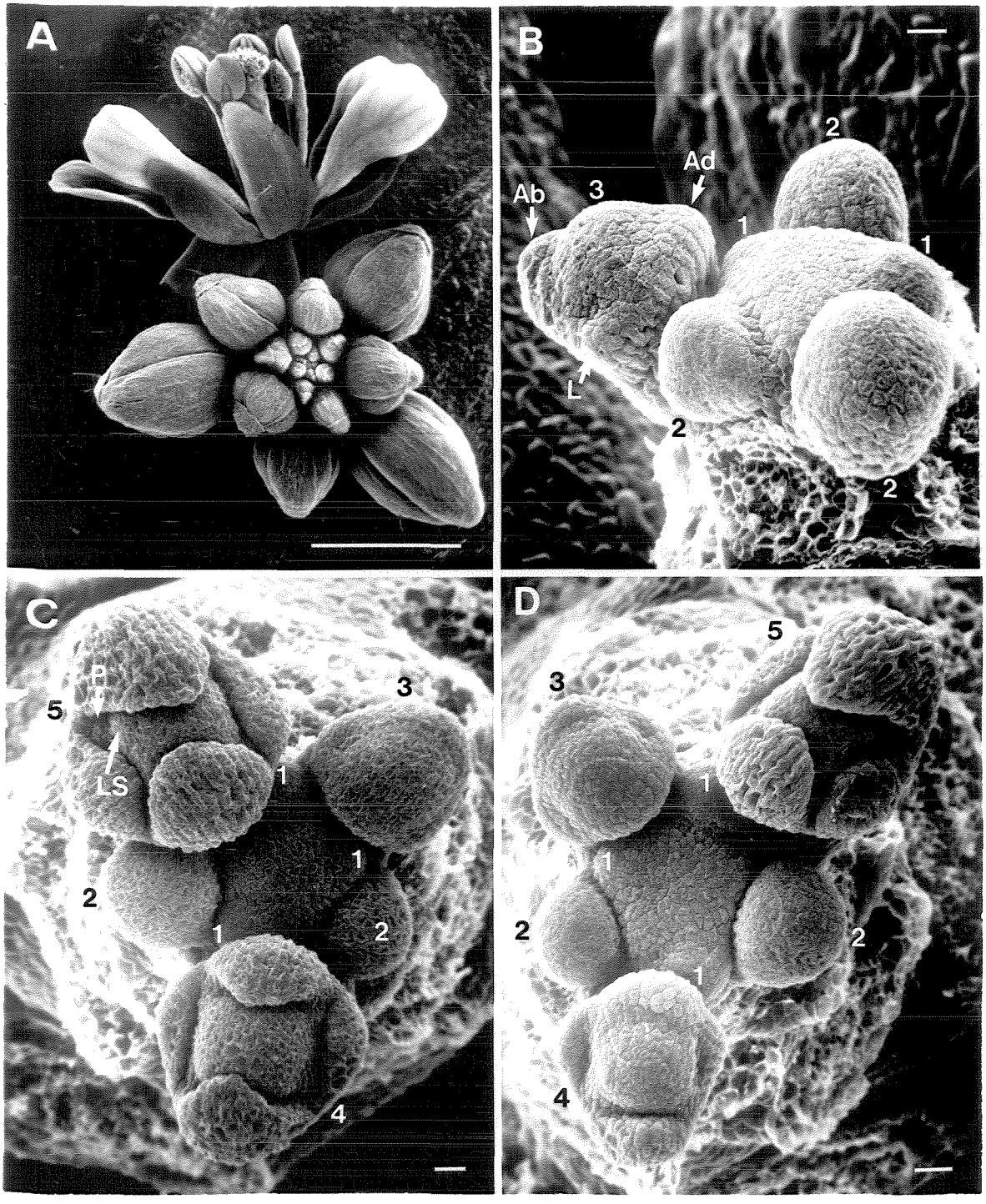
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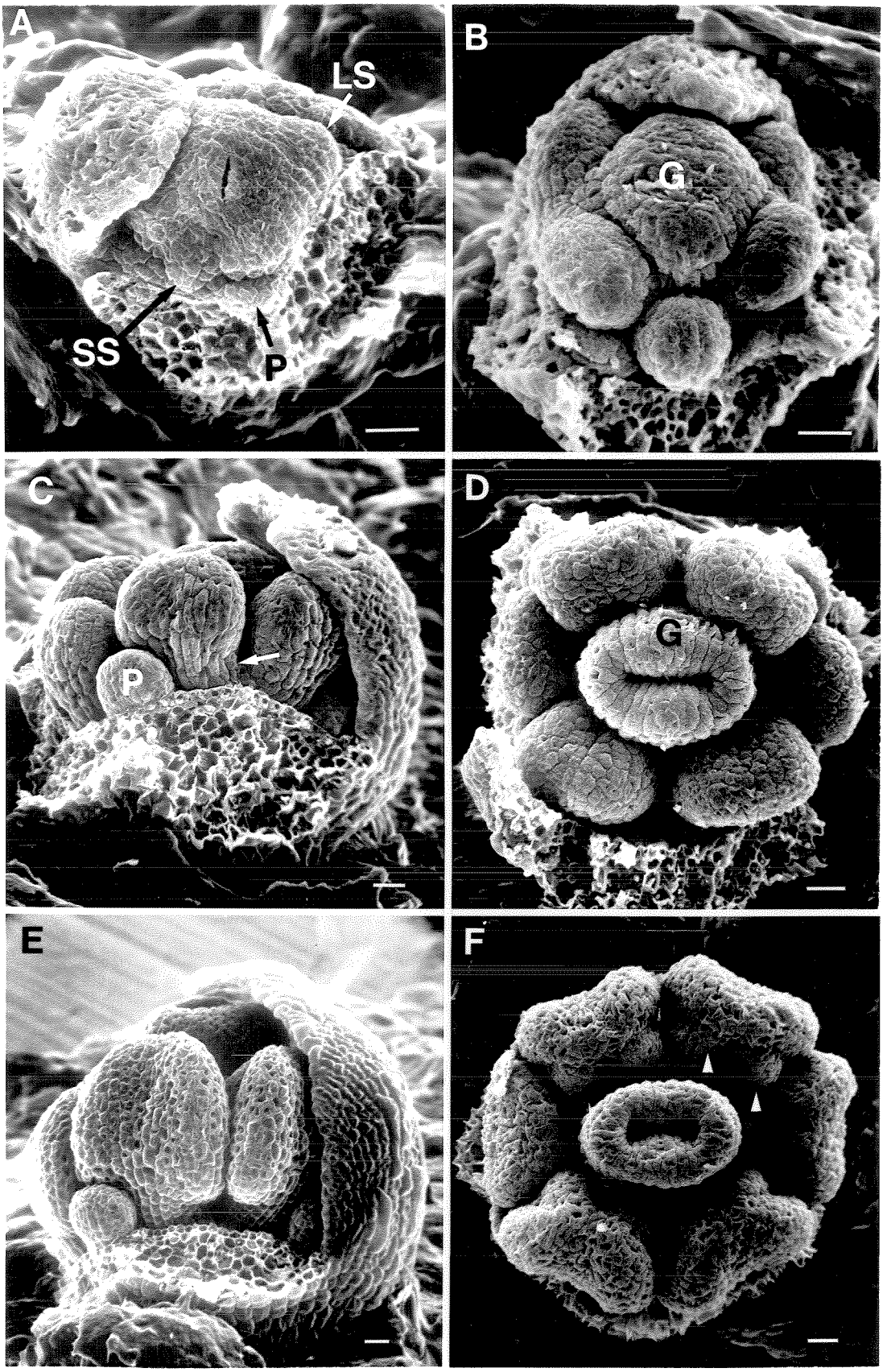
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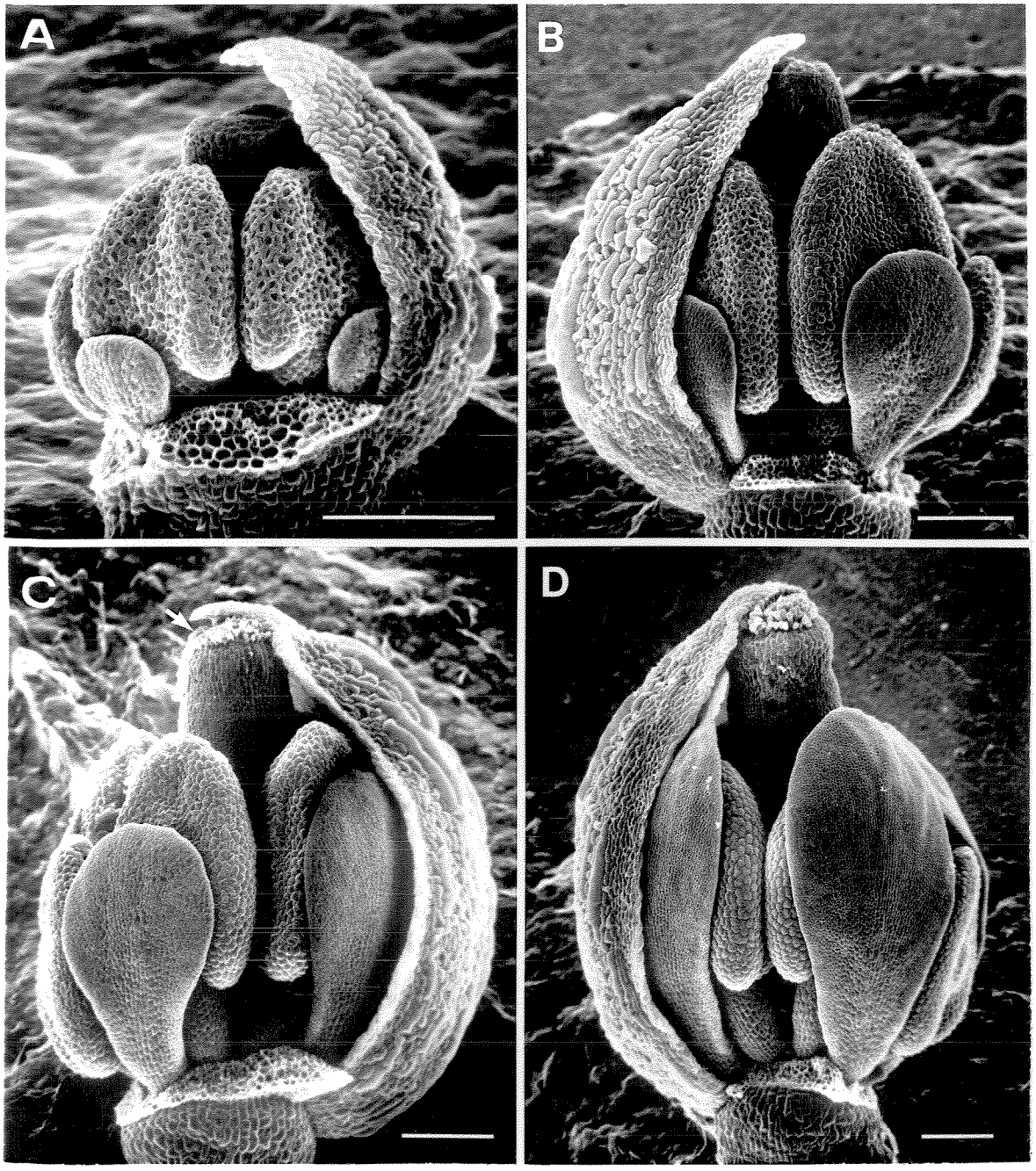
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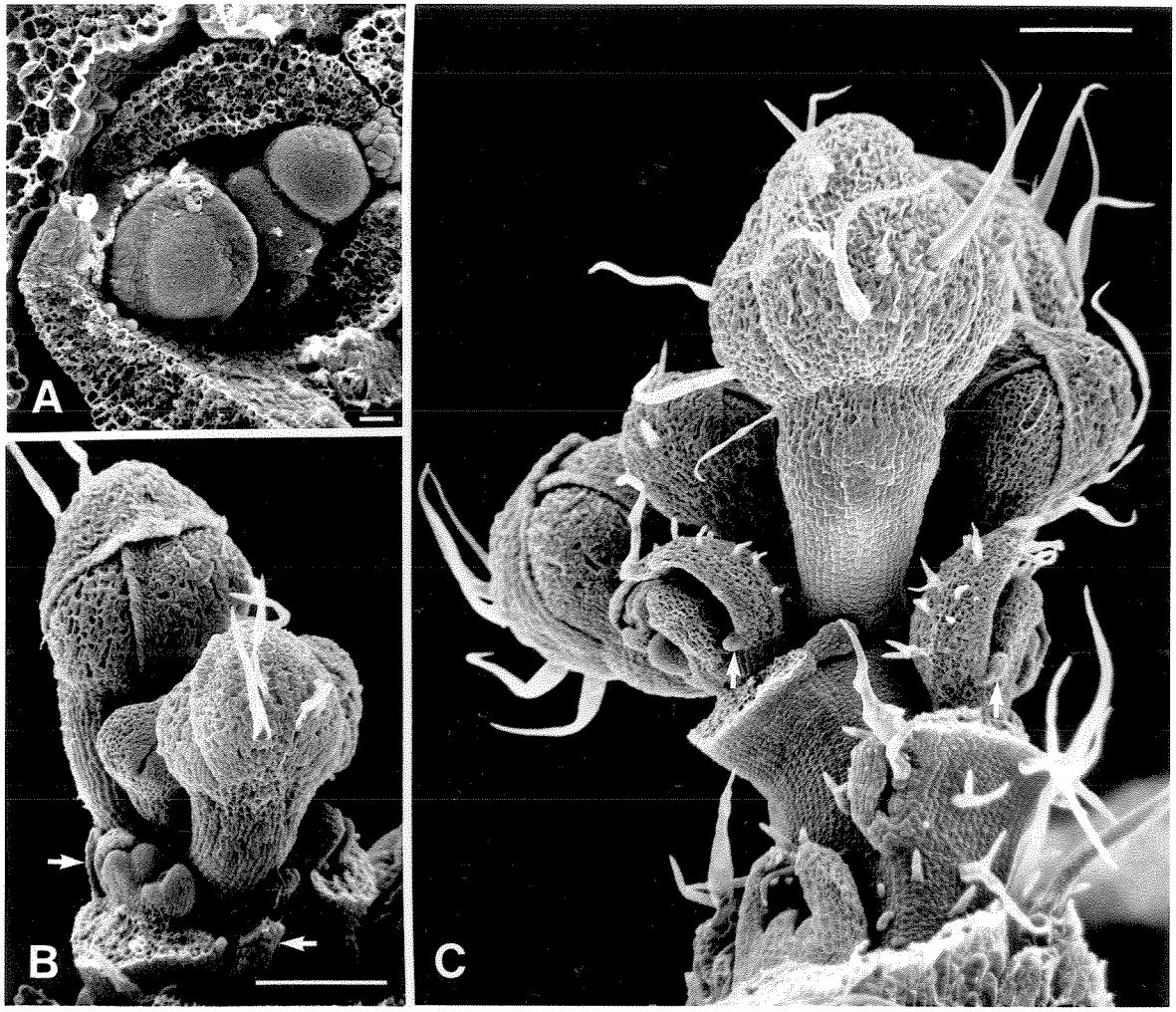
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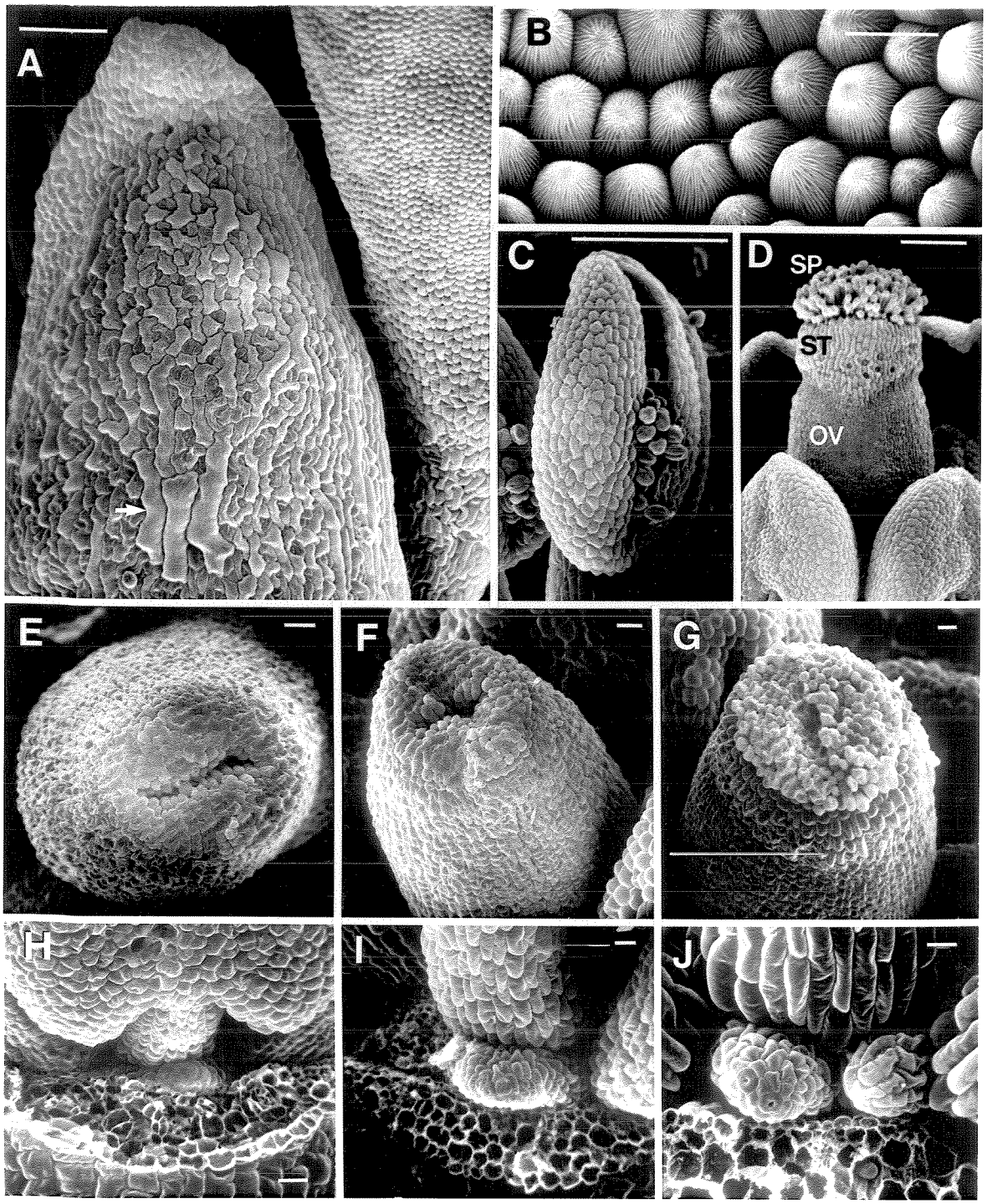
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CHAPTER TWO

GENES DIRECTING FLOWER DEVELOPMENT IN *ARABIDOPSIS*

John L. Bowman, David R. Smyth, and Elliot M. Meyerowitz

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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).

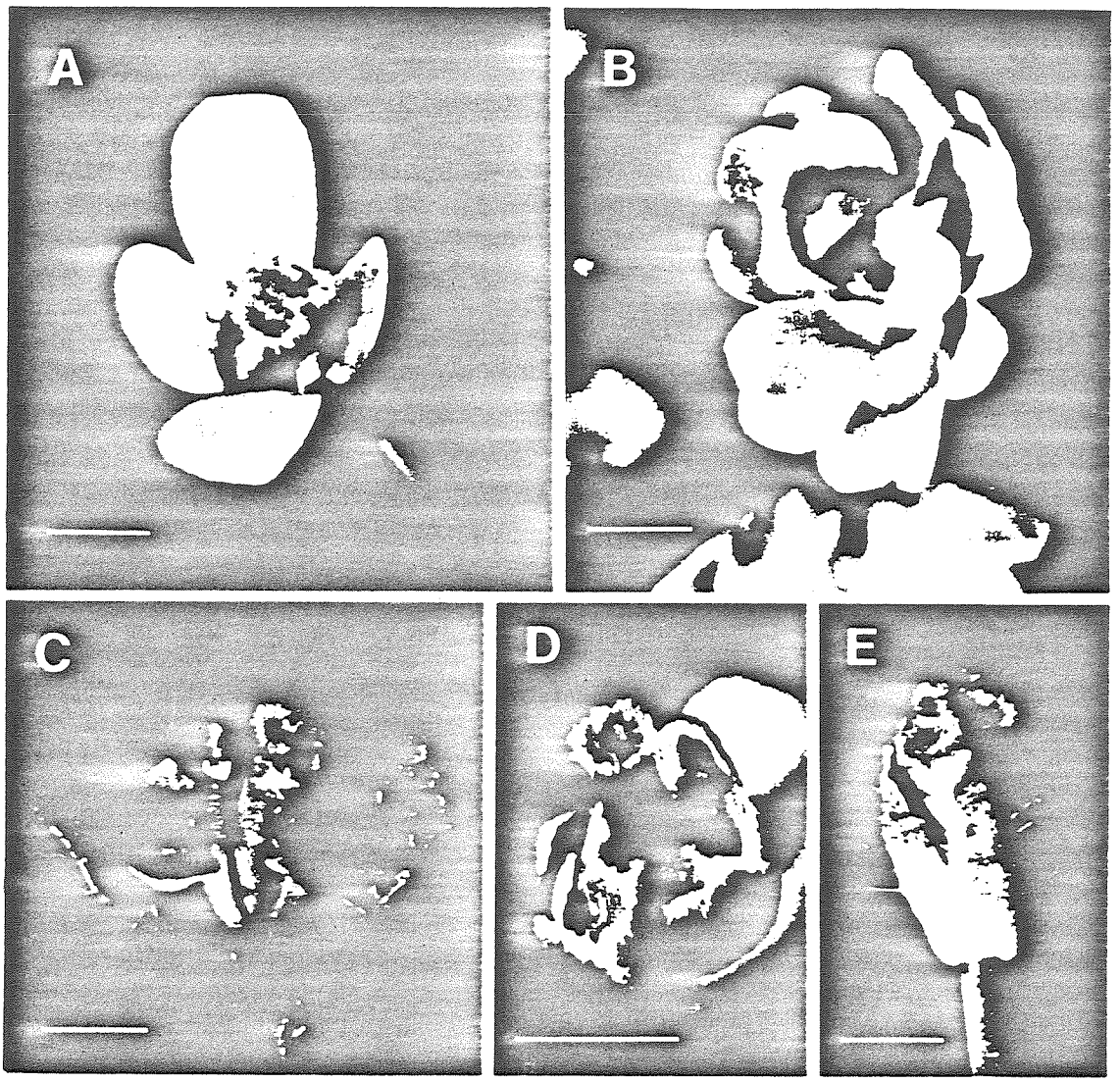

Figure 1. Phenotypes of Wild-Type and Mutant *A. thaliana* Flowers.

(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-

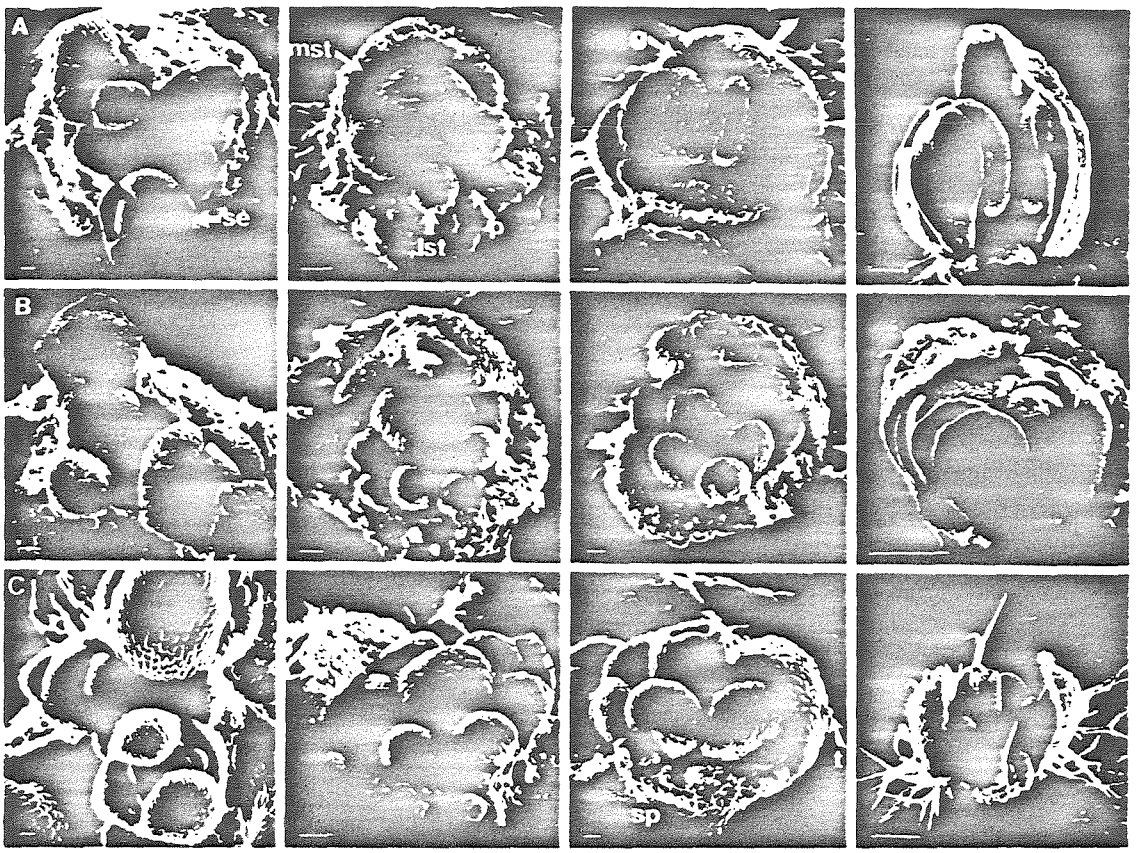

Figure 2. SEM Micrographs of Early Flower Development in Wild-Type and Mutant *Arabidopsis* Plants Grown at 25°C.

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

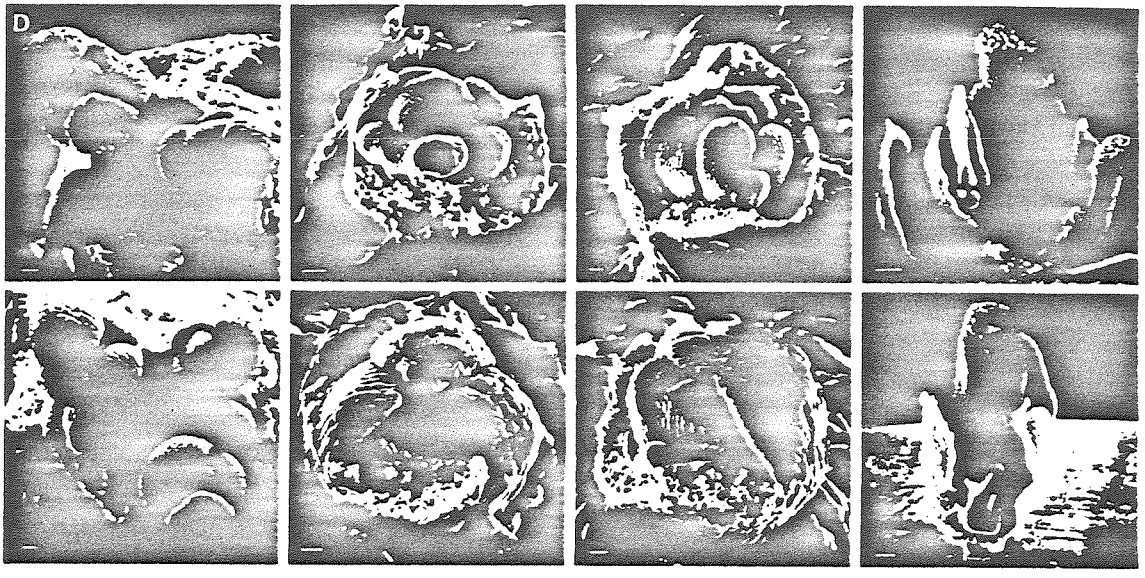

Figure 2 (continued).

(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.

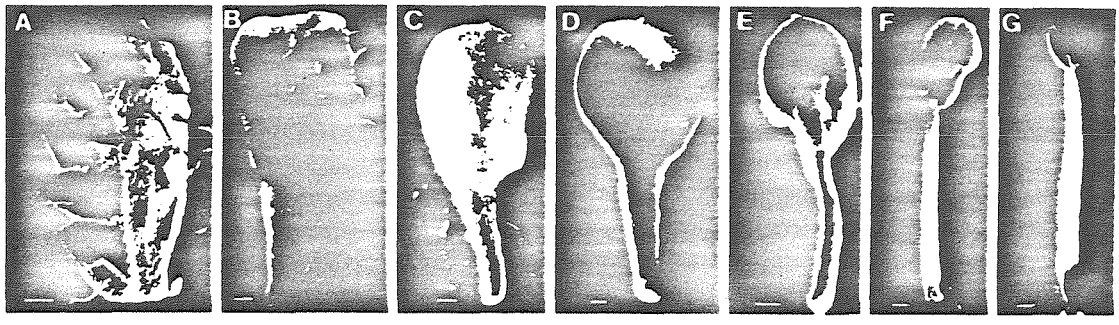

Figure 4. SEM Micrographs of Organs Observed in the Second Whorl of *apetala2* Flowers.

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	46			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	18	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.

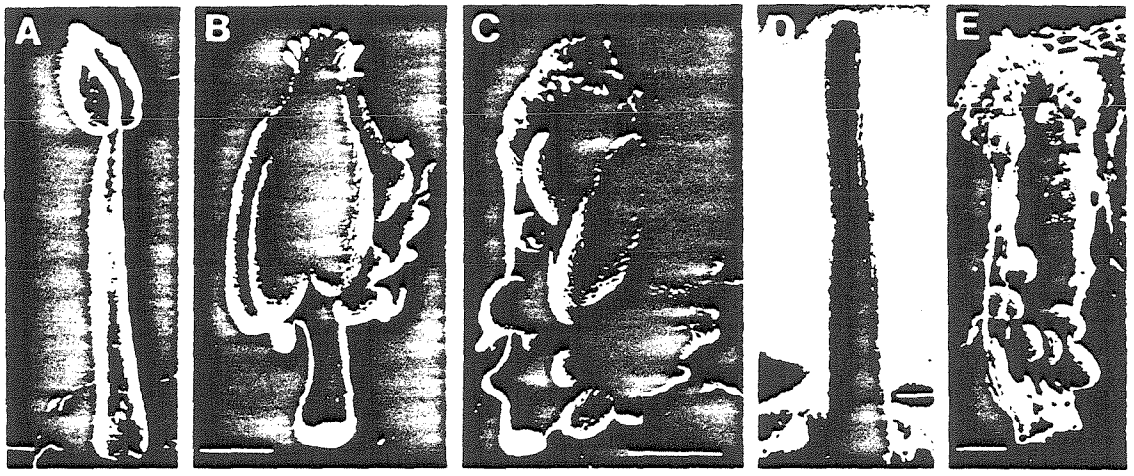

Figure 7. SEM Micrographs of the Inner Surface of Organs Observed in the Third Whorl of *apetala3* Flowers.

(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

(A) *apetala3* 16°C to 29°C						
absent	9	2	2			
carpel	10	5	13			
filament	5	6				
staminoid carpel		1	1	4		
carpelloid stamen					10	
deformed stamen					6	
stamen						36
stage of flower at time of shift	m/b/p (1,2)	se (3,4)	se+ (4,5)	1st bud (6)	2nd, 3rd buds (7,8?)	older buds (6+)

(B) *apetala3* 29°C to 16°C						
absent			2	2	1	
carpel			1		2	16
filament						1
staminoid carpel					2	15
carpelloid stamen	2				4	18
deformed stamen					3	1
stamen	74	36	21	18	4	
stage of flower at time of shift	m/b (1)	p (2)	se (3,4)	se+ (4,5)	1st bud (6)	bud (6+)

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.

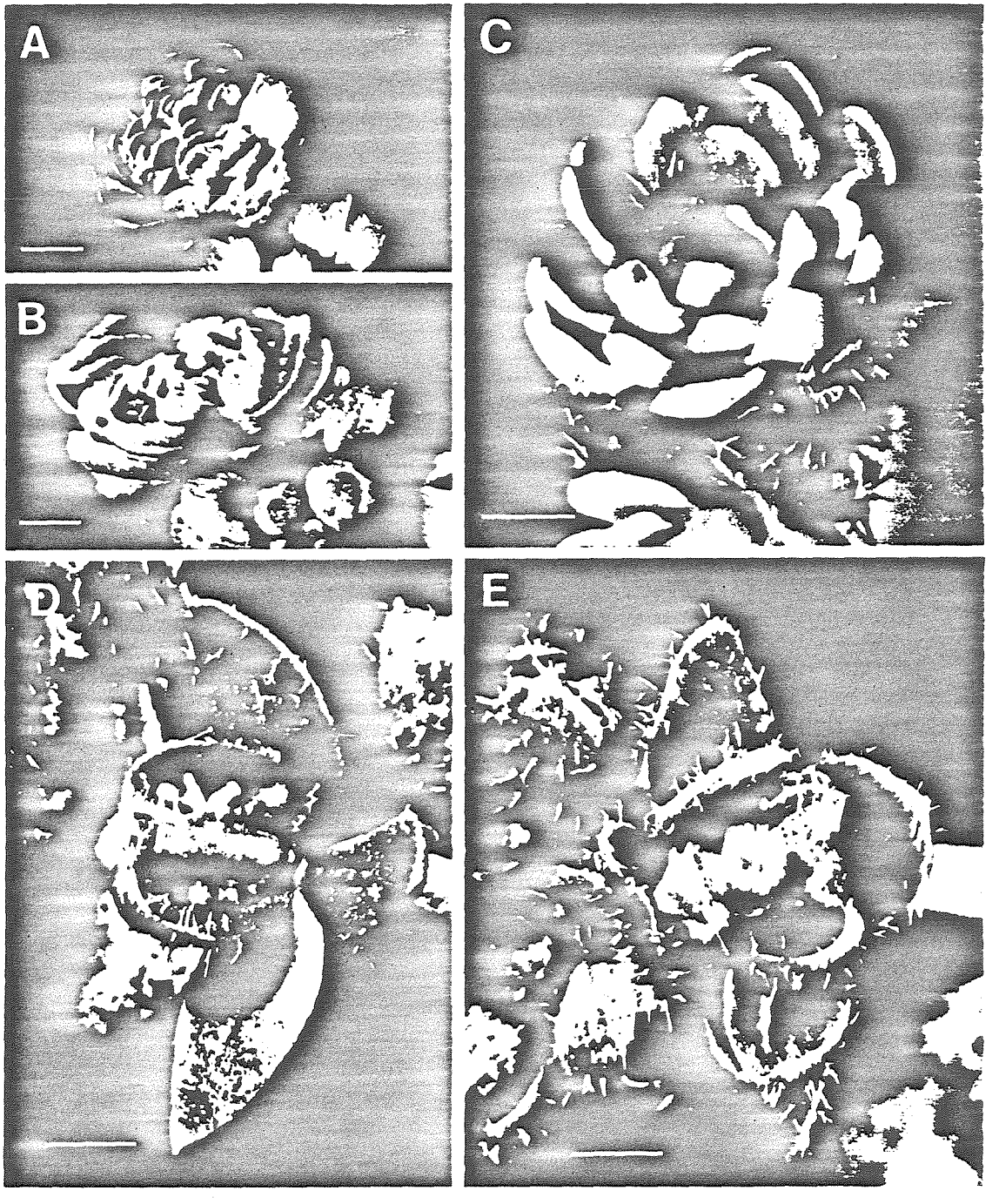

Figure 9. Phenotypes of Double Mutant Combinations Grown at 25°C.

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

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 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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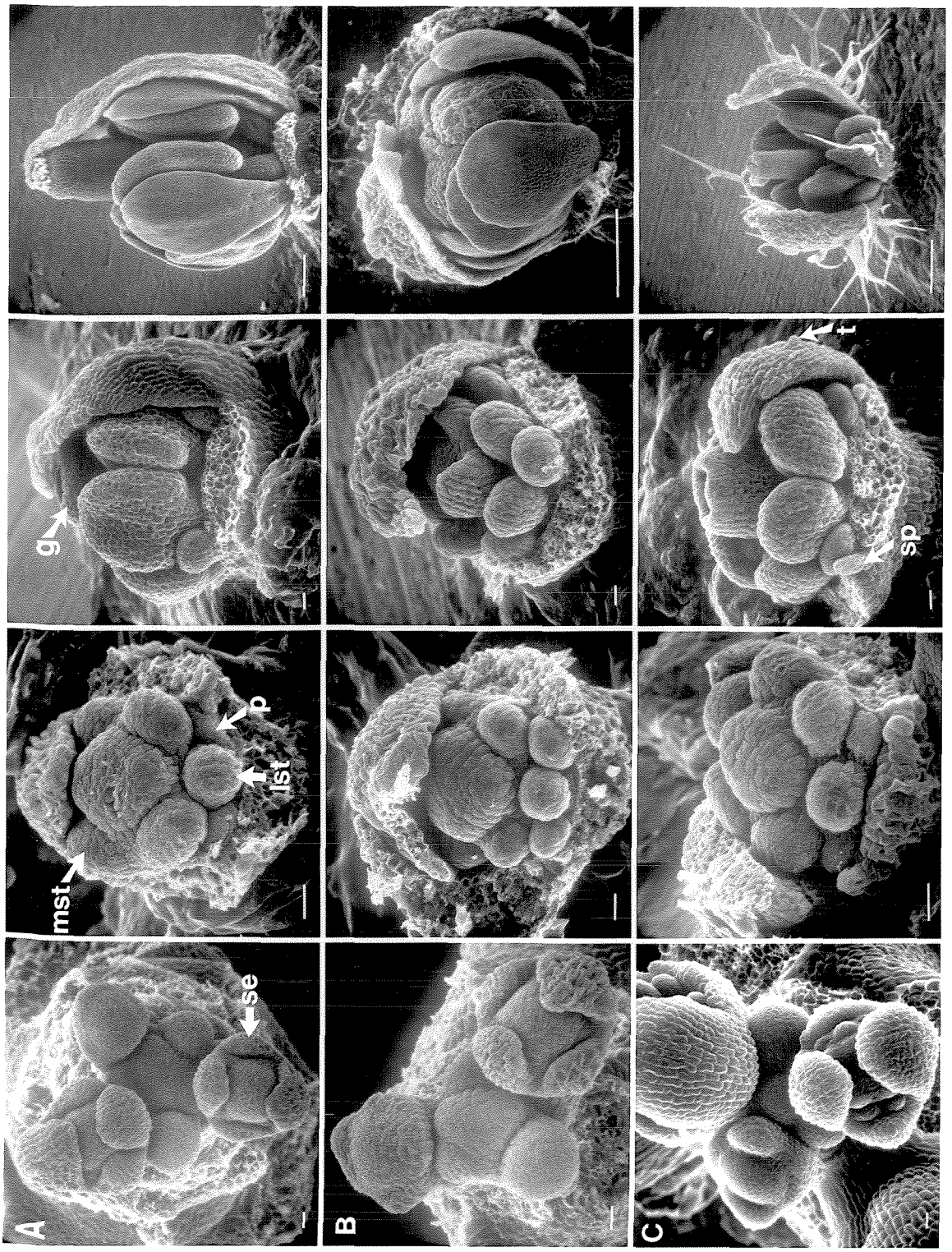
NOTE ADDED IN PROOF

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).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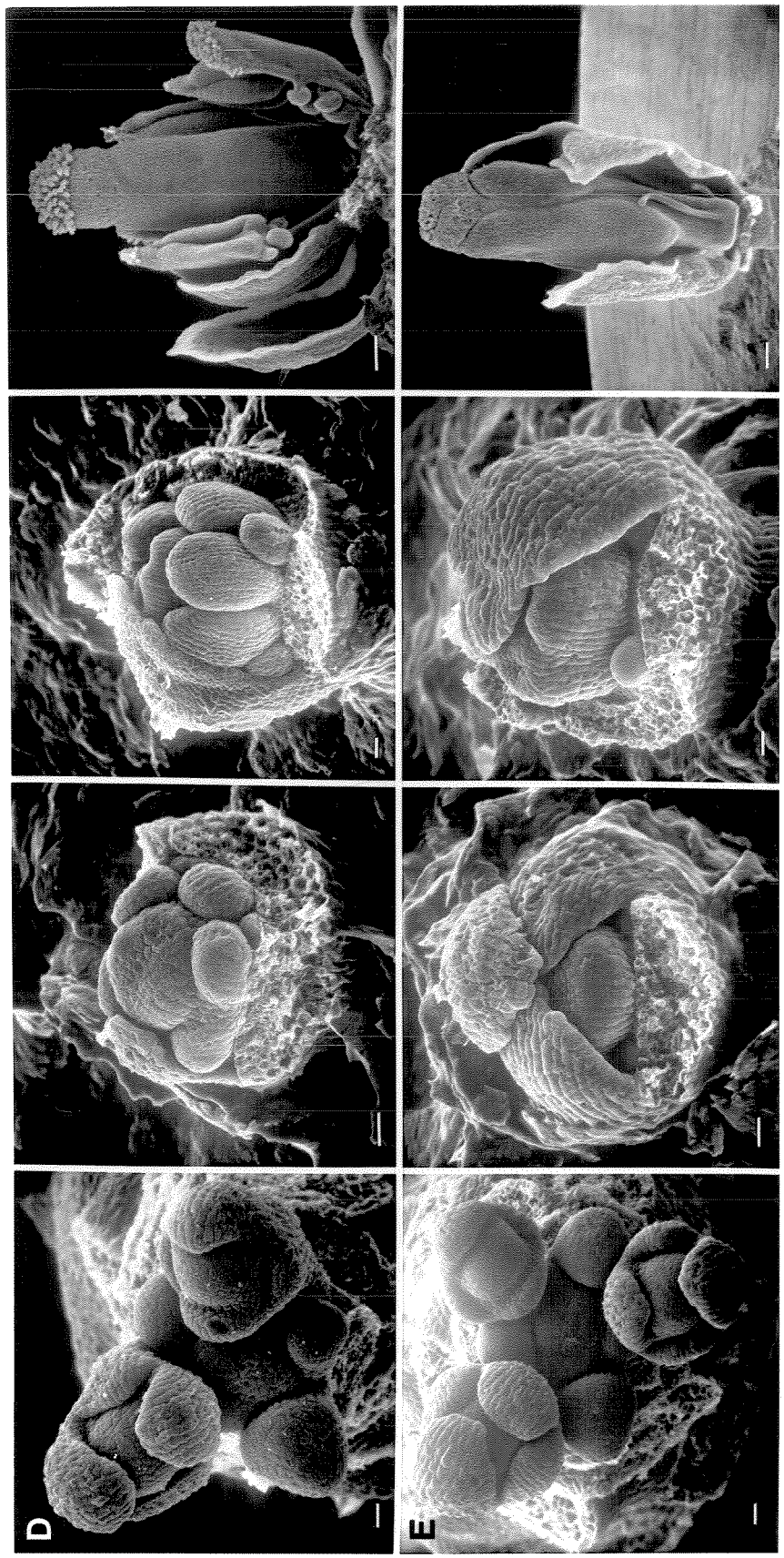
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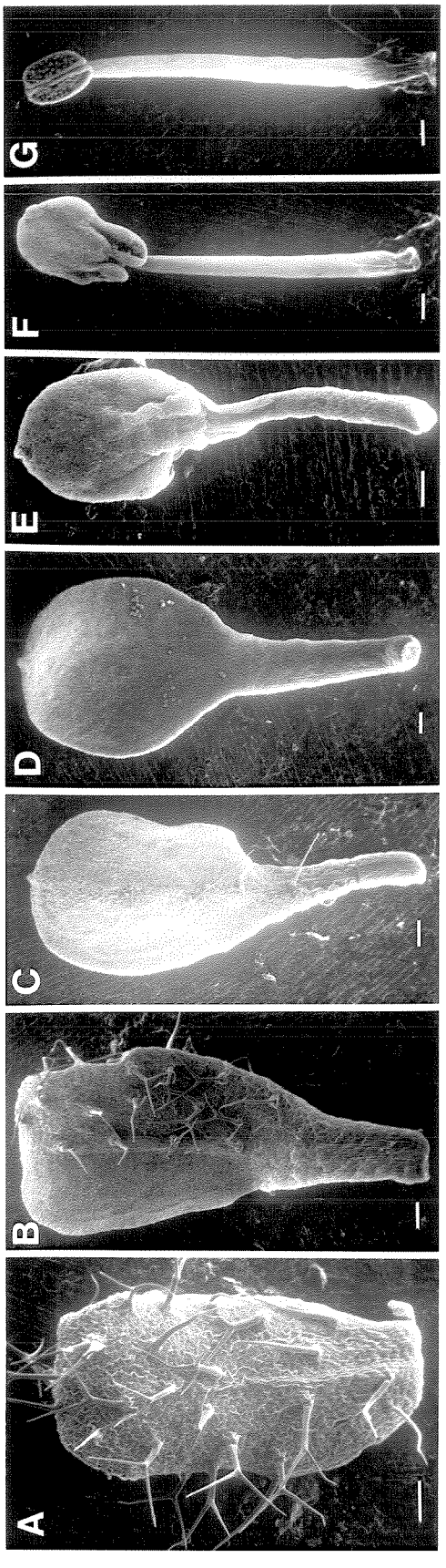
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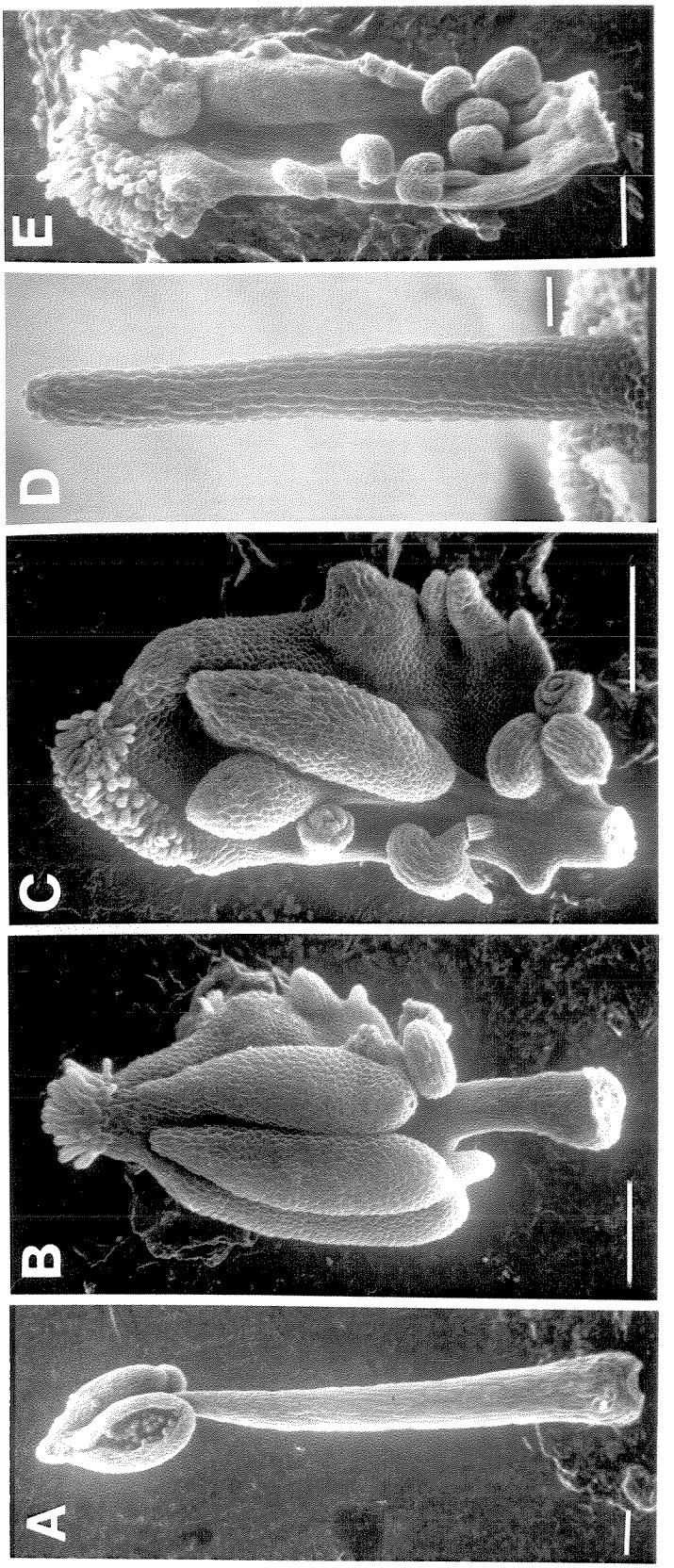
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CHAPTER THREE

GENETIC INTERACTIONS AMONG FLORAL HOMEOTIC GENES OF *ARABIDOPSIS*

John L. Bowman, David R. Smyth, and Elliot M. Meyerowitz

(*Development* **112**, in press, May 1991)

SUMMARY

We describe allelic series for three loci, mutations in which result in homeotic conversions in two adjacent whorls in the *Arabidopsis thaliana* flower. Both the structure of the mature flower and its development from the initial primordium are described by scanning electron microscopy. New mutations at the *APETALA2* locus, *ap2-2*, *ap2-8* and *ap2-9*, cause homeotic conversions in the outer two whorls: sepals to carpels (or leaves) and petals to stamens. Two new mutations of *PISTILLATA*, *pi-2* and *pi-3*, cause second and third whorl organs to differentiate incorrectly. Homeotic conversions are petals to sepals and stamens to carpels, a pattern similar to that previously described for the *apetala3-1* mutation. The *AGAMOUS* mutations, *ag-2* and *ag-3*, affect the third and fourth whorls and cause petals to develop instead of stamens and another flower to arise in place of the gynoecium. In addition to homeotic changes, mutations at the *APETALA2*, *APETALA3* and *PISTILLATA* loci may lead to reduced numbers of organs, or even their absence, in specific whorls. The bud and flower phenotypes of doubly and triply mutant strains, constructed with these and previously described alleles, are also described. Based on these results, a model is proposed that suggests that the products of these homeotic genes are each active in fields occupying two adjacent whorls, *AP2* in the two outer whorls, *PI* and *AP3* in whorls two and three, and *AG* in the two inner whorls. In combination, therefore, the gene products in these three concentric, overlapping fields specify the four types of organs in the wild-type flower. Further, the phenotypes of multiple mutant lines indicate that the wild-type products of the *AGAMOUS* and *APETALA2* genes interact antagonistically. *AP2* seems to keep the *AG* gene inactive in the two outer whorls while the converse is likely in the two inner whorls. This field model successfully predicts the phenotypes of all the singly, doubly and triply mutant flowers described.

INTRODUCTION

Flowers of *Arabidopsis thaliana* originate as small outgrowths of cells on the flanks of the florally induced shoot apical meristem. These cells divide and differentiate, eventually producing a flower with a precisely defined pattern of four types of floral organs, with each type found in one of the four concentric whorls of the wild-type flower (Bowman et al., 1989; Smyth et al., 1990). During the developmental process, the cells in each flower primordium must in some way assess their positions, either globally or in reference to nearby cells, and they or their descendents must subsequently differentiate to the appropriate cell types. As an approach to finding the molecular mechanisms by which cells in developing flowers recognize and realize their fates, we study genes whose products are necessary for proper pattern formation in *Arabidopsis* flowers.

Several such genes have been described (Pruitt et al., 1987; Meyerowitz, 1987; Bowman et al., 1988, 1989; Komaki et al., 1988; Haughn and Somerville, 1988; Hill and Lord, 1989; Meyerowitz et al., 1989; Kunst et al., 1989; Yanofsky et al., 1990). The best-studied of them are a group of four genes whose mutant phenotypes include homeotic conversions of floral organs. These are the *AGAMOUS* (*AG*) gene, mutants of which have petals where stamens are found in wild type (the third whorl), and internal flowers in the place of the ovary (the fourth whorl in wild type); the *APETALA2* (*AP2*) gene, different mutant alleles of which cause different conversions in the outer two of the four whorls of the flower; and the *APETALA3* (*AP3*) and *PISTILLATA* (*PI*) genes. Mutations in the *AP3* and *PI* genes cause sepals to develop in the positions occupied by petals in wild-type flowers (the second whorl), and have variable effects in the third (in wild type, stamen) whorl. To date, there are published descriptions of two mutant *ag* alleles, *ag-1* (Pruitt et al, 1987; Bowman et al., 1988; Bowman et al., 1989) and *ag-2* (Yanofsky et al., 1990), seven mutant *ap2* alleles that show a broad range of related phenotypes (*ap2-1* through *ap2-7*: Pruitt et al., 1987; Bowman et al., 1988, 1989; Komaki et al., 1988;

Meyerowitz et al., 1989; Kunst et al., 1989), and one mutant allele each of *ap3* (Bowman et al., 1989) and *pi* (Bowman et al., 1989; Hill and Lord, 1989).

Given the range of phenotypes seen in *ap2* mutants, in which first whorl organs can be leaves or carpels, second whorl organs petaloid stamens, stamens, or absent, and third whorl organs normal or absent, it seemed worthwhile to extend the allelic series for each of the homeotic loci. In this paper, we report the phenotypes of one new *ag* allele, three alleles of *ap2*, each with phenotypes different from those described before; and two new alleles of *pi*. We also describe the mature phenotype and early development of a range of doubly and triply mutant combinations made with these new and previously described alleles.

These new data, along with those already published, have led us to a general and testable model of organ specification in *Arabidopsis* flowers. This model details the roles of the wild-type products of each of the homeotic genes in specifying organ identity in different regions of the developing flower. These data also provide new information on the roles of the wild-type products of the homeotic genes in establishing organ number and pattern in the flower.

MATERIALS AND METHODS

The mutant alleles studied are listed in Table 1. All are recessive. The new mutations are in the Landsberg ecotype, homozygous for the *erecta* mutation, and were generated by mutagenesis of seeds with ethylmethane sulfonate (EMS). *ag-1*, *ap2-1*, *pi-1*, and *ap3-1* were obtained from Maarten Koornneef (Department of Genetics,Wageningen Agricultural University, The Netherlands). 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. Doubly and triply mutant strains were constructed by manual cross-pollination, using as parents strains

homozygous for individual mutations, except in the strains involving *agamous* alleles, which are sterile when homozygous, thus necessitating the use of heterozygotes as parents. The resulting F_1 plants were allowed to self-pollinate, and double and triple mutants were selected from the F_2 plants. Seeds were planted on a peat moss/potting soil/sand (3:3:1, v:v:v) mixture. The plants were grown in incubators under constant cool-white fluorescent light at 25°C (unless otherwise stated) and 70% relative humidity.

For scanning electron microscopy (SEM), young primary inflorescences were fixed in 4% glutaraldehyde in 0.025 M sodium phosphate (pH 7.0) at 4°C overnight, and then transferred to 1% osmium tetroxide in the same buffer at 4°C overnight. They were rinsed in the same buffer 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 using glass needles. The mounted specimens were coated with gold and palladium (4:1) in a Technics Hummer V sputter coater following each dissection. SEM was performed on an ETEC Autoscan scanning electron microscope at an accelerating voltage of 20 kV, and the images recorded on Kodak 4127 or Polaroid 55 film.

RESULTS

Wild type

Wild-type *Arabidopsis thaliana* flowers (Figure 1A) contain four concentric regions (whorls), each occupied by a different organ type (Bowman et al., 1989). The first (outermost) whorl of the wild-type flower contains four sepals, two medial and two lateral (with respect to the stem of the inflorescence). The second whorl holds four petals, which are in alternate positions with the sepals. The third whorl includes six stamens, four long medial ones, and two short lateral. The fourth whorl is occupied by the gynoecium, which consists of a two-chambered ovary topped with a short style, and capped with a stigma.

Nectaries, which appear as small cellular mounds with stomata on top, are formed at the base of the stamens, though their presence is variable, so that in any flower some stamens may have them, and others may not. The individual cells that constitute each organ are characteristic of the organ type, so that both overall structure and cellular identity can be used as criteria for organ type.

The development of the flowers has been described in detail, and divided into twelve stages from the first appearance of a flower primordium, to anthesis (Müller, 1961; Bowman et al., 1989; Hill and Lord, 1989; Smyth et al., 1990; Figure 2A). New flower primordia are added at the top of the inflorescence, where the growing point is, in a spiral phyllotactic pattern. The inflorescence is thus a raceme, and an individual inflorescence may contain a complete developmental series of flowers, from the youngest primordium at the apex, to mature fruits toward the base.

Single mutant strains

agamous

Two *agamous* alleles have been described previously, *ag-1* (Bowman et al., 1989) and *ag-2* (Figure 1B, Yanofsky et al., 1990) and we report here the isolation of a third allele, *ag-3* (Table 1) The *ag-1* and *ag-3* mutants have very similar phenotypes; the *ag-2* mutant has a somewhat different appearance. In all the mutants, whorls one and two appear normal, while in whorl three the positions occupied by the six stamens in wild type are occupied by six petals, and in whorl four the cells where the gynoecium normally develops differentiate into a new flower, consisting generally of an outer whorl of sepals, and two whorls of petals. Since this process of formation of flowers within flowers continues, mutant flowers can consist of at least five nested flowers, with a total of 70 or more organs (Bowman et al., 1989). All of the mutants also show a less than perfect repetition in the inner flowers. Some inner flowers have fewer than 10 petals, and some of

the organs may arise in ectopic positions. The internal sepalloid organs are often not perfect sepals, but can be mosaics of petal and sepal tissue, with the petal tissue always forming in longitudinal sectors on the outer margins of the organs. Development of *ag-2* flowers resembles that of *ag-1* flowers (Bowman et al., 1989), as can be seen in Figure 2B. The one difference so far observed is that *ag-2* homozygous flowers show a greater degree of pedicel elongation of the internal flowers than *ag-1* and *ag-3*. Very likely this is due to a difference at the *erecta* locus, which when mutant reduces internode elongation throughout the plant. The *ag-1* and *ag-3* mutants are in the *erecta* background; *ag-2* is not (Table 1). None of these *ag* alleles is temperature sensitive.

apetala2

Detailed descriptions are available for *ap2-1* (Bowman et al., 1989) and *ap2-5, -6,* and *-7* (Kunst et al., 1989), and briefer descriptions of *ap2-2* (Meyerowitz et al., 1989) and *ap2-3* and *-4* (Komaki et al., 1988) have also been published. We give here a detailed description of the flowers and of their development in homozygotes for three further alleles, *ap2-2*, *ap2-8*, and *ap2-9* (Table 1). All of these show a greater departure from wild type than any of the mutants previously described. These descriptions extend the allelic series, and indicate new functions for the wild-type *AP2* gene product. In addition, we found that the three alleles are all temperature-sensitive, as has been previously shown for *ap2-1* (Bowman et al., 1989), implying that the underlying process or the wild-type protein, rather than the mutant gene product specifically, is affected by temperature changes.

apetala2-2

The most extreme allele characterized to date is *ap2-2*. Flowers of plants homozygous for this allele consist mostly of carpelloid and staminoid organs (Figure 1C, Tables 2-4). The identities of the outer two whorls of organs are altered and, in addition,

the numbers and/or positions of organs in all four whorls may be altered. Phenotypic differences are observed between the lateral and medial first whorl organ positions and thus they are treated separately (Table 2). About half of the time, the lateral positions have no organ development. When organs do form they tend to be cauline leaf-like organs with stellate trichomes on their abaxial surfaces and stipules at their bases (both characteristics of leaf development) or carpelloid leaf-like organs with stigmatic tissue at their tips and rudimentary ovules along their margins (Table 2). Each of these organs may have the long abaxial epidermal cells characteristic of sepals. The lateral first whorl positions can also be occupied by filamentous structures, some of which have leaf-like character (such as stellate trichomes) while others are squamule-like structures, thin filamentous outgrowths of cells that have been observed in other species of Brassicaceae (Arber, 1931) and in *ap1-1* flowers of *Arabidopsis* (Irish and Sussex, 1990; D. R. S. and J. L. B.). In contrast, the medial first whorl positions are always occupied and exhibit a greater degree of carpellody. Solitary carpels capped with stigmatic tissue, and exhibiting marginal ovules are the most common organ in these positions (Table 2). Scanning electron microscope analysis of the epidermal cells of these organs shows cellular morphologies ranging from carpel-like (with a lower ovary-like portion of regular cellular files, and an upper stylar portion) to cauline leaf-like (consisting of irregularly-shaped cells with occasional stellate trichomes). On occasion these carpelloid medial first whorl organs are fused to the central, fourth whorl gynoecium. In many cases, the medial first whorl organs are mixtures of stamen and carpel tissue (Table 2, Figures 1C, 2C). The staminal portion is always positioned at the outer margins of the organs, while the carpelloid tissue is central. The stamen sectors have both epidermal and internal cellular morphologies resembling those of wild-type stamens, but the central carpelloid sectors have cellular morphologies ranging from those characteristic of carpels to leaf-like. Individual, phenotypically mosaic organs of this type can contain both ovules and pollen.

There are no organs in the positions occupied by second whorl organs in wild-type flowers in *ap2-2* homozygotes (Table 3). This is due to a failure to form organ primordia. The third and fourth whorls are affected with respect to position and number but their identities are not altered. Third whorl positions are largely unoccupied (Table 4, Figure 1C). When the third whorl organs are present, they are normal and fertile stamens and tend to occupy the lateral positions. Two carpels occupy the fourth whorl but they often fail to fuse properly (Table 4). In addition, when they do fuse correctly, the resulting gynoecia are usually not oriented as they would be in wild type (Figure 2C). In 14 out of 15 fused carpel gynoecia of *ap2-2* homozygotes scored, they were either twisted, or oriented 90° from the wild-type orientation. The gynoecium is nonetheless functional, setting seed when manually pollinated.

The development of *ap2-2* flowers diverges morphologically from that of wild type as early as stage three, the stage when the organ primordia of the first whorl arise (Figure 2C). This is slightly after the beginning of the temperature-sensitive period (stage two) that was determined for second whorl organ development in *ap2-1* homozygotes (Bowman et al., 1989). The medial first whorl organs are enlarged relative to wild type, and grow rapidly, with the cell files typical of gynoecial development. These organs develop ovules on their margins and stigmatic papillae at their apices prior to the formation of the same features in the central gynoecium. Stipules, which are characteristic of early leaf development (Smyth et al., 1990), and which are seen at the base of developing first whorl organs of *ap2-1* homozygotes (Bowman et al., 1989), are not seen in medial first whorl *ap2-2* organs. Lateral first whorl primordia, when present, appear to originate lower on the pedicel of the flower primordium than organs in the same position in wild-type flowers (Figure 2C) and the organs that develop in these positions often have stipules at their bases. The occasional stamens that are observed developing in third whorl positions occupy the lateral positions, and there are a maximum of two stamens in any developing flower. In wild type, nectaries are always associated with stamens, while in *ap2-2* homozygotes

nectaries can be present regardless of the occurrence of stamens. The remaining floral meristem gives rise to the gynoecial cylinder. Its growth is often irregular, and the division between the two carpels occurs in an abnormal orientation. Observations on developing mutant flowers show that the absence of organs in positions normally occupied in the second and third whorls does not result from failure of initiated organ primordia to develop, but rather results from the failure of primordia to appear in these positions. This also occurs in most of the lateral first whorl positions that lack organs; occasionally, however, a small mound of cells may occur in these positions in which no organ develops. It is unclear whether these outgrowths represent aborted organ primordia or some other structure. Thus, in addition to changes in organ identity, mutations in the *ap2* locus change basic organ number in flowers.

When *ap2-2* plants are grown at low temperature (16°C), the frequency of occurrence of lateral first whorl and all third whorl organs is increased compared to 25°C. Nonetheless, second whorl organs fail to develop. In addition, the first whorl organs may fuse to each other as described below for *ap2-9* flowers grown at 16°C. When they are grown at 29°C, *ap2-2* homozygotes frequently fail to produce any first, second or third whorl primordia at all. This results in flowers that contain only a gynoecium, which is typically composed of two carpels oriented at 90° from the positions occupied by the two carpels in wild type.

apetala2-8

Flowers of plants homozygous for the *ap2-8* allele are similar to those of *ap2-2* homozygotes in both their mature phenotype (Tables 2-4) and their ontogeny (Figure 2D). The *ap2-8* homozygotes are also similar to those of *ap2-2* in their response to different growth temperatures, with greater organ loss and more complete organ conversions at higher temperatures, and less departure from wild type at lower temperatures (Figure 3).

apetala2-9

Plants homozygous for the *ap2-9* mutation have flowers with a phenotype slightly closer to wild type than plants homozygous for *ap2-2* or *ap2-8* (Figure 1D). Briefly, the medial first whorl organs are carpelloid and the lateral first whorl organs often fail to develop (Table 2). No organs form in the second whorl positions (Table 3) and the third whorl is also largely unoccupied (Table 4). When third whorl organs are present they form normal fertile stamens. Two carpels occupy the fourth whorl, but they often fail to be properly fused (Table 4).

When grown at 16°C, flowers homozygous for *ap2-9* exhibit both numerical and positional abnormalities (Figure 2E). Although fewer organs are missing than in flowers grown at higher temperatures, those organs that are present tend to occupy ectopic positions and are often fused to each other. For example, lateral first whorl primordia are frequently present, though they appear to arise lower on the pedicel of the developing flower than do lateral first whorl organ primordia in wild type. These organ primordia differentiate to become carpelloid leaves, which are often fused to the more-carpelloid medial first whorl organs to form a connate ring, with marginal ovules and stigmatic tissue at its top (Figure 2E). Sometimes a fifth organ primordium arises between the positions ordinarily occupied by first and second whorl organs (Figure 2E). Second whorl organs appear to be absent, though there are stamens, carpelloid stamens and solitary carpels whose positions do not allow their assignment to the second or third whorl. Third whorl organs, which also arise in ectopic positions, are normal stamens, and the ovary is usually normal.

trans-heterozygotes

In addition to characterizing the phenotype of homozygotes of each of the three extreme *ap2* alleles, we have made all possible heterozygous combinations of these alleles

with each other, and with *ap2-1*, the allele whose homozygotes show the least departure from wild type (at 25°C, four first whorl leaves, four second whorl petalloid stamens, normal third and fourth whorls). In each case, the phenotype of the heterozygotes is intermediate between that of the homozygotes for each allele involved. *ap2-1/ap2-2* heterozygotes (Figure 2F, Tables 2-4), representing a combination of the two extremes of the *ap2* allelic series, are discussed because they emphasize aspects of the *ap2* phenotype, in particular the difference in phenotype between the lateral and medial first whorl organs, the failure of organ primordium formation in the second and third whorls, and the abnormal positions of the remaining second and third whorl organ primordia. *ap2-1/ap2-2* flowers have lateral first whorl organs that resemble cauline leaves, usually (76% of 72 organs scored) with stigmatic tissue at their tips, whereas the medial first whorl organs are much more carpelloid (Table 2, Figure 2F). These positions are occupied by solitary carpels or carpelloid leaves, with ovules at their margins in addition to stigmatic papillae at their tips. The carpellody of the first whorl organs increases with the age of the inflorescence, with the cauline leaf-like organs present only on the first few flowers, and the solitary carpels generally present in later flowers. This trend has been observed for all the *ap2* alleles. Second whorl organs are usually absent and their absence is due to the failure of organ primordia to form, not to failure of initiated primordia to develop (Figure 2F). When they are present, the second whorl organs range in phenotype from morphologically normal stamens to staminoid petals. Third whorl organs are normal, and occupy the same positions as in wild type when all six are present. When fewer than six are formed some may be in ectopic positions. The nectaries are normal, as is the gynoecium. The phenotype of *ap2-1/ap2-9* heterozygotes (Tables 2-4) is slightly closer to wild type than that of *ap2-1/ap2-2* heterozygotes. Figure 3 illustrates the *ap2* allelic series, as a summary of the very broad range of phenotypes that result from the different mutant alleles at this locus.

pistillata and *apetala3*

Mutants homozygous for one allele each of these two unlinked loci have been described before (Bowman et al., 1989; Hill and Lord, 1989). Homozygotes for *pi-1* have a normal first whorl of four sepals but the organs of the second whorl develop as sepals rather than petals. The cells that would in wild type form the organs of the third whorl appear to be largely incorporated into the gynoecium, which is as a consequence abnormal in size and irregular in structure. The gynoecium usually has more than two carpels and often there are thin, filamentous structures partly or fully fused to its sides (Bowman et al., 1989; Hill and Lord, 1989). Homozygotes for *ap3-1*, when raised at 25°C, have the same second whorl abnormality seen in *pi-1*, but the six third whorl organs are usually carpels or staminoid carpels, either solitary or connate with other members of the same whorl (Table 5). At 29°C the third whorl organs in *ap3-1* flowers can also be free filamentous structures which are thinner than the filaments associated with wild-type stamens but usually thicker than the filamentous structures of *pi-1* flowers (see Figure 7D of Bowman et al, 1989). The two new alleles of *pistillata*, *pi-2* and *pi-3* (Table 1) have phenotypes intermediate between those of *ap3-1* and *pi-1*, with the allelic series (in order of increasing departure from wild-type organ number and gynoecial morphology) *pi-3*, then *pi-2*, then *pi-1*. None of these *pi* alleles is temperature sensitive.

pi-2

Flowers homozygous for *pi-2* (Figure 2G) have normal first whorl of sepals, and like *pi-1* homozygotes, have four additional sepals in the positions normally occupied by petals in wild type. The six third whorl organ positions may be occupied by filamentous structures, by carpelloid organs, or organs may be absent (Table 5). The filamentous structures, which may be capped with stigmatic papillae, are thinner than the filaments associated with wild-type stamens but usually thicker than those observed in *pi-1* flowers.

The third whorl organs present are often congenitally fused to the central gynoecium, producing an irregular gynoecial morphology (Figure 2G, second and third panels). In other cases, the gynoecium is normal.

pi-3

Homozygotes for *pi-3* are very similar to *ap3-1* mutants. They have second whorl sepals and, in the third whorl positions, either solitary carpels, staminoid carpels, or filamentous structures such as those described for *pi-2* flowers (Table 5). The lateral third whorl organs are more staminoid than those occupying medial positions, and the extent of carpellody in all third whorl positions increases with the age of the inflorescence (as in *ap3-1*).

pi-1/pi-2

Heterozygotes of genotype *pi-1/pi-2* (Figure 1E) are intermediate between the two homozygous types in third whorl organ identity. Many (33%) of these organs are fused to the central gynoecium, regardless of their identity. A summary of the changes in organ identity in the third whorl, with increasing departure of the *pi* phenotype from normal, is staminoid carpel, carpel, filamentous structure; with fusion to the fourth whorl organs also increasing with increasing departure from wild type (Table 5).

In all *pi* mutants, the nectaries are normal and occupy their wild-type positions (see Smyth et al., 1990). When (as in *pi-1* and some *pi-2* positions) there is no third whorl organ, the nectaries are still present between the sepals occupying the second whorl and the gynoecium. The presence of nectaries thus does not depend on the development of stamens or any third whorl organ. In all *pi* mutants, and in *ap3-1* mutants, the sepals in the second whorl are smaller than the sepals occupying the first whorl, and they develop on a relatively delayed time course, as do petals relative to sepals in wild-type flowers (Bowman et al., 1989; Hill and Lord, 1989).

Doubly mutant strains

Many of our current conclusions about the domains of action of the various homeotic gene products, and about their interactions, come from study of the phenotypes of strains homozygous for different pairs of the homeotic mutations. We have previously described the phenotypes of the doubly mutant strains *ag-1 pi-1*; *ag-1 ap3-1*; *ag-1 ap2-1*; *ap2-1 ap3-1*; and *ap2-1 pi-1* (Bowman et al., 1989). The alleles of *ag* and *pi* used in these studies display the most extreme phenotypes of those so far observed in their respective allelic series; they may thus represent null or close to null phenotypes. In contrast, the allele of *ap2* used, *ap2-1*, is the weakest allele in the series of mutations known at this locus. Consequently, we have constructed more doubly mutant strains, using the allele at the *ap2* locus that shows the greatest departure from wild type, *ap2-2*. We have also constructed an *ap2-1 pi-2* strain, to observe the effect of using a weaker *pi* allele in one combination. In addition, we present here new information on flower development in the doubly mutant strains whose mature phenotypes were previously described (Bowman et al., 1989).

ap2-1 pi-1, *ap2-1 pi-2*, and *ap2-1 ap3-1*

Flowers homozygous both for *ap2-1* and *pi-1* have a first whorl of four slightly carpelloid leaves (as observed in *ap2-1* alone, but with a slightly increased degree of carpellody). The second whorl contains 1 to 4 organs that range from cauline leaf to solitary carpel, with most mixtures of the cell types characteristic of these organs, and none showing staminody. Third whorl organs are absent, as in *pi-1* alone, and the gynoecium resembles that of *pi-1* flowers (Bowman et al., 1989). When a weaker *pi* mutant is used, such as in *ap2-1 pi-2*, flowers exhibit a phenotype more similar to that of *ap2-1 ap3-1* flowers (Bowman et al., 1989) than to that of *ap2-1 pi-1*. The outer whorl is occupied by

cauline leaf-like organs that can have stigmatic tissue at their tips. Second whorl organs range in phenotype from cauline leaf-like organs to organs that have characteristics of leaves, stamens and carpels. The third whorl organs are solitary carpels or filamentous structures such as those described for the third whorl of *pi-2* flowers.

In each of the genotypes, both the second and third whorl organs are frequently absent. When all the positions in a whorl are occupied by an organ, the organs occupy the correct positions. When one or more are missing, however, the remaining ones develop from organ primordia that are often enlarged and occur in ectopic positions (Figure 4A, B). In contrast, when second whorl organs are absent from *ap2-1* flowers the positions of the remaining organs is not altered and second whorl organs are never absent in *ap3-1*, *pi-1*, or *pi-2* flowers. The development of the gynoecial cylinder is often abnormal, resulting in an unfused ovary that may also be adnate with both second and third whorl organs. The fusion between second and third whorl organs and the central gynoecium is congenital. Observations on developing flowers show that missing organs result from failure of organ primordium formation (Figure 4A, B).

ap2-2 pi-1

When a plant is homozygous for both the strong alleles, *ap2-2* and *pi-1*, many organs are absent (Figure 5A). The lateral first whorl positions may be occupied by cauline leaf-like organs (11 out of 66 positions scored), carpelloid leaves, (9/66), or filamentous structures (22/66). In 24 of 66 positions, no organ was observed. The filamentous structures observed are either filamentous leaf-like structures or thinner squamule-like structures, such as those described in the lateral first whorl positions of *ap2-2* flowers. An example of a squamule-like structure is shown in the fourth panel of Figure 4C. The remainder of the *ap2-2 pi-1* flower consists only of a central gynoecium, which usually consists of four carpels (30 out of 33 flowers analyzed; Figure 4C). In the first five or so flowers produced on any inflorescence, the carpels in the medial positions have stellate

trichomes on their abaxial surfaces, while the lateral carpels are bare (Figure 5A). Small domes of cells may be present between the positions of the lateral outer whorl organs (or the positions where they would be expected to be, were they present) and the central ovary. These are apparently nectaries, since they arise late in development, when the gynoecium develops its stigmatic papillae, and since stomata occur at the dome apices (Figure 4C).

Observations on the development of *ap2-2 pi-1* flowers show that the two medial first whorl organ primordia do form, and are enlarged relative to the size of wild-type medial first whorl primordia. These primordia are fused to each other as they develop, forming a cylinder that will later be a part of the central gynoecium (Figure 4C). The cells central to these primordia, which in wild type would develop into the second, third and fourth whorls, appear to be recruited into the growing cylinder formed by the fused medial first whorl primordia. Thus, the mature gynoecium appears to consist of two medial (and slightly phylloid) carpels that originate in the first whorl, and two lateral fourth-whorl carpels. The absence of any organs developing from the second and third whorls is consistent with the phenotypes of the single mutant homozygotes, since *ap2-2* mutants lack second whorl organs, and *pi-1* mutants lack those of the third whorl. As seen in *ap2-2* flowers, the lateral first whorl organs that form develop from primordia produced lower on the pedicel of the flower primordium than do first whorl organ primordia in wild-type flowers.

ap2-2 ap3-1

Homozygotes for the strong *ap2-2* allele and *ap3-1* produce flowers that consist primarily of carpelloid organs, with occasional cauline leaf-like organs in the lateral first whorl positions. All of the carpelloid organs may be fused, such that the flower resembles that of *ap2-2 pi-1* homozygotes, or the medial first whorl organs may be separate from the central gynoecium. Organs do sometimes form between the organs of the first and fourth whorls. These may be third whorl organs, as sometimes form in *ap2-2*, but they are

carpels and not stamens (as in *ap2-2*) as a result of the absence of the wild-type action of the *AP3* gene.

ap2-1 ag-1

Our earlier observations of *ap2-1 ag-1* double homozygotes showed that the flowers retain the indeterminate growth characteristic of *ag-1* homozygotes, but have additional changes in organ identity (Bowman et al., 1989). The first floral whorl contains four cauline leaves with stellate trichomes and stipules, as well as an epidermal cellular morphology typical of leaves (Figure 4D). These organs have stigmatic tissue at their tips very infrequently in contrast to the first whorl organs of *ap2-1* flowers that are often capped with stigmatic papillae. The second and third whorl organs are intermediate between petals and stamens, both at the organ level, and in the appearance of individual epidermal cells. Observations of the development of these flowers shows that all organs of the first three whorls originate from primordia that are in the positions and numbers of the similar primordia in wild type (Figure 4D). The cells that would ordinarily develop into the gynoecium form, as in *ag-1* homozygotes, an inner flower, whose organ number and positions may differ from those typical of wild-type flowers.

ap2-2 ag-1

Remarkably, *ap2-2 ag-1* double homozygotes (Figure 5B) strongly resemble *ap2-1 ag-1* double homozygotes: the absence of wild-type *AG* activity eliminates many of the phenotypic differences between flowers homozygous for the different *ap2* alleles. This strongly implies that these differences are due to different interactions of the products of the *ap2* alleles with the wild-type *AG* gene or its product, a model that will be developed in the Discussion.

The phenotype of the *ap2-2 ag-1* double homozygotes includes lateral first whorl organs (which were present in 32 out of 44 positions counted) resembling either cauline

leaves (22/32 organs) or filamentous structures (10/32 organs). The filamentous structures may be either leaf-like or squamule-like structures, such as those described for lateral first whorl positions of *ap2-2* flowers Six of the 22 phylloid organs had stigmatic tissue at their apices. The medial first whorl organs are carpelloid leaves (in contrast to the solitary carpels, with few leaf-like characteristics, found in the *ap2-2* single mutant). These carpelloid leaves have an epidermal cellular morphology of leaves, often develop with stipules at their bases, and frequently have stellate trichomes on their abaxial surfaces (Figure 5B). Long epidermal cells of the type found only on the abaxial surface of sepals also are present, however, and stigmatic papillae and ovules may develop on the tips and margins, respectively.

Second whorl organs do occur in the double mutant, again in contrast to *ap2-2* single homozygotes. Those second and third whorl organs that form are indistinguishable from each other and have both an overall morphology and individual epidermal morphologies intermediate between those ordinarily seen in petals and stamens (Figure 4E). The organs have rudimentary locules in which pollen grains are produced, but they do not dehisce. It is of note that the number of second and third whorl organs that develop (7.6 on average) is closer to the wild-type number (10) than the number of these organs found in flowers homozygous for *ap2-2* alone (average 0.25). The second and third whorl organs in the double homozygote arise in roughly the normal pattern (Figure 4E).

The remaining floral meristem, which in wild type would give rise to the gynoecium, develops into another flower primordium, as in *ag-1* flowers. The organ primordia in this (and subsequent) inner flowers differentiate in the same patterns as those of the outermost flower, with a whorl of carpelloid leaves surrounding two whorls of stamen-petal intermediate organs. Organs that resemble carpelloid leaves (in whorls 1, 4, 7, etc.) may have longitudinal stamen-petal hybrid sectors along their margins, similar in pattern to the hybrid organs observed in the analogous positions in *ag-1* flowers.

The *ag-1* mutation thus suppresses both the extreme carpellody observed in the first whorl organs of *ap2-2* flowers, and also suppresses the loss of second and third whorl organs characteristic of *ap2-2* flowers. The fact that the *ag-1* mutation exhibits phenotypic effects in the first and second whorls in a background homozygous for *ap2-2*, but shows no effects in these whorls when alone, suggests that the *AG* and *AP2* genes interact at some level.

ag-1 pi-1 and *ag-1 ap3-1*

Finally, we will consider two other doubly mutant combinations involving *agamous*. Both *ag-1 pi-1* and *ag-1 ap3-1* flowers consist of an indeterminate number of whorls of sepals (Bowman et al., 1989; Figure 5C, D). The developmental basis of the phenotypes in the two different genotypes is somewhat different; *ag-1 pi-1* homozygotes consist of a repeating pattern of two whorls, whereas *ag-1 ap3-1* flowers have a repeating pattern of three whorls. An *ag-1 pi-1* double homozygote is shown in Figure 6A. The four outer whorl and four second whorl organ primordia are initiated in the correct positions and all of these primordia develop into sepals. The four second whorl primordia are small and develop much more slowly than the outer whorl sepals, with the result that the second whorl of sepals remains smaller than the outer whorl sepals. The cells that would normally give rise to the third and fourth whorl organ primordia behave as if they were another flower primordium, developing four organ primordia on the margins of the remaining dome of meristematic cells. This process repeats indeterminately, as seen in *ag-1* flowers, thus producing a flower with many whorls, all of which have four sepals. *ag-1 ap3-1* flowers also consist of an indeterminate number of sepals (Figure 5D) but, in contrast to *ag-1 pi-1* flowers, the pattern of organ primordia formation is indistinguishable from that observed in *ag-1* (Figure 6B), thus consisting of repeats of three whorls, with four organs in each of the outer two whorls, then six organs in the third. Each of the organs in both

ag-1 pi-1 and *ag-1 ap3-1* flowers consist of cell types characteristic of wild-type sepals, complete with long epidermal cells on their abaxial surfaces.

Triply Mutant Strains

To extend our genetic analysis of the *Arabidopsis* homeotic genes, we have constructed four different strains, each homozygous for mutant alleles of three of the four homeotic loci. We will first discuss three of these strains together, since their phenotypes are similar. These strains involve *ap2-1* and *ag-1* in combination with *ap3-1*, *pi-1*, or *pi-2*.

ap2-1 ag-1 pi-1, *ap2-1 ag-1 ap3-1*, and *ap2-1 ag-1 pi-2*

Each of these strains produce flowers that exhibit the indeterminate growth caused by the *ag* mutation and, in addition, all of the organs of the flower resemble cauline leaves (Figures 7A, B). The organs are cauline leaf-like in several aspects: they have stellate trichomes on their abaxial and, less frequently, on their adaxial surfaces, stipules are present at the base of most of the organs (Figures 8A, B), they have an epidermal cellular morphology characteristic of leaves, and they senesce on the time course characteristic of leaves, not that typical of sepals (which begin to senesce soon after anthesis). They do, however, have the long epidermal cells on their abaxial surface that are characteristic of wild-type sepals.

Occasionally (in about 5% of the flowers) in each triply mutant genotype a secondary flower is formed in the axil of a lateral first whorl organ. These flowers have their own pedicel and the same phenotype as the flower in which they arise. This phenomena has also been observed in *ap2-1*, *ap2-1 ap3-1*, *ap2-1 ag-1*, and *ap2-1 pi-1* flowers at a lower frequency, and occurs in almost every flower with the *ap1-1* genotype (McKelvie, 1962; Irish and Sussex, 1990).

In *ap2-1 ag-1 ap3-1* flowers, outer whorl organ primordia are initiated in correct positions and the second whorl organ primordia are usually in wild-type positions and

numbers (Figure 8A). By contrast, the third whorl organ primordia are variable in number, position, and size (Figure 8A). The remaining floral meristem continues to produce organ primordia but in an ill-defined pattern. All of these organ primordia develop into cauline leaf-like organs (Figure 7A).

In *ap2-1 ag-1 pi-1* flowers, the identity of each organ is also that of leaf (Figure 7B), but the pattern of the organs is different from that of *ap2-1 ag-1 ap3-1*. The pattern in which the organs appear deviates from that of wild type at stage 5, when the second and third whorl primordia are normally formed (Figure 8B). The pattern initially resembles that seen in *ag-1 pi-1* flowers. The four first whorl primordia are initiated in the correct positions, as are the second whorl organ primordia in most cases. The remaining floral meristem then behaves as if it were a new flower, producing four more primordia on the margins of the remaining dome of cells. At this point, the floral meristem continues to produce organ primordia, but in no easily defined pattern.

Flowers of genotype *ap2-1 ag-1 pi-2* have been analyzed at the resolution of light microscopy. At this level, they appear similar to the flowers of the *ap2-1 ag-1 ap3-1* strain.

ap2-2 ag-1 pi-1

The final genotype constructed, *ap2-2 ag-1 pi-1*, involved the strongest mutant alleles thus far isolated. Flowers on these triple homozygotes are composed of an indeterminate number of slightly carpelloid leaf-like organs (Figure 7C) similar to those observed in the medial first whorl positions of *ap2-2 ag-1* flowers. Each of the organs has an overall morphology intermediate between cauline leaves and carpels. The epidermal cellular morphology is characterized by irregularly shaped cells interspersed frequently with stomata, which is a phylloid appearance, though the abaxial surface also has many of the very long cells that are normally characteristic of sepals. Stellate trichomes, again a leaf character, may form on the abaxial surfaces, with their frequency decreasing on the inner organs. The most cauline leaf-like organs, which usually develop in what appears to be the

lateral first whorl positions, sometimes have stipules at their bases. Stigmatic papillae develop at the tips of most organs, and ovules may develop along the margins of the organs from what resembles placental tissue.

Development is altered as early as stage 3, when the first whorl organ primordia appear (Figure 8C). In *ap2-2 ag-1 pi-1* flowers the medial first whorl organ primordia appear in the correct positions, but their growth is abnormal, and they differentiate into carpelloid leaves. The lateral first whorl primordia sometimes fail to form (they are present only in 11/18 positions analyzed) and when they do form they appear to arise lower on the flower primordium than the position of the analogous organs in wild-type flowers. These lateral primordia differentiate either into cauline leaf-like organs with few carpelloid features (7/11 organs), or into filamentous structures (4/11 organs) like those described for lateral first whorl positions of *ap2-2* flowers. The remaining floral meristem produces two more organ primordia in lateral positions, directly inside the lateral first whorl organs when these are present, and interior to all of the first whorl organs (Figure 8C). This is followed by the production of two additional primordia in medial positions, interior to all of the previously produced primordia. This pattern of pairs of lateral, then medial organ primordium formation repeats indefinitely, and eventually each of the primordia develops into a carpelloid leaf. Occasionally, the medial first whorl organs and the next two inner organs are congenitally fused, forming a ring of organs. The lateral first whorl organs have not been observed to fuse with any other organs.

The phenotypes of each of the singly, doubly and triply mutant flowers are summarized in Table 6.

DISCUSSION

Each of the genes, *AG*, *AP2*, *AP3*, and *PI*, seems to act by allowing cells to determine their positions within the developing flower, and thus to act in allowing the cells

to differentiate (or to direct their progeny to differentiate) appropriately. In addition, the *AP2*, *AG*, and *PI* products appear to be involved in the initial production of floral organ primordia, which precedes their differentiation. Mutations in these genes thus result in both the misinterpretation of positional information, causing organ primordia to differentiate improperly, and the disruption of floral organ number and pattern. We have derived a model, presented below, which explains how these four genes could specify the identity of the floral organs. The model has been successful in predicting the phenotypes of doubly and triply mutant flowers, and in predicting the pattern of expression of one homeotic gene that we have cloned and analyzed so far (*AG*, Yanofsky et al., 1990). The model does not, however, address the issue of how these and other genes act to specify the position and number of organs in the developing flower. The model is also insufficient to explain all of the organ identity phenotypes seen. It is clear, then, that what follows is an overly simple, first-order model, which will require considerable modification as new mutations, and new phenomena, are described.

A simple model

The model is depicted in Figure 9. The *Arabidopsis* wild-type flower consists of four concentric whorls of organs, arranged in a stereotypic pattern in terms of number and position. Each of the four mutations affects the differentiation of two adjacent whorls of organs, and thus falls into one of three classes: those affecting the first and second whorls, those affecting the second and third whorls, and those affecting the third and fourth whorls. The flower primordium can, consequently, be divided into concentric fields made up of pairs of adjacent whorls. The term "field" is introduced here to refer to pairs of adjacent whorls, since these pairs of whorls, rather than single whorls, appear to be the domains of action of each of the classes of homeotic genes described. Field A is made up of whorls 1 and 2, and is the domain of action of AP2, the product of the *AP2* gene. Whorls 2 and 3 constitute field B, which is the domain of AP3 and PI; and field C is made

of whorls 3 and 4, the domain of AG action. AP3 and PI are grouped together for the remaining discussion because a mutation in either one results in a similar phenotype with respect to organ differentiation, and because the double mutant phenotype is indistinguishable from the single mutant phenotypes (Bowman et al., 1989). In what follows, it is important to recognize that by "whorl" we mean a geographic location, within which organs of any identity can arise, and not the group of organs themselves. Whorls are thus identified by their position in the developing flower, and by the number and disposition of the organs within them, but not by the identity of these organs (see Bowman et al., 1989 for a longer discussion of this point).

Underlying our model is the assumption that the combination of homeotic gene products present in each whorl is responsible for specifying the developmental fates of the organ primordia that form in that whorl. The three overlapping fields of gene action thus specify each of the four different whorls of organs in the flower: in any whorl in which AP2 alone is active, sepals form. If both AP3 and PI are also present in combination with AP2, as in the wild-type second whorl, any organ primordia that are present differentiate into petals. Similarly, if AG alone is present, it directs any primordia in that whorl to develop into carpels, as is the case for the wild-type fourth whorl. If AP3 and PI are present in combination with AG, stamens are specified, as in the wild-type third whorl.

The final aspect of this simple model is that the *AP2* and *AG* products are mutually antagonistic, such that AG prevents the action of AP2 in the third and fourth whorls of a wild-type flower, and AP2 prevents the action of AG in the first and second whorls. This proposal is based on observations that the *ag-1* mutation has phenotypic effects in the first and second whorls in an *ap2-2* mutant background, while no first or second whorl effects are seen in *ag-1* singly-mutant flowers. In *ap2-2 ag* flowers the first whorl develops as carpelloid leaves rather than carpels as in *ap2-2* flowers, and second whorl organs, which are missing in *ap2-2* flowers, are partially restored in the double mutant and have a phenotype different from that observed in any *ap2* allele alone. Likewise, the *ap2*

mutations have third and fourth whorl homeotic effects when combined with *ag-1*, but not when alone; *ap2-2 ag-1* flowers have third whorl organs intermediate between petals and stamens, while *ag-1* alone has normal petals in the third whorl. If the first whorl of the inner flower in an *ag* mutant is considered equivalent to a wild-type fourth whorl, *ap2-2* also has a fourth whorl effect in an *ag* background, namely, conversion of these fourth whorl sepals into leaves or carpelloid leaves. According to this part of the model, then, if the *AP2* product is missing, the phenotypic expression of AG expands into field A (whorls 1 and 2) and conversely, if the *AG* product is absent, the phenotypic expression of AP2 expands to include field C (whorls 3 and 4).

If one or more of the homeotic gene products is missing, the distribution of remaining products determines organ identity. For example, if either the *AP3* or *PI* gene product is missing, leaving only those products specifying fields A and C, then the outer two whorls should develop into sepals and the inner two whorls into carpels. This is indeed the case in *ap3-1*, and *pi-3*. If the *AP2* product is missing, AG is active in both fields A and C. The first and fourth whorl organs should then be carpels, and the second and third whorl organs, which develop in the presence of AG and AP3/PI, should be stamens. This is observed in the *ap2* allelic series; all those organs arising from cells that would normally give rise to second and third whorl organs are staminoid, whereas those arising from cells normally forming first and fourth whorl organs are carpelloid. Conversely, if the *AG* product is missing, the *AP2* product should be active in both fields A and C (with, as in wild-type, AP3/PI active in field B), and a flower in which the first and fourth whorl organs are sepals, and the second and third whorl organs are petals should (and does) develop.

Predictions for double and triple mutants

The model makes clear predictions for the phenotypes of doubly and triply mutant flowers. For example, in *ap3 ap2* and *pi ap2* flowers, the *AG* product (and none of the

others) should be present in all four whorls, since the *AP2* product is not present to repress the activity of AG in the outer two whorls. Therefore, the flowers are expected to consist entirely of carpels. This is the case in such doubly mutant flowers (Figures 4C, 5A). A similar argument can be made to predict flowers composed entirely of sepals for *ag pi* and *ag ap3* double mutants, and this prediction is confirmed by the phenotypes of these flowers (Figures 5C, D; Bowman et al., 1989).

The prediction for *ag ap2* flowers is more difficult to make, because if both the *AG* and *AP2* products are absent, only the *AP3/PI* products remain in field B. This is a distribution of homeotic gene products that does not match that thought to be found in any whorl of wild-type flowers. In wild type, the formation of both petals and stamens is dependent on the *AP3/PI* products. The decision as to whether to produce either a petal or a stamen, however, is dependent on the presence of either the *AP2* product or the *AG* product, respectively. Thus the second and third whorl organs might be expected to have characteristics of both petals and stamens, but to be neither wild-type petals nor wild-type stamens. This is what is observed in *ap2-2 ag-1* flowers (Figure 4E). The phenotype of first and fourth whorl organs in the *ap2-2 ag* double mutants is also difficult to predict, since whorls 1 and 4 are predicted to have none of the homeotic gene products present in them, a situation with no precedent in the wild-type flower. Thus, the organs occupying these positions should not be of a type normally found in flowers. Indeed, the organs of these whorls in *ap2-2 ag-1* flowers have characteristics of leaves (Figure 5B).

It is tempting to speculate that the known three homeotic pathways are sufficient to specify floral organs and that, in their absence, only vegetative organs can form. The developmental ground state, however, appears not to be entirely vegetative, since the organs in the first and fourth whorls of *ap2-2 ag-1* flowers are carpelloid leaves. If these organs indeed exhibit the developmental ground state, all floral organs should be carpelloid leaves in the triple mutant, in which all three of the homeotic pathways are blocked. This

prediction is also confirmed; all organs in *ap2-2 ag-1 pi-1* are carpelloid leaves (Figures 7C, 8C).

Each of these genes appears to control the specification of organ identity by regulating genes that are involved in the production of cellular and morphological characteristics of the different organ types, and not by directly specifying the final differentiation products. This is clear from the fact that organ types removed by single mutations may be partly restored by additional mutations. For example, *ag* mutants have no stamens, but in *ap2 ag* doubly mutant strains, the second and third whorl organs are in many respects staminoid. Another example is that single *ag* mutants have no carpelloid structures, but such structures are found in the *ap2-2 ag-1 pi-1* triply mutant strain.

ap2-1: a special case

The phenotypes of *ap2-1* flowers seem to refute the organ specification model: the first whorl of *ap2-1* flowers is composed of leaves with few carpel characteristics, not the carpels predicted by the model. This apparent exception could be explained by proposing that in the *ap2-1* allele, only one of the two proposed functions of AP2 has been significantly affected. According to our model, AP2 has two functions: one is to prevent AG from acting in the outer two whorls (field A), and the other is to affect directly the expression of the genes that lead to the differentiated phenotypes of sepals and petals. If the *ap2-1* gene product fails to activate downstream genes, yet still prevents activity of AG in whorl 1, none of the three classes of homeotic genes would be active in whorl 1. The model would therefore predict the development of carpelloid leaves in these positions; this is what is observed in *ap2-1* flowers grown at 29°C (Bowman et al., 1989).

First whorl positions in *ap2-1* flowers grown at temperatures below 25°C, however, are occupied by leaves with little or no carpel characteristics. In addition, first and fourth whorl positions in *ap2-1 ag-1* double mutants, and all whorls in *ap2-1*-containing triple mutant strains, leaves are found where carpelloid leaves are expected. The activity of AG

is clearly reduced (or possibly absent) in the double and triple mutant flowers. Since the phenotype of the first whorl organs of *ap2-1* flowers is nearly identical with that of the organs of the double and triple mutant flowers, the activity of AG must also be reduced in *ap2-1* flowers. This implies a third function for the *AP2* product, to repress an as yet-unknown homeotic activity similar to that of AG, which when expressed in leaves adds some carpel characteristics. Greater loss of AP2 function, as in *ap2-2*, allows this activity to function, thus producing carpelloid leaves, while a low level of AP2 activity, as in *ap2-1*, represses it (variably) and results in leaves with few or no carpel characteristics. Quadruple mutants for the unidentified gene and all three of the known homeotic paths would have true leaves in all floral organ positions, and this collection of gene classes would be sufficient for specifying all of the differences between vegetative and floral organs.

This proposal predicts either that the *AP2* product has two or more separable domains of function, or that different levels of the *AP2* product are required for its different functions. The staminoid petals in the second whorl of *ap2-1* flowers are unlike those in the second and third whorls of *ap2-2 ag-1* flowers (they consist of sectors of stamen tissue, sectors of petal tissue, and sectors that appear intermediate between petal and stamen tissue, Pruitt et al., 1987; Bowman et al., 1989). This implies variable repression of AG in the second whorl in *ap2-1*, and a variable ability of the *AP2* product in this whorl to affect downstream genes. In the stamen tissue the *AP2* product appears not to accomplish either of its functions; in the petal tissue it performs both of its functions; and, in the hybrid sectors, it is repressing AG, but is not contributing to the differentiation process.

Unexplained complications

Mosaic organs and whorls

As stated previously, the proposed model does not address questions of how the homeotic genes affect organ number and organ position. Beyond this shortcoming, there are other observations relating to organ identity, which is within the realm of our model, that are not incorporated.

The presence of phenotypically mosaic organs in a number of the genotypes examined presents one aspect not readily explained by the model. Prominent examples are organs consisting of carpel and stamen sectors in the first whorl positions of *ap2-2*, *ap2-8* and *ap2-9* flowers. These *ap2* alleles alter the number, size and shape of all four whorls of organ primordia, as well as the pattern of differentiation of the organs in the outer two whorls. The first whorl organ primordia from which the mosaic organs develop appear to be larger, and to involve a greater proportion of the flower primordium, than do the first whorl organ primordia in wild-type flowers (Figure 2C, D). It could be that these organ primordia are large enough to encompass more than a single geographic whorl within the developing flower. The shape of these primordia is such that their margins could arise from cells that would normally be part of the second or third whorls while the central parts of the primordia arise from cells of the outer whorl. This would explain why the stamen sectors are always on the margins, and the carpel sectors always occupy the central portion of the phenotypically mosaic organs. A similar argument may be made for the phenotypically mosaic (mixed sepal and petal) inner whorl organs in *ag-1*, *-2*, and *-3* flowers. This would suggest that the formation of fields specifying differentiation of cell types, and hence organ types, is to some degree independent of the formation of organ primordia.

The importance of organ primordium position within the developing flower is emphasized by the different fates of the medial and lateral first whorl organ primordia. As noted in this and in other studies (Komaki et al., 1988; Kunst et al., 1989) of *ap2*, the medial first whorl organs are generally carpelloid, while the lateral first whorl organs are quite leaf-like, even in the stronger alleles. In wild-type flowers, the lateral primordia are only slightly lower on the pedicel than the medial first whorl primordia (Smyth et al., 1990). By contrast, in *ap2* flowers the lateral first whorl organ primordia are initiated much lower on the pedicel than the medial first whorl primordia. If the activity of the genes that specify floral organ identity is not present in the lower cells, which would normally give rise to the receptacle and pedicel, organ primordia arising ectopically from these cells might be expected to differentiate, inappropriately, into leaf-like organs.

Secondary flowers

Another observation that does not immediately fit with the model is that of secondary flowers that develop in the axils of the lateral first whorl organs of singly, doubly, and triply mutant flowers with the *ap2-1* genotype. The lateral first whorl organs in *ap2-1* resemble cauline leaves. They have stellate trichomes on their abaxial surfaces, leaf-like epidermal and internal cellular morphologies, and stipules at their base. These are all characteristics of leaves and not of floral organs. Another characteristic of *Arabidopsis* leaves, both radical (rosette) and cauline, is that each has an associated meristem in its axil. Thus, it may not be surprising that organs that have the identity of leaves develop meristems in their axils. For genuine radical and cauline leaves, the meristem is an inflorescence meristem. In *ap2-1* floral leaf-like organs, it is a floral meristem, indicating the possibility that meristem identity, like organ identity, can be determined independently of position.

Second whorl leaves

A final detail not easily explained by the model is the occurrence of leaf-like organs in the second whorl of *ap2-1* flowers grown at 16°C (Bowman et al., 1989). Regardless of the activity of the product of the *ap2-1* allele, it is expected that the presence of wild-type PI and AP3 activities in this field B position would modify the leaf fate of these organs. It apparently does not, at least in some instances.

Interpretation of the *ag* fourth whorl and the problem of indeterminacy

The cells that would ordinarily give rise to the fourth whorl gynoecium in wild-type flowers form another flower in *ag*. The fourth whorl of organs in *ag* flowers may thus be interpreted as either a fourth whorl of sepals, replacing the fourth whorl carpels of wild-type flowers (the explanation used above), or as the first whorl of sepals in an internal flower. The number and positions of the organs support the second interpretation, while the identity is, in either case, consistent with our model. Therefore, the structure of *ag* flowers is most easily described as a reiterated pattern: (first whorl, second whorl, third whorl)$_n$ with no fourth whorl organs ever forming. Likewise, the structures of *ag-1 pi-1* flowers and *ap2-2 ag-1 pi-1* flowers could be described as the reiterated patterns of organs: (first whorl, second whorl)n and (medial first whorl)n, respectively. This interpretation does not affect the organ differentiation model presented, except to show that we may not know what the phenotype of *AP2* expression in a fourth whorl organ might be.

The larger question of the nature of action of the wild-type *AG* product in suppressing indeterminate growth of the floral meristem is left unanswered. It seems that this activity could partially explain the absent organs in whorls 1 and 2 in *ap2* mutants; the absence of AP2 activity in these whorls allows AG to act in them, which could then suppress cell division during their development. This is consistent with the observation that removal of the *AG* product from these *ap2* whorls (in the *ap2-2 ag-1* double mutants) partially restores organ number toward wild-type. The number of second and third whorl

primordia in *ap2-2 ag-1* flowers (average number of 7.6) is closer to the wild-type number (10) than the number found in flowers homozygous for *ap2-2* alone (average 0.25).

Missing organs in mutant flowers result from the failure to initiate organ primordia

In several *ap2* and *pi* alleles, organs fail to develop. For example, second and third whorl organs may be missing in *ap2-2*, *ap2-8*, and *ap2-9* flowers and second whorl organs are often absent in *ap2-1/ap2-2* flowers and *ap2-1* flowers grown at 29°C (Bowman et al., 1989). In each of these instances, as well as in the *pi* alleles, there is no evidence of organ primordia at any stage of development in those positions lacking organs (Figure 2C-F). Previous descriptions of the *ap2-6* and *ap2-7* alleles, however, (Kunst et al., 1989) suggested that second whorl organ primordia of flowers homozygous for these alleles form in wild-type positions and numbers, but fail to develop properly (*ap2-7*) or fuse to the first whorl organs (*ap2-6*). However, the outgrowths of cells described as second whorl organ primordia in *ap2-6* and *ap2-7* flowers may not be organ primordia, but rather stipules (due to the phylloid character of the outer whorl organs; see Figure 11B of Kunst et al., 1989) or squamules, small filamentous outgrowths of cells that have been documented in other species of Brassicaceae (see Figures 5A, B and 11C, D of Kunst et al., 1989; Arber 1931; Smyth et al., 1990) and *ap1-1* flowers (Irish and Sussex, 1990; D. R. S. and J. L. B. unpublished). Stipules arise early in development (stage 3-4 for first whorl organs) while squamules arise much later. Further analysis of developing *ap2-6* and *ap2-7* flowers should clarify this uncertainty.

Predicted spatial and temporal expression patterns

If the homeotic genes act autonomously, certain predictions can be made about their expression patterns. *AP2* should be expressed in the outer two whorls and as early as stage two, prior to the appearance of the first whorl primordia (Bowman et al.,1989).

Likewise, *AG* expression could be restricted to the inner two whorls. The expression of either *AP3* or *PI* or both should be localized in the middle two whorls. *AP3* should be expressed as early as stage five and should continue at least through stage seven and possibly much later (Bowman et al.,1989). The lack of third whorl primordia in *pi-1* flowers suggests that its expression should start prior to stage five, the time at which the third whorl primordia arise. An important prediction is that the expression pattern of *AG* should expand to include all four whorls in an *ap2* background and conversely the expression of *AP2* should be in all whorls in an *ag* background. It should be noted that what is meant by expression is not restricted to the level of transcription since translational or post-translational control are plausible options. In addition, only one of the products needs be restricted to achieve the phenotypic results if one of the products is functionally inactive in the presence of the other.

One of these genes, *AG*, has been recently cloned and encodes a putative transcription factor (Yanofsky et al., 1990). mRNA tissue *in situ* hybridization shows that its expression in wild-type plants is localized to whorls three and four (Yanofsky et al., 1990; Gary N. Drews, J. L. Bowman, and E. M. Meyerowitz, in prep.). Furthermore, in an *ap2-2* mutant background, the range of expression expands to include all whorls, supporting the hypothesis that AP2 negatively regulates *AG* in the outer whorls in the developing flower (G. N. Drews, J. L. Bowman, and E. M. Meyerowitz, in prep.). Another flower homeotic gene, *DEFICIENS*, which when mutated results in a phenotype similar to those of *ap3* and *pi*, has been cloned from *Antirrhinum* (Sommer et al., 1990). This gene is predominantly expressed in petals and stamens although a low level of expression has been reported to occur in sepals and carpels (Schwarz-Sommer et al.,1990).

The timing of initial *AG* and *AP2* expression could play a role in their localization. Tissue *in situs* to mRNA show that *AG* expression is not detectable until stage three, when the first whorl organ primordia become morphologically distinct from the remainder of the developing flower. In contrast, the tsp of the *ap2-1* allele encompasses stages two through four, prior to and during the formation of the first whorl primordia, suggesting that the

initial expression of *AP2* precedes that of *AG*. Since the formation of the four whorls of floral organ primordia is a sequential process, it is likely that the expression patterns of the genes responsible for pattern formation in the developing flower are influenced by this sequential nature. For example, *AP2* could initially be expressed in cells that will give rise to the outer two whorls of organs prior to and during the time of their formation. This would preclude the expression of *AG* in these whorls. *AP2* expression would then be curtailed in the remaining floral meristem due to either loss of positive regulation by some factor that was responsible for its expression in the outer two whorls or negative regulation by a newly expressed factor, such as AG. In this case, *AG* would be expressed in those cells that will give rise to the third and fourth whorl organs precluding *AP2* expression in the inner two whorls. The spatial regulation of AP3 and PI could be attained by having their expression commence only after the first whorl is formed and having a gene such as *SUPERMAN* (Bowman and Meyerowitz, 1990; Meyerowitz et al., 1990) negatively regulate their expression in the fourth whorl. Thus, mutations in *AP2* would be expected to have the most dramatic effects since it is one of the first genes expressed in sequential but overlapping gene pathways specifying both cellular identities and organ primordium patterns.

A candidate for a gene responsible for the regulation of the homeotic genes is *LEAFY* (Haughn and Sommerville, 1988; Detlef Weigel, John Alvarez, and D. R. Smyth, unpublished). When this locus is mutated, the floral meristem behaves as if it were an inflorescence meristem. Each floral meristem produces a number of cauline leaf-like organs in a phyllotactic spiral; the later ones produced are often carpelloid. This phenotype is similar to that of the triple mutant *ap2-2 pi-1 ag-1* flowers in which all organs are carpelloid leaves but, in the case of the triple mutant strain, the organs are arranged in a radial phyllotaxy. Other genes must be involved as well, to produce the complex patterns of expression expected of the later-acting homeotic genes, and the change in phyllotaxy from spiral to whorled.

Evolutionary considerations

Finally it should be noted that mutations similar to those described here exist in many other species of plants. For example, the *agamous* phenotype, which is commonly referred to as a double flower, has been described in *Matthiola* (Dodoens, 1568: see Saunders, 1921), *Cheiranthus* (Masters, 1869), *Petunia* (Sink, 1973), and *Antirrhinum* (Carpenter and Coen, 1990; Schwarz-Sommer et al., 1990) as well as many others (see Penzig 1890-4; Meyer, 1966; Reynolds and Tampion, 1983; Meyerowitz et al., 1989; for reviews). *ap2* homologues have been reported in *Capsella* (Dahlgren, 1919; Shull, 1929) and *Antirrhinum* (Carpenter and Coen, 1990; Schwarz-Sommer et al., 1990) and *ap3/pi* homologues have been described in *Cheiranthus* (Sirks, 1924; Nelson, 1929) and *Primula* (Brieger, 1935) in addition to *Antirrhinum* (Stubbe, 1966; Sommer et al., 1990; Carpenter and Coen, 1990). That numerous mutants homologous to those described here occur in other plant families suggests that the mechanisms governing flower development in *Arabidopsis* are likely to operate in many flowering plants. When genetic experiments similar to those reported here have been done with other species, direct comparisons of the actions and interactions of homeotic genes in these species will be possible, and an assessment can be made of the degree to which these mutations are homologous with those of *Arabidopsis*.

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Table 1. Description of mutants studied.

Gene	Mutant allele	Reference
AGAMOUS	*ag-1*	Bowman *et al.* 1989
	ag-2	Yanofsky *et al.* 1990
	ag-3	Present study
APETALA2	*ap2-1*	Bowman *et al.* 1989, Kunst *et al.* 1989
	ap2-2	Meyerowitz *et al.* 1989
	ap2-8	Present study
	ap2-9	Present study
PISTILLATA	*pi-1*	Bowman *et al.* 1989, Hill & Lord 1989
	pi-2	Present study
	pi-3	Present study
APETALA3	*ap3-1*	Bowman *et al.* 1989

All mutations were induced in the Landsberg *erecta* background except for *ag-2* which resulted from a T-DNA insertion in the Wassilewskija ecotype.

Table 2. Percentage of organs of various types in the first whorl positions of *ap2* mutants

Organ	Genotype (No. of flowers scored)						
	ap2-2 (39)	*ap2-8* (24)	*ap2-9* (48)	*ap2-1/ ap2-2* (36)	*ap2-1/ ap2-9* (26)	*ap2-1** (41)	wild type** (47)
A. Medial first whorl positions							
sepals	0%	0%	0%	0%	0%	0%	100%
leaves	0%	0%	0%	14%	19%	100%	0%
carpelloid leaves	0%	2%	5%	32%	50%	0%	0%
staminoid carpels	37%	60%	40%	0%	0%	0%	0%
carpels	63%	35%	45%	54%	31%	0%	0%
absent	0%	0%	0%	0%	0%	0%	0%
other	0%	2%	9%	0%	0%	0%	0%
B. Lateral first whorl positions.							
sepals	0%	0%	0%	0%	0%	0%	100%
leaves	32%	10%	13%	93%	96%	100%	0%
carpelloid leaves	17%	23%	2%	0%	2%	0%	0%
staminoid carpels	0%	0%	0%	0%	0%	0%	0%
carpels	0%	0%	0%	0%	0%	0%	0%
absent	44%	56%	76%	6%	2%	0%	0%
other	7%	10%	9%	1%	0%	0%	0%

Flowers scored were the first 10-15 produced on the primary inflorescence of plants raised at 25°C.

In the medial positions the organs in the "other" category usually had characteristics of leaves, carpels, and stamens; organs in the lateral positions classified as "other" were usually filamentous structures, either thin leaf-like structures with occasional stellate trichomes or much thinner squamule-like structures (for example, see Fig. 4C).

* from Bowman et al., 1989; ** from Smyth et al., 1990.

Table 3. Percentage of organs of various types in the second whorl positions of *ap2* mutants.

Organ	Genotype (No. of flowers scored)						
	ap2-2 (39)	*ap2-8* (24)	*ap2-9* (48)	*ap2-1*/ *ap2-2* (36)	*ap2-1*/ *ap2-9* (26)	*ap2-1** (41)	wild type** (47)
petals	0%	0%	0%	0%	0%	9%	100%
staminoid petals	0%	0%	0%	1%	3%	37%	0%
petaloid stamens	0%	0%	0%	8%	4%	24%	0%
stamens	0%	0%	0%	4%	24%	2%	0%
absent	100%	100%	100%	87%	69%	29%	0%

Flowers scored were the first 10-15 produced on the primary inflorescence of plants raised at 25°C. White petal shaped organs possessing rudimentary locules were termed staminoid petals and organs shaped like stamens, but with some white petal tissue, usually near the tip, were classified as petaloid stamens. Those organs classified as petals and stamens were morphologically wild type.

* from Bowman et al., 1989; ** from Smyth et al., 1990.

Table 4. Percentage of organs of various dispositions in the third and fourth whorl positions of *apetala2* mutants.

	Genotype (No. of flowers scored)						
	ap2-2 (39)	*ap2-8* (24)	*ap2-9* (48)	*ap2-1/ ap2-2* (36)	*ap2-1/ ap2-9* (26)	*ap2-1** (41)	wild type** (47)
third whorl stamens (% present)	8%	4%	14%	93%	95%	87%	96%
fourth whorl carpels fused normally	28%	42%	42%	100%	100%	100%	100%

Flowers scored were the first 10-15 produced on the primary inflorescence of plants raised at 25°C.

* from Bowman et al., 1989; ** from Smyth et al., 1990.

Table 5. Percentage of organs of various types in the six third whorl positions of *pistillata* and *apetala3* mutants.

Organ	Genotype (No. of flowers scored)					
	*pi-1** (75)	*pi-1*/*pi-2* (40)	*pi-2* (15)	*pi-3* (27)	*ap3-1** (15)	wild type** (47)
stamens	0%	0%	0%	0%	29%	96%
filamentous structures	14%	83%	54%	16%	<1%	0%
carpelloid organs	0%	5%	12%	66%	53%	0%
absent	86%	12%	33%	16%	18%	4%

Flowers scored were the first 10-15 produced on the primary inflorescence of plants raised at 25°C. In the case of the *pi* mutants, many of the organs were fused with the central gynoecium. The apparent absence of many third whorl organs in the strongest allele, pi-1, may reflect their complete incorporation into the fourth whorl gynoecium.

* from Bowman et al., 1989; ** from Smyth et al., 1990.

Table 6. Summary of Phenotypes of *Arabidopsis* Flowers

whorl:	first (medial/ lateral)	second	third	fourth
wild type	sepals	petals	stamens	carpels
ag-1, -2, -3	sepals	petals	petals	another flower
ap2-1	leaves	staminoid petals	stamens	carpels
ap2-2, -8, -9	carpels/ absent	absent	absent or stamens	carpels
ap2-1/ap2-2	carpels/ leaves	absent	stamens	carpels
pi-1	sepals	sepals	absent	extra carpels
ap3-1, pi-2, -3	sepals	sepals	carpels	carpels
ap2-1 ap3-1, ap2-1 pi-2, ap2-1 pi-1	leaves	carpelloid leaves	carpels or absent	carpels
ap2-2 pi-1,	carpels/ absent	absent	absent	carpels
ap2-2 ap3-1	carpels/ absent	absent	absent or carpels	carpels
ap2-1 ag-1	leaves	staminoid petals	staminoid petals	another flower
ap2-2 ag-1	carpelloid leaves/ absent	petaloid stamens	petaloid stamens	another flower
ag-1 pi-1	sepals	sepals	absent	another flower
ag-1 ap3-1, ag-1 pi-2	sepals	sepals	sepals	another flower
ap2-1 ag-1 ap3-1, ap2-1 ag-1 pi-2, ap2-1 ag-1 pi-1	leaves	leaves	leaves or absent	another flower
ap2-2 ag-1 pi-1	carpelloid leaves/ absent	absent	absent	another flower

Figure 1. Phenotypes of wild-type and mutant *Arabidopsis* flowers.

(A) *wild type*

(B) *agamous-2*

(C) *apetala2-2*

(D) *apetala2-9*

(E) *pistillata-1/pistillata-2*

The plants were grown at 25°C. Bar = 1 mm.


A
B
D
C
E

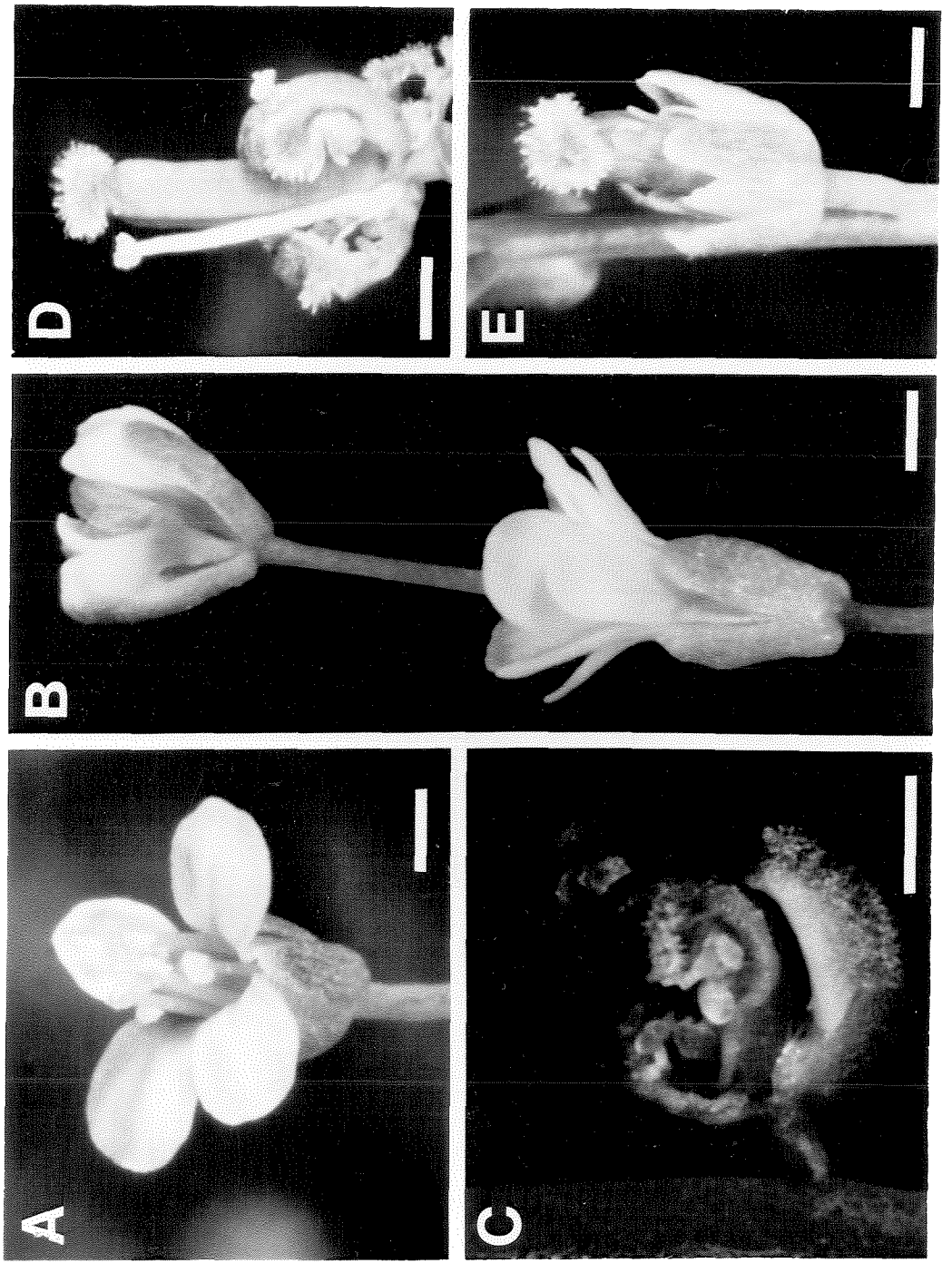
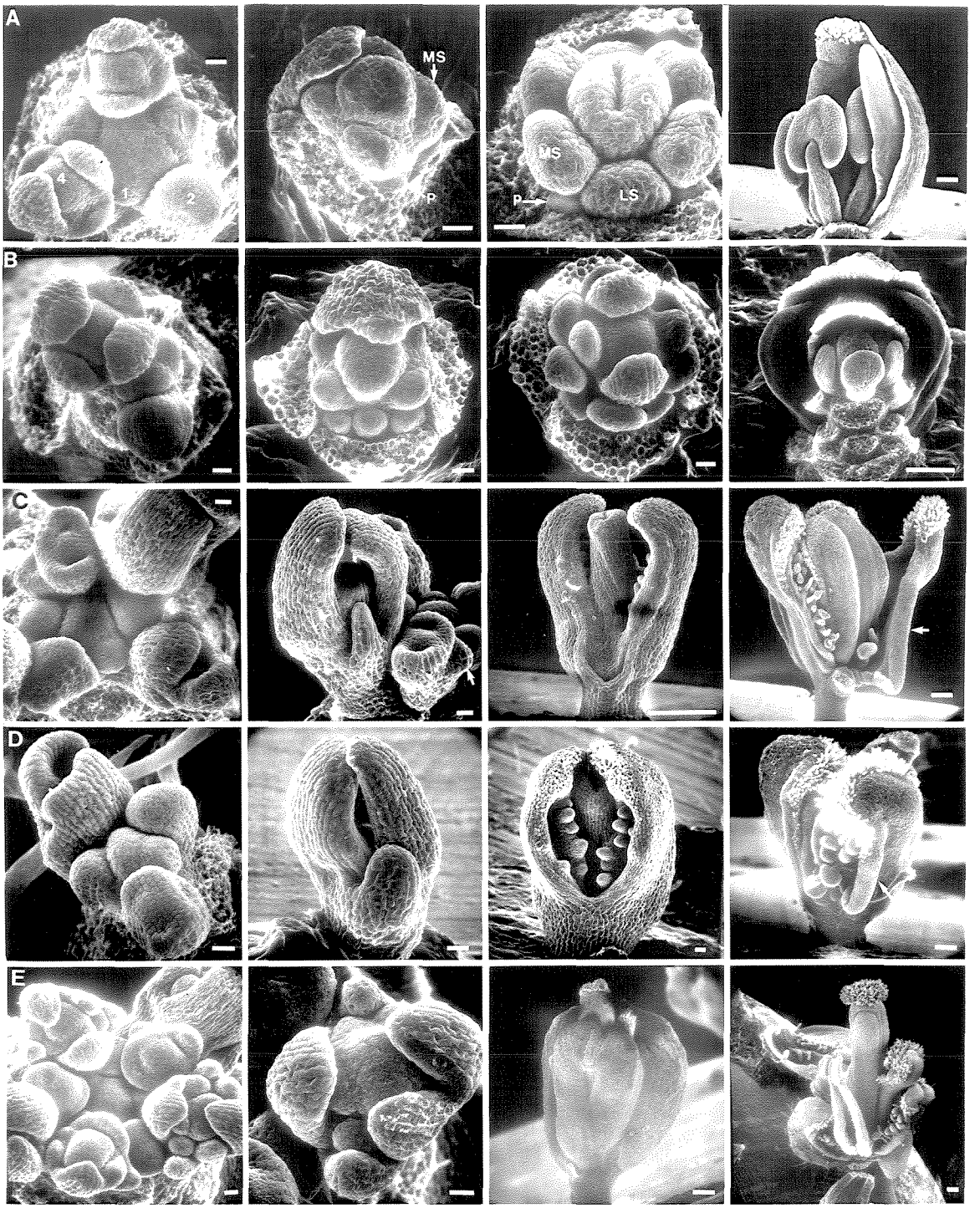

A
B
C
D
E

Figure 2. Scanning electron micrographs depicting the development of wild-type and mutant *Arabidopsis* flowers. The first (left) panel in each row displays the apical meristem and the early stages of flower development (stages 1 to 4 or 5). The second and third panels show successively older developing flowers, and the fourth panel shows flowers nearing maturity. First and second whorl organs have been removed in most cases to reveal the underlying organs. The positions of medial and lateral are with respect to the apical (primary inflorescence) meristem. Growth conditions for each were 25°C and 24 hour illumination except E which was 16°C. Bar = 10 mm in the first two panels ; 10 mm in the third panels of A, B and D; 100 mm in the third panels of C, E, F and G; 50 mm in the fourth panel of B and 100 mm in the remaining fourth panels.

(A) Wild type. In the first panel, the stage of some of the individual flower primordia is indicated. Petal (P) and medial stamen (MS) primordia are indicated on the stage six flower in the second panel. One lateral stamen primordium is not present, as often seen in wild-type flowers (Müller, 1961; Smyth et al., 1990). Petal (P), medial stamen (MS), lateral stamen (LS), and the gynoecial (G) primordia are indicated on the stage 7 flower in the third panel. The fourth panel shows a stage 12 flower (3 sepals and 2 petals have been removed) in which the cell types of each of the four floral organs are visible.

(B) *agamous-2*. Note the internal flowers in panels three and four. First whorl organs have been removed in the second and third panels and organs in the first four whorls were removed from the flower in the fourth panel.

(C) *apetala2-2*. Medial first whorl organ primordia are enlarged relative to wild type (first panel). A lateral first whorl primordium (arrow) is indicated in the second panel; its position is lower on the pedicel than is seen in wild type (A). The central gynoecium of *ap2-2* flowers is often twisted (third panel) and usually fails to develop normally (fourth panel). A stamen-carpel mosaic in a medial first whorl position is indicated in the fourth panel (arrow).


A
MS
4
1
2
P
MS
P
LS
B
C
D
E

(D) *apetala2-8*. The two medial first whorl organs are enlarged relative to wild type (first panel) and develop into solitary carpels with prominent ovules and stigmatic tissue (third panel) or develop into carpel-stamen mosaic organs (arrow in fourth panel).

(E) *apetala2-9*. Organ primordia of *ap2-9* flowers grown at 16°C do not appear in the normal number or position, as seen in the second panel. The outer whorl organ primordia may congenitally fuse to form a ring-like structure as shown in the third panel.

(F) *apetala2-1/apetala2-2*. When fewer than the normal number of organ primordia form in the second and third whorls, those that do arise occur in ectopic positions as seen in the second panel. The lack of organs in most second whorl positions is due to the failure to form organ primordia (second panel). A nectary is noted in the third panel (arrow) in the normal lateral position; the lateral first whorl organ has been removed. Note the different phenotypes of the first whorl organs of the flower in panel four; the lateral ones are leaf-like (L) while the medial ones are solitary carpels (C).

(G) *pistillata-2*. Some third whorl organ primordia are apparently congenitally fused to the developing gynoecial cylinder, as seen in the second panel. When the third whorl organ primordia are free, they often develop into filamentous structures, indicated in the fourth panel (arrow). Second whorl sepals are visible in the third and fourth panels.

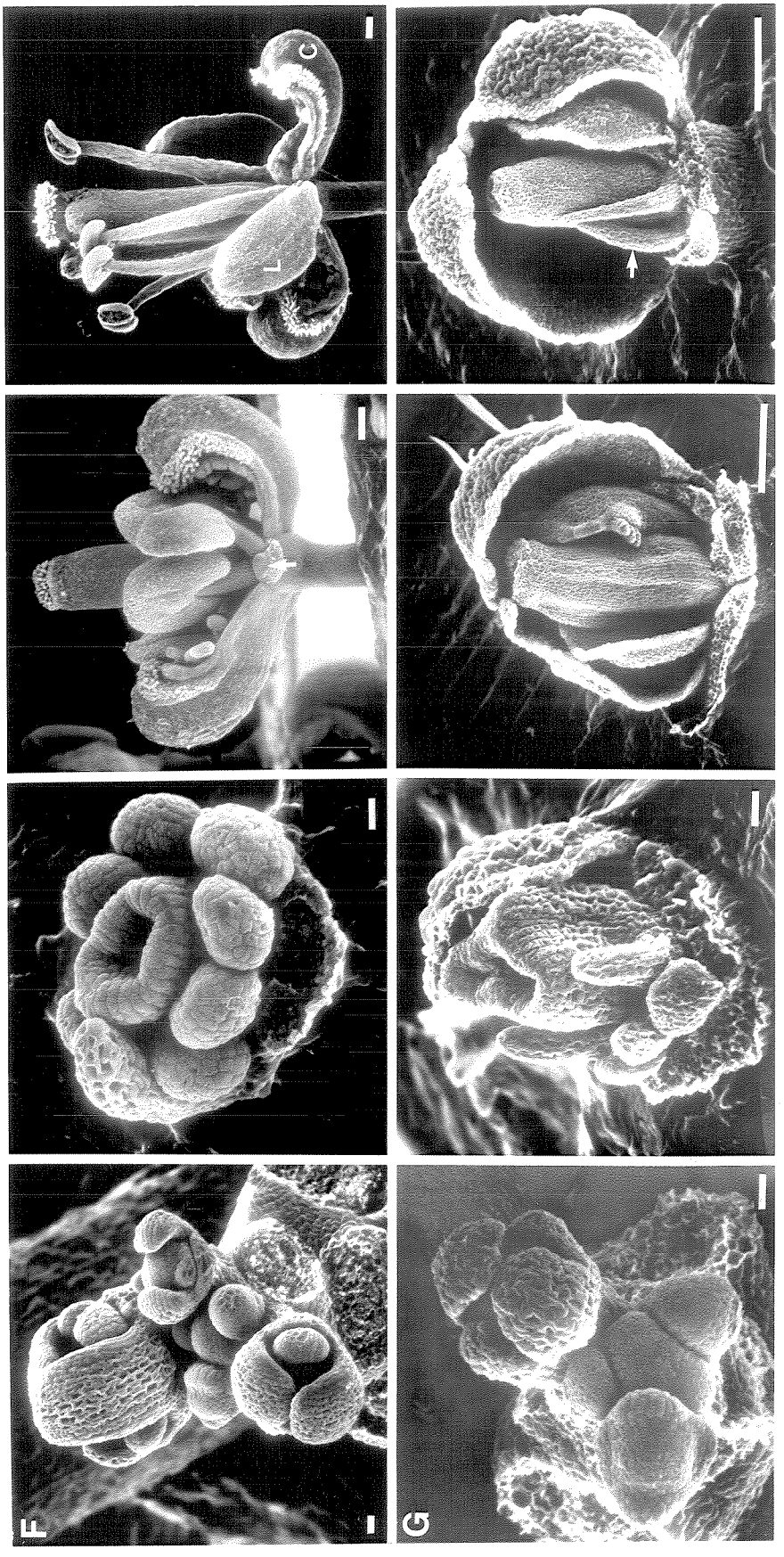
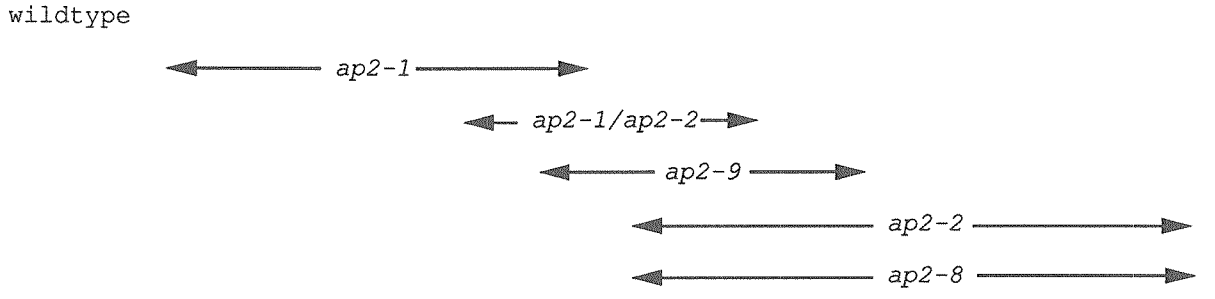

F
G

Figure 3. The range of phenotypes of each of the described *ap2* alleles is displayed. The left end of the arrow for each allele represents its phenotype at 16°C whereas the right end represents its phenotype at 29°C. The phenotype (medial first whorl, lateral first whorl, second whorl, third whorl, fourth whorl) for any particular allele is read from top to bottom. For example, the phenotype of *ap2-1/ap2-2* flowers ranges from (carpelloid leaves, leaves, stamens/absent, stamens, carpels) at 16°C to (carpels, leaves/absent, absent, stamens/absent, carpels) at 29°C.

Figure 3. ***ap2*** **allelic series.**



medial first whorl	sepals leaves carpelloid leaves phylloid carpels carpels absent
lateral first whorl	sepals leaves leaves/absent absent
second whorl	petals petaloid stamens stamens/ absent absent
third whorl	stamens stamens/absent absent
fourth whorl	carpels unfused carpels

Figure 4. Scanning electron micrographs of *apetala2* double mutant combinations. The developmental series displayed in the four panels are the same format as those described for Figure 2. All plants grown at 25°C. Bar = 10 mm in the first three panels (except the third panel of C = 50 mm), 100 mm in the fourth panel, and 100 μm in the inset of the fourth panel of C.

(A) *apetala2-1 pistillata-1*. The pattern of organ primordia is severely disrupted; all of the cells that would ordinarily give rise to the second and third whorl primordia can be recruited into an abnormal structure composed of both leaf (L) and carpel (C) tissue like that seen in the second and fourth panels. The outer whorl organs are cauline leaf-like as evidenced by the presence of stellate trichomes and stipules (arrows in third panel).

(B) *apetala2-1 apetala3-1*. The pattern of second and third whorl organ primordia is disrupted (second panel).

(C) *apetala2-2 pistillata-1*. Medial first whorl primordia are enlarged relative to wild type and the lateral first whorl organs arise lower on the flower primordium. The medial first whorl organs are fused to each other very early in development (panels one and two) to form a cylinder, and the remaining floral meristem appears to be incorporated into this gynoecial cylinder. An ovary composed of four carpels usually results, as seen in the second and fourth panels. Lateral first whorl organs may be missing (panels one and four), or they may be leaf-like organs (panels two and three), or filamentous structures (arrow in panel four). Nectaries may occur in the axils of these organs (arrow in the inset of panel four). The apical meristem is visible at the lower left of the fourth panel.

(D) *apetala2-1 agamous-1*. The positions of the organ primordia are similar to that of *ag-1* flowers. Those primordia arising in the second and third whorls develop into staminoid petals (P/S).

(E) *apetala2-2 agamous-1*. The number of organs in the second and third whorls (8 in each of the flowers shown in the second and third panels) is greater than ever seen in *ap2-2* flowers (Figure 2C). These organs do not always arise in the proper positions; in both the

second and the third panels, four of the organs seem to occupy the medial third whorl positions (arrows) but the other four organs are not in positions normally occupied in wild-type flowers. The organ primordia arising from these positions develop into petaloid stamens with conspicuous locules (panel four). These organs are much more stamen-like than those in the corresponding positions of *ap2-1 ag-1* flowers. The medial first whorl organs are carpelloid leaves with stigmatic tissue at their tips (panel four). The apical meristem is visible in the lower left of the fourth panel. The flower in panel two appears to have only two fourth whorl organs, in lateral positions, while the fourth whorl of the flower in the third panel consists of four organs.

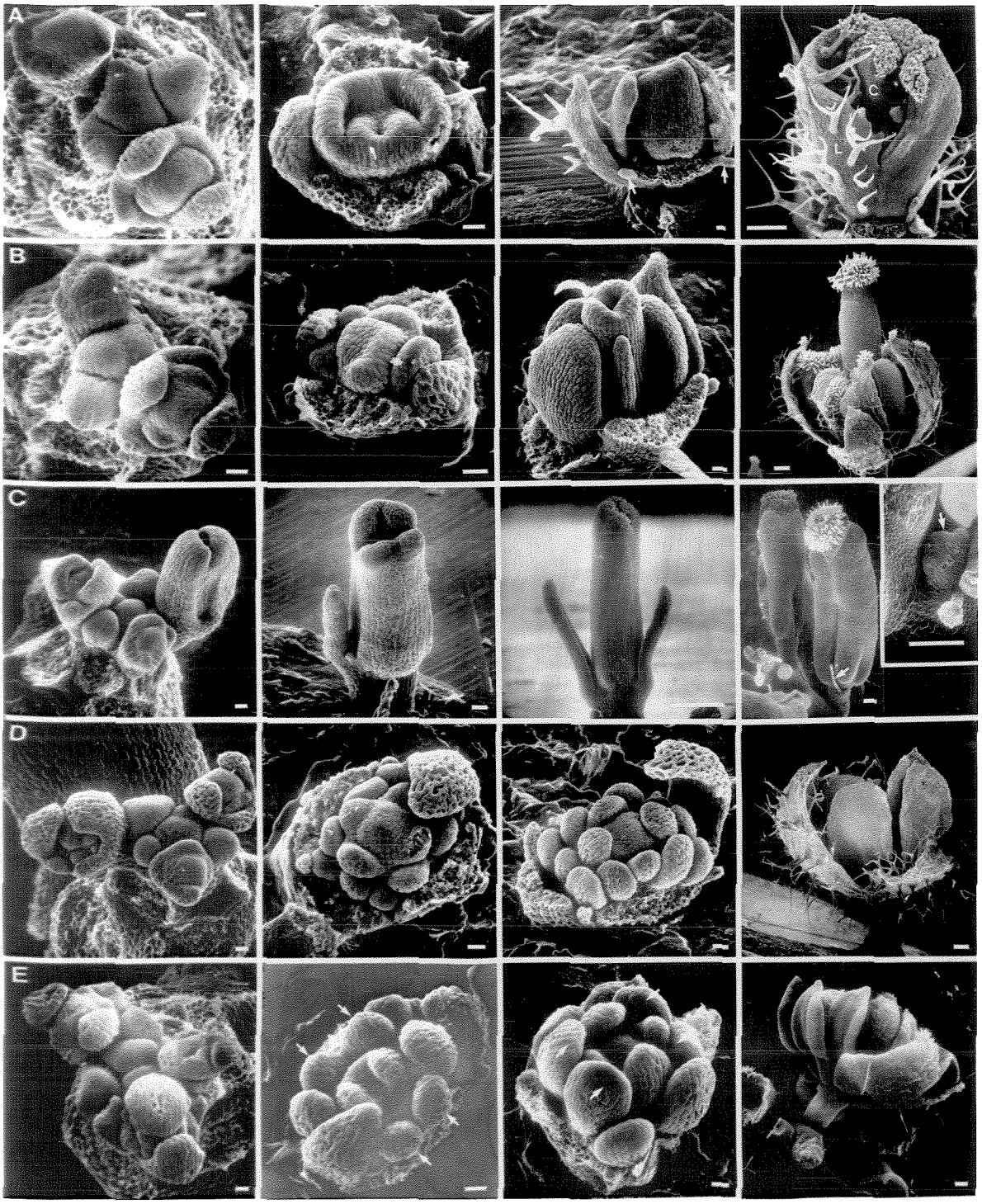

A
B
C
D
E

Figure 5. Phenotypes of double mutant strains.

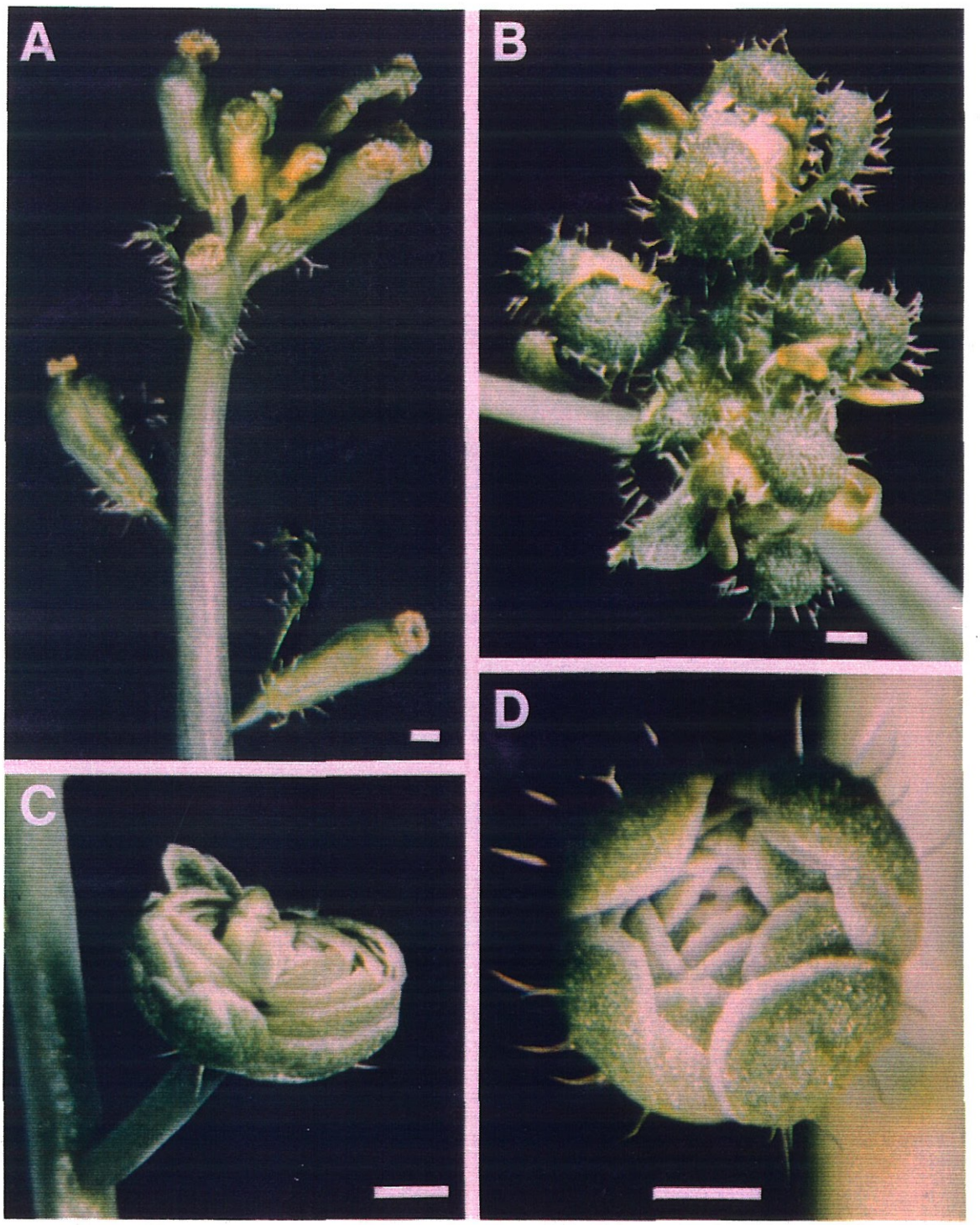
(A) *apetala2-2 pistillata-1*

(B) *apetala2-2 agamous-1*

(C) *agamous-1 pistillata-1*

(D) *agamous-1 apetala3-1*

Plants grown at 25°C. Bar = 1 mm.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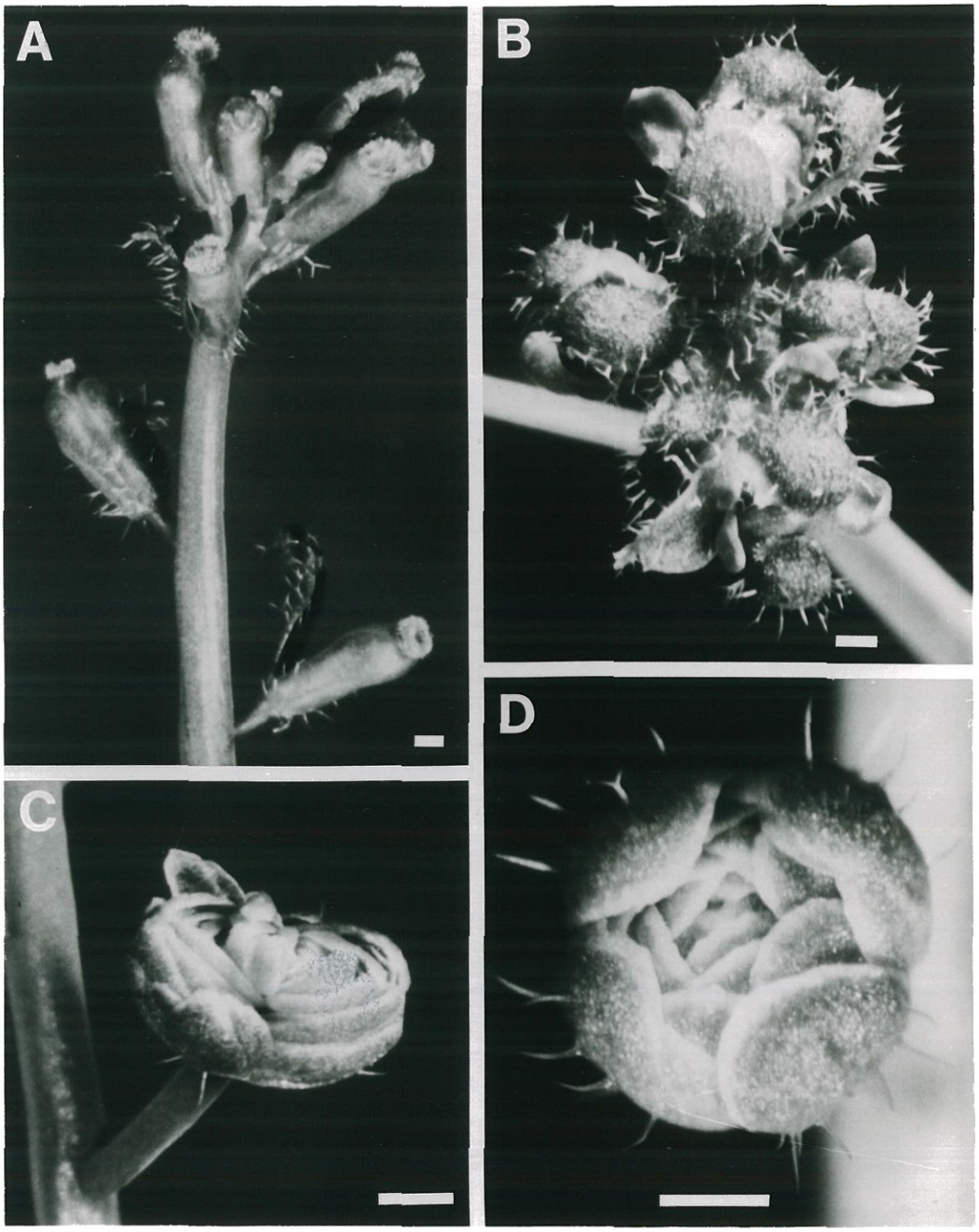
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Figure 6. Development of *agamous-1 pistillata-1* and *agamous-1 apetala3-1* flowers. The developmental series displayed are the same format as those described for Figure 2. *ag-1 pi-1* plants were grown at 25°C while the *ag-1 ap3-1* plants were grown at 20°C. Bar = 10 mm in the first three panels and 100 mm in the fourth panel.

(A) *ag-1 pi-1*. After proper formation of the first two whorls of organ primordia, the third whorl organ primordia fail to form (as observed in *pi-1* flowers) and the remaining floral meristem behaves as if it is another flower primordium, as seen in the third panel. This process repeats indeterminately producing a flower with multiple whorls of sepals in alternate positions (fourth panel).

(B) *ag-1 ap3-1*. Pattern of organ primordia is the same as that seen in *ag-1* flowers (panel three). Due to the temperature sensitivity of the *ap3-1* mutation, the second and third whorl organs are petaloid sepals (panel four) at the intermediate growth temperature (20°C) used.

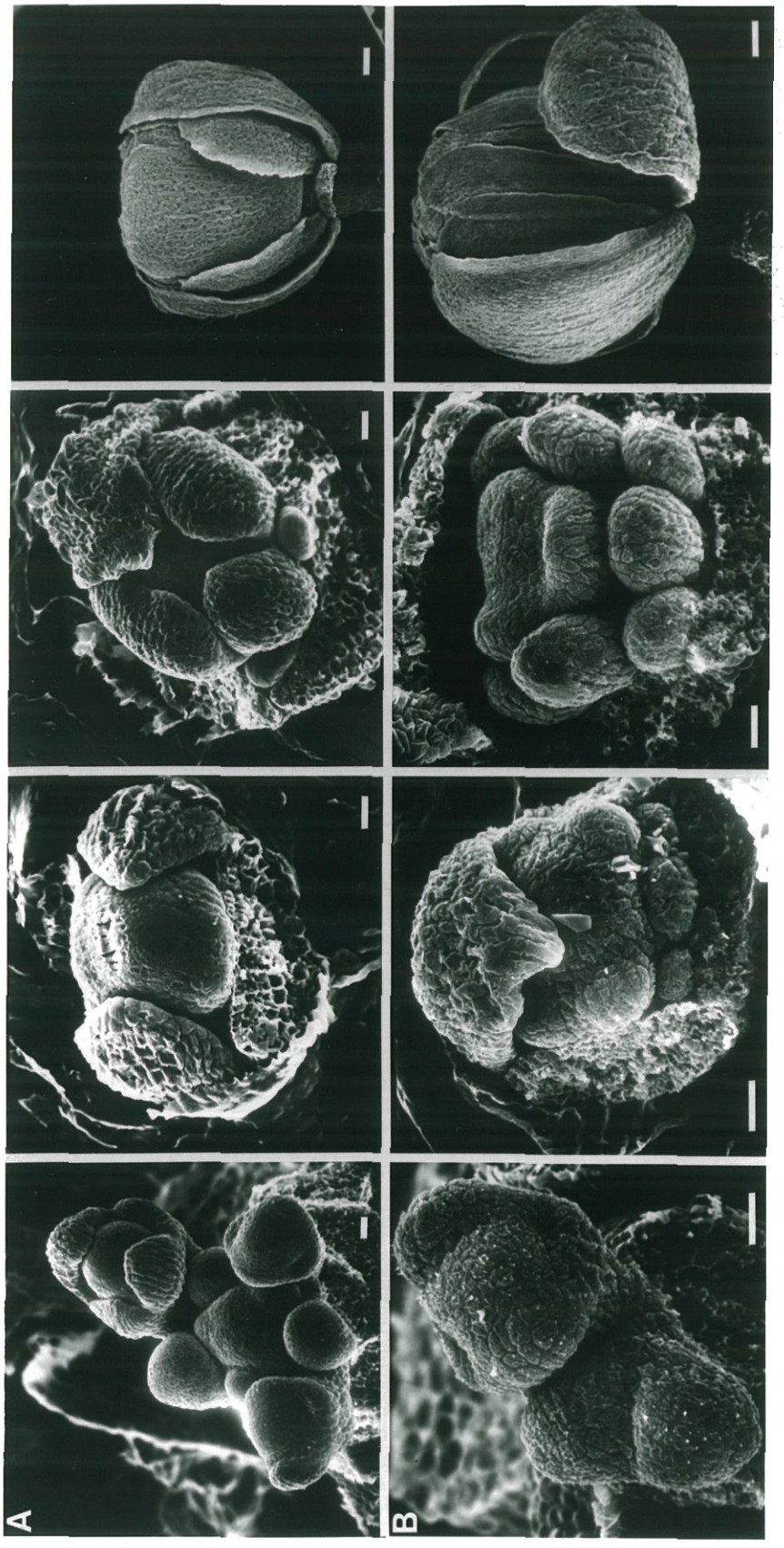

A
B

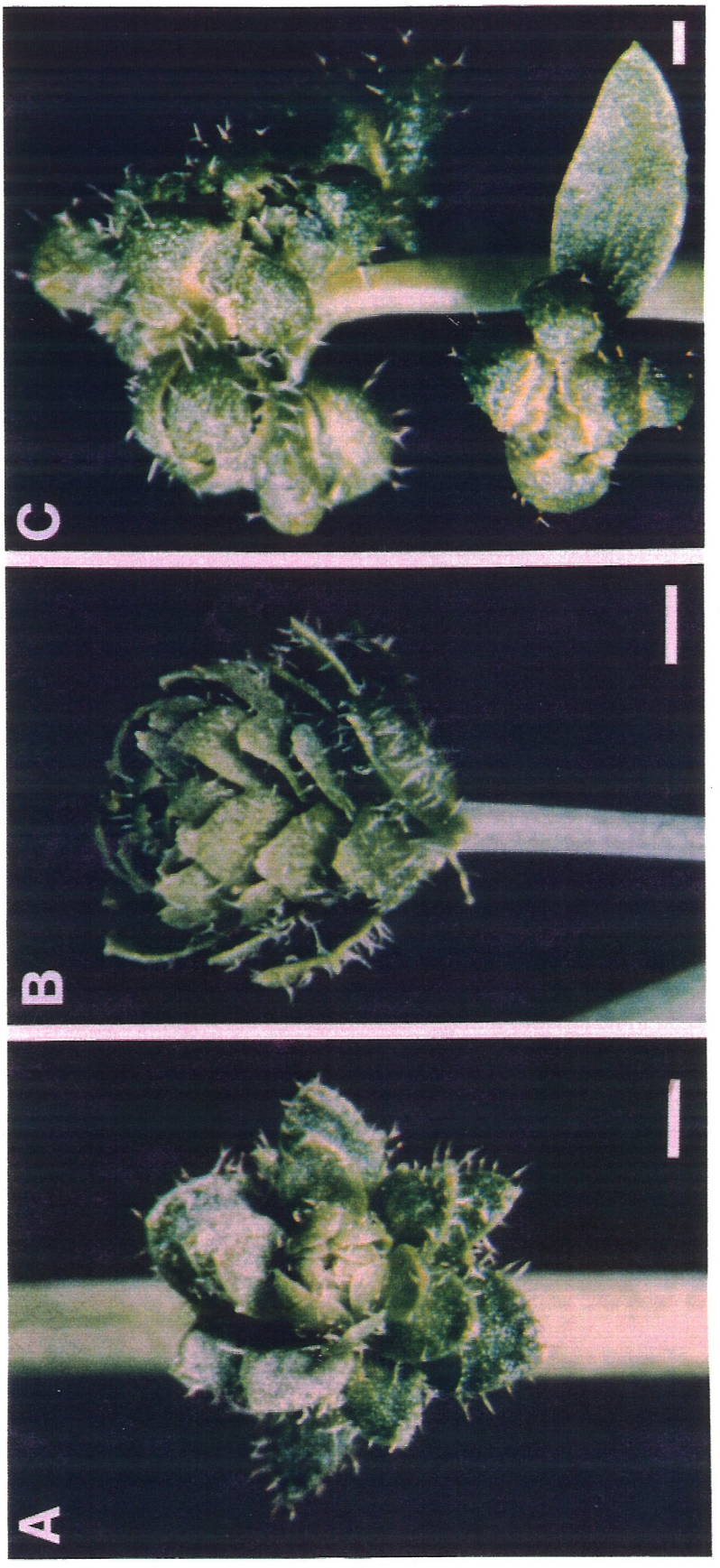
Figure 7. Phenotypes of triply mutant flowers.

(A) *apetala2-1 agamous-1 apetala3-1*

(B) *apetala2-1 agamous-1 pistillata-1*

(C) *apetala2-2 agamous-1 pistillata-1*

All plants grown at 25°C. Bar = 1 mm.


A
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C

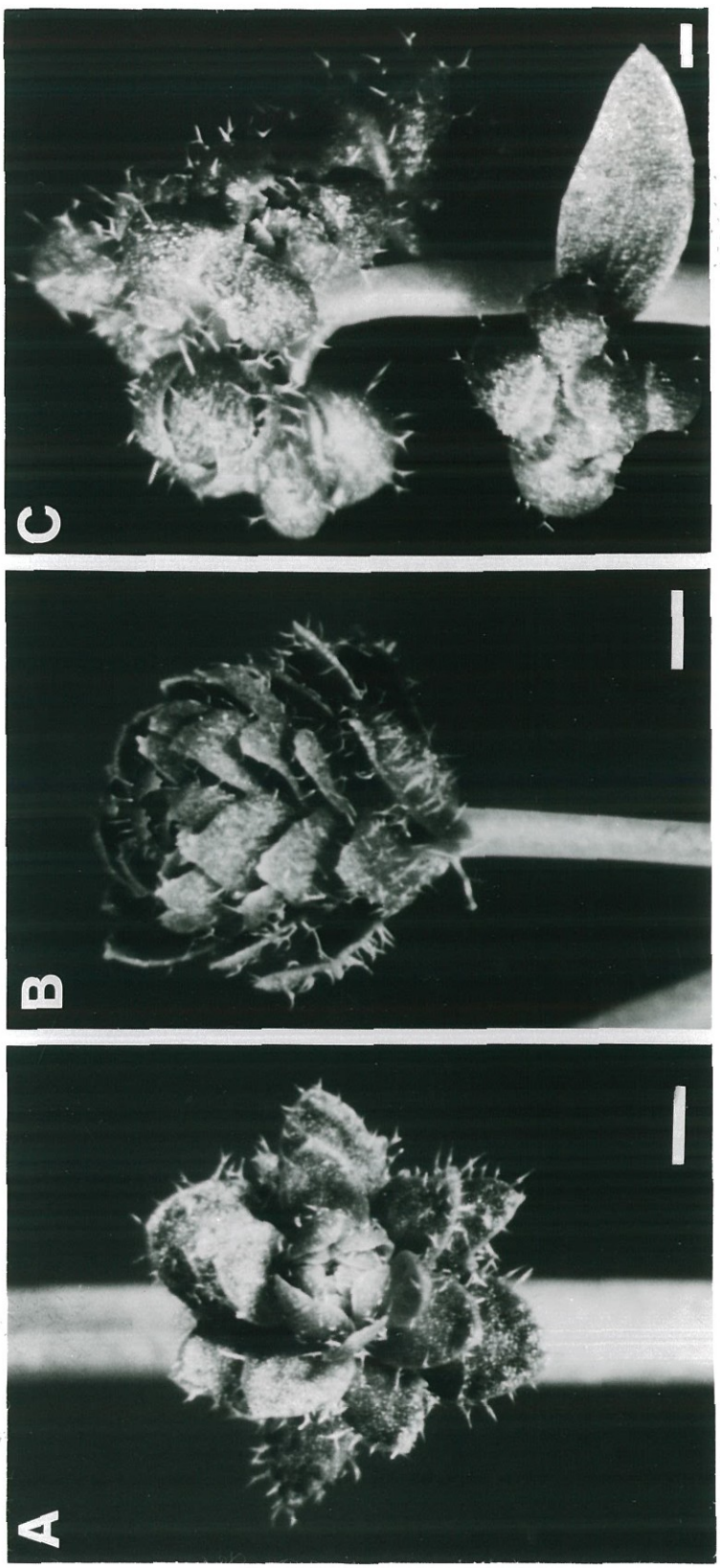

A
B
C

Figure 8. Development of triply mutant flowers. The developmental series displayed for each genotype is the same format as that described in Figure 2. All plants were grown at 25°C. Bar = 10 mm in the first three panels and 100 mm in the fourth panel.

(A) *apetala2-1 agamous-1 apetala3-1.* Second and third whorl organ primordia may form in abnormal positions and sometimes appear to be fused, as seen in the second panel. Each develops into a cauline leaf-like organ with stellate trichomes on its abaxial surface and stipules at its base (arrows in third panel). Developing stipules can also be seen in the third panel.

(B) *apetala2-1 agamous-1 pistillata-1.* The pattern of organ primordia is similar to that seen in *ag-1 pi-1* flowers. Stipules can be seen at the base of both the outer whorl organs in panel three and at the base of organs in the inner whorls in panel four (arrows).

(C) *apetala2-2 agamous-1 pistillata-1.* A flower with two medial and two lower lateral first whorl primordia can be seen in the first panel. The second panel shows a flower from the lateral view; two medial first whorl organs have been removed to expose the two lateral primordia (arrows) arising interior to the first whorl. A lateral view of a slightly older flower is shown in the third panel; two medial first whorl organs have been removed as well as a lateral organ interior to the first whorl. Two additional medial organ primordia (M) and another lateral organ primordia (L) interior to all the previously produced primordia are visible. The fourth panel displays a mature flower with one lateral first whorl leaf-like organ (L), and all of the remaining organs carpelloid leaves.

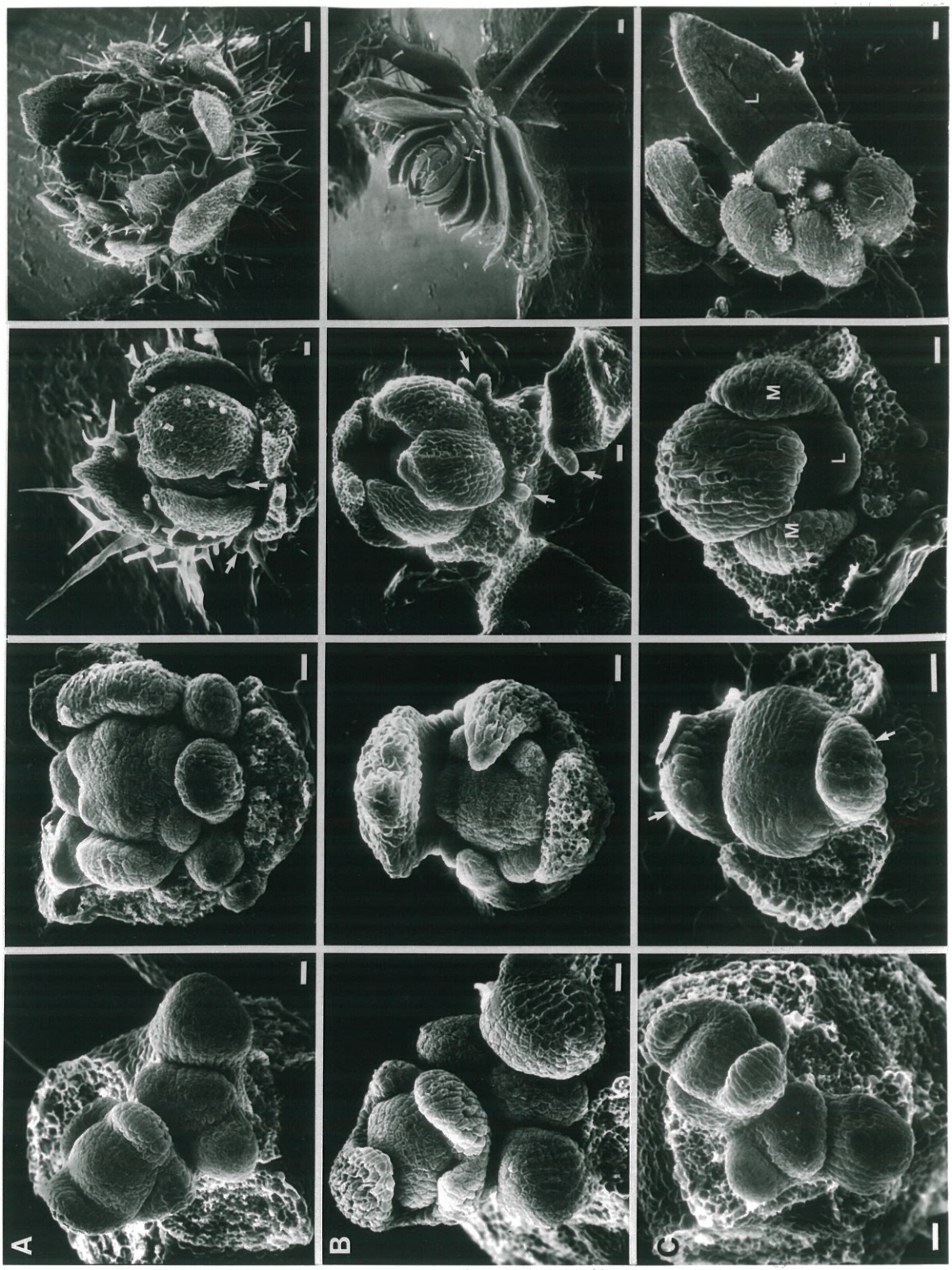
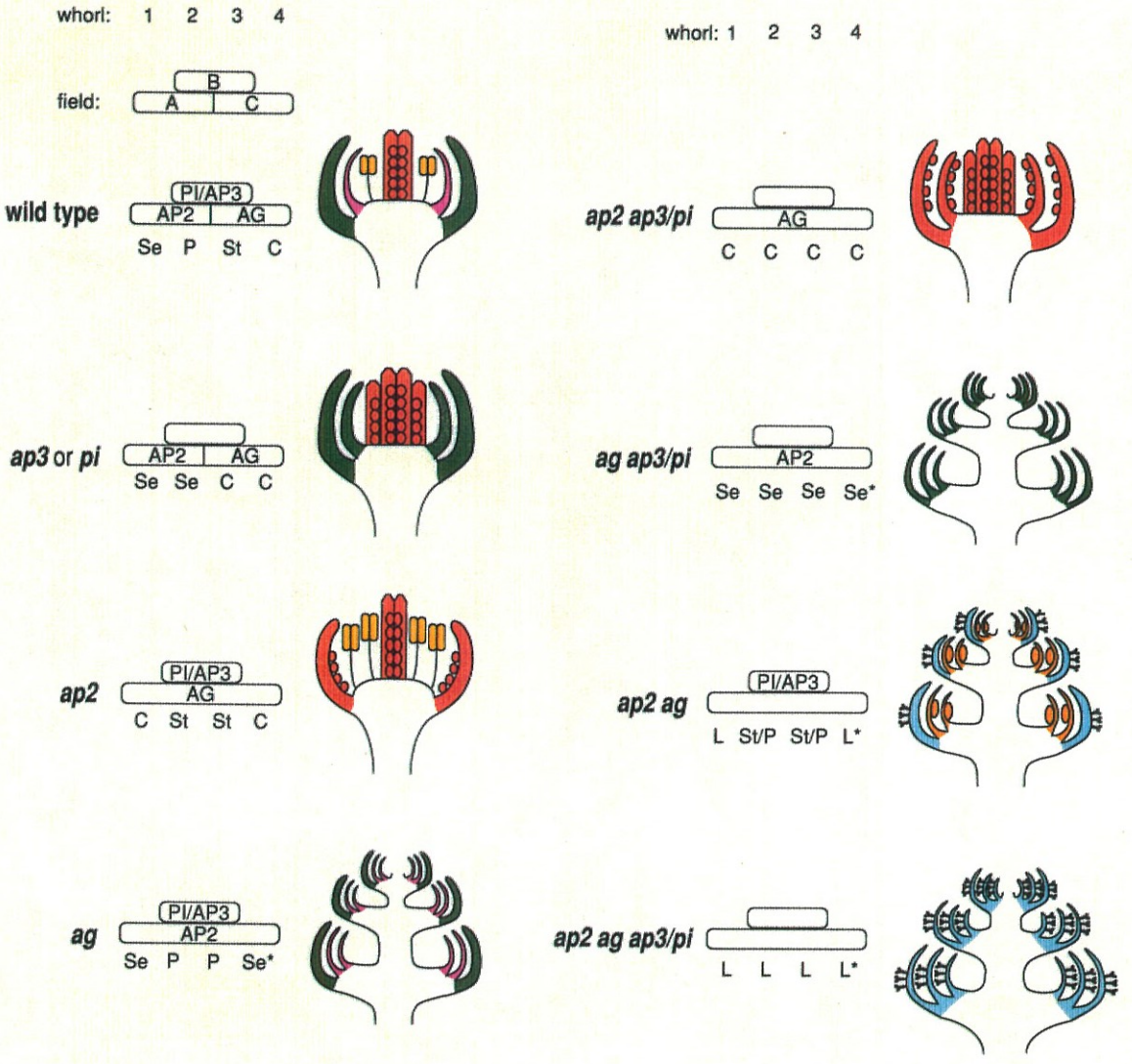

A
B
C
L
M
M
L

Figure 9. A schematic representation of the model depicting how three classes of floral homeotic genes could specify the identity of each of the four whorls of floral organs. A section through one-half of a floral primordium is represented as a set of boxes, with the regions representing each whorl shown at the top of each column. Each box represents a single field (defined in the Discussion); these are also shown at the top of the first column. The genotype under consideration is listed at the left with the predicted distribution of gene products present in each genotype indicated by uppercase letters within the boxes. The predicted phenotype of the organs in each whorl are shown under the diagrams. Se = sepal, P = petal, St = stamen, C = carpel. P/St = petaloid stamen, an organ, present in *ap2 ag* flowers, with characteristics of both petals and stamens. L = leaf or carpelloid leaf, either can be found in the positions indicated depending upon the allele of *ap2* present. The * is a reminder that in each genotype containing *ag*, there are several whorls of organs interior to the fourth whorl. A schematic drawing of a longitudinal cross section of each genotype of flower is depicted on the right. Each of the organ types is color coded: sepals = green, petals = pink, stamens = yellow, carpels = orange, petaloid stamens = gold, and leaves and carpelloid leaves = blue. The whorls interior to whorl nine in the *ag* containing genotypes are not shown.


whorl: 1 2 3 4
field:
B
A
C
wild type
PI/AP3
AP2
AG
Se P St C
ap3 or pi
AP2
AG
Se Se C C
ap2
PI/AP3
AG
C St St C
ag
PI/AP3
AP2
Se P P Se*
whorl: 1 2 3 4
ap2 ap3/pi
AG
C C C C
ag ap3/pi
AP2
Se Se Se Se*
ap2 ag
PI/AP3
L St/P St/P L*
ap2 ag ap3/pi
L L L L*

CHAPTER FOUR

THE PROTEIN ENCODED BY THE *ARABIDOPSIS* HOMEOTIC GENE *AGAMOUS* RESEMBLES TRANSCRIPTION FACTORS

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ARTICLES

The protein encoded by the *Arabidopsis* homeotic gene *agamous* resembles transcription factors

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Mutations in the homeotic gene *agamous* of the plant *Arabidopsis* cause the transformation of the floral sex organs. Cloning and sequence analysis of *agamous* suggest that it encodes a protein with a high degree of sequence similarity to the DNA-binding region of transcription factors from yeast and humans and to the product of a homeotic gene from *Antirrhinum*. The *agamous* gene therefore probably encodes a transcription factor that regulates genes determining stamen and carpel development in wild-type flowers.

ARABIDOPSIS THALIANA is widely used for molecular and genetic studies of many developmental processes in plants[1,2]. We have focused on the molecular basis of pattern formation in developing *Arabidopsis* flowers. Flower development remains one of the most complex processes unique to plants, and little is known about the molecular mechanisms involved. Flowers develop from a few undifferentiated cells into a structure that has several types of organs occupying precisely defined positions. *Arabidopsis* has typical mustard flowers (Fig. 1*a*) consisting of four concentric whorls of organs[3]. The first or outermost whorl consists of four green sepals. Interior to and alternating with the sepals are four white petals which make up the second whorl. The third whorl consists of six stamens, four long and two short, each of which has a filament capped by a pollen-bearing anther. The fourth and innermost whorl consists of two fused carpels capped by a short style and stigmatic papillae.

Many mutations affecting floral morphogenesis have been identified in *Arabidopsis*. Mutations in at least four genes result in homeotic transformations of floral organs[1,3-7]. In general, these homeotic mutations affect the development of adjacent whorls of floral organs. For example, the *ap2-1* mutation results in the conversion of the sepals into leaf-like organs, and in the conversion of petals into stamen-like organs[3]. Mutations in the *AP3* (*ap3-1*) or *PI* (*pi-2*) genes result in the conversion of petals into sepals and stamens into carpels. Mutations in the *agamous* (*AG*) gene result in the overall phenotype of a flower within a flower and the absence of stamens and carpels. The six stamens have been replaced in *ag* mutant flowers by six petals, and the carpels have been replaced by a new flower. Thus, *ag* flowers consist of ten petals (four normal and six in the place where stamens are normally found) inside of the four sepals, and inside the petals are again four sepals and ten petals (Fig. 1*c*). This pattern repeats itself and the resulting flower can consist of more than 70 organs. Other species of plants with similar double flower phenotypes were recognized as long ago as 2,000 years[8]. The first published report of *Arabidopsis* flowers with an *ag* mutant phenotype was more than a century ago[9], and another *Arabidopsis* mutant having similar flowers has been described by Conrad[10]. The extensively characterized[3] mutant allele, *ag-1*, was isolated after ethylmethane sulphonate (EMS) mutagenesis and was first described by Koornneef *et al.*[11]. The *AG* locus has been mapped to chromosome 4 (ref. 11).

Here we describe the molecular cloning and characterization of the *AG* gene, which was facilitated by a T-DNA insertion mutation[12]. The deduced AG protein product is similar to transcription factors from humans (SRF) and yeast (MCM1, ARG80), and to the product, DEF A, of a recently isolated homeotic gene from the snapdragon *Antirrhinum majus*.

Insertion mutant of *agamous*

Recently, a method has been developed to tag genes by insertion of the T-DNA from *Agrobacterium tumefaciens*[13]. One of the

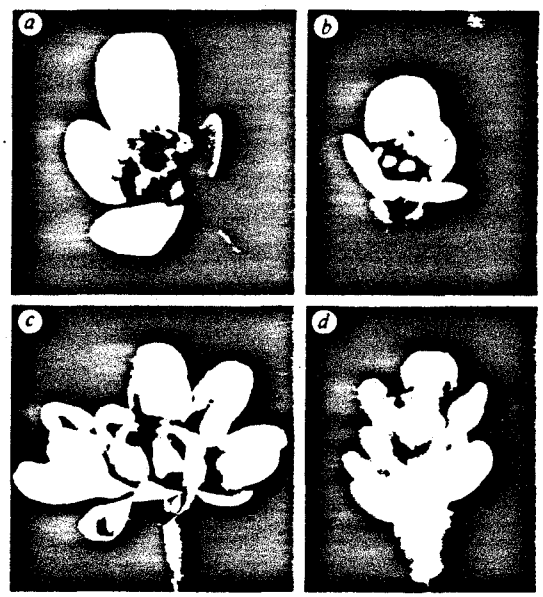

FIG. 1 Photographs of *Arabidopsis* flowers. *a*, Wild-type; *b*, *ag-2* plants transformed with pCIT540; *c*, *ag-1*; *d*, *ag-2*. Complementation of the *ag-2* mutation was tested by introducing the cosmid pCIT540 into the *Agrobacterium* strain ASE (ref. 26). This strain was used to infect leaf pieces of homozygous *ag-2* mutant plants by standard methods[27]. A regenerated plant that displayed wild-type flower morphology is shown here.

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mutants isolated by this approach (Fig. 1*d*) displays a phenotype that closely resembles that of *ag-1* mutant plants (Fig. 1*c*). Like *ag-1*, this insertional mutant lacks stamens and carpels and has the overall phenotype of a flower within a flower. The only noticeable difference between the insertional mutant and *ag-1* mutant plants is that the pedicels of inner flowers in the former are elongated, resulting in a greater separation of internal flowers. The elongated pedicel is not due to the insertional mutation, but is the result of different genetic backgrounds (M.F.Y., H.M., J.L.B., G.N.D., K.A.F. and E.M.M., unpublished results). To demonstrate whether the insertional mutation is an allele of *AG*, plants heterozygous for *ag-1* were crossed to plants heterozygous for the insertional mutation (homozygous mutant plants could not be used because they are sterile). As the *ag-1* and insertional mutants are both recessive to wild-type, and as a quarter of the resulting F1 progeny produced *ag* mutant flowers, this confirms that the two mutations are allelic. We have named the insertional allele *ag-2*.

Genetic analyses indicate that the kanamycin resistance marker encoded by the T-DNA co-segregates with the mutant flower phenotype (data not shown). This, together with the demonstration that the insertion mutation is allelic to *ag-1*, indicates that the T-DNA can be used as a molecular probe to isolate the *AG* gene. Plasmid rescue was used to isolate sequences flanking the insertion site and one of the resulting clones, pCIT505, contained plant sequences and was analysed further (Fig. 2*a*). To determine whether these sequences were derived from the *AG* genomic region, the genetic map position of the

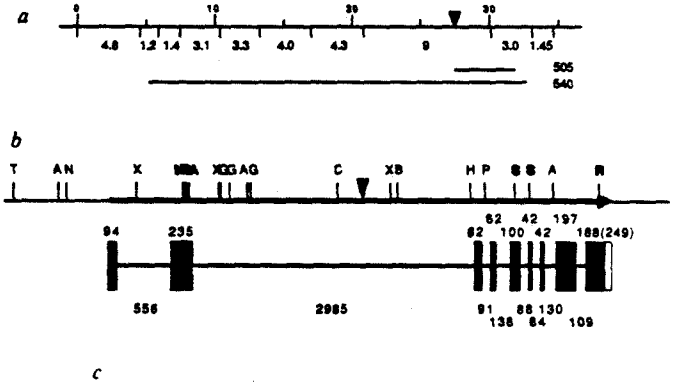

FIG. 2 *a*, Map of the *agamous* region. *Eco*RI restriction sites are shown for a 35-kb region from the Columbia ecotype flanking the *AG* gene. Size of fragments is in kb. ▼, site of T-DNA insertion in the *ag-2* mutant. Below the restriction map, horizontal lines indicate the region contained in the plasmid-rescued clone pCIT505, and the cosmid pCIT540. *b*, Structure and restriction map of *AG* gene. The diagram below the restriction map is of introns (lines) and exons (shaded boxes); size in nucleotides is given. One cDNA extended an additional 61 nucleotides (open box). Restriction sites include: A, *Sca*I; B, *Bam*HI; C, *Nco*I; H, *Hin*dIII; N, *Nde*I; S, *Sac*I; R, *Eco*RI; T, *Pst*I; X, *Xba*I. Restriction sites in outline indicate sites in exon sequences. ▼, site of the 35-kb T-DNA insertion in *ag-2* allele. Arrow, direction of transcription. *c*, Nucleotide sequence of 4*G* cDNAs and the deduced amino-acid sequence for the open reading frame. Nucleotide 1 corresponds to the first nucleotide in the longest isolated cDNA and nucleotide 1,043 is the site of polyadenylation. The region preceding nucleotide 1 was derived from genomic sequence. ▼, position of intron sequences as deduced from a comparison of cDNA and genomic sequence data. *, position of the translational initiation codon for each of the *B. napus* genes.

METHODS. Cosmids were isolated from genomic libraries of nuclear DNA prepared from the Columbia and Landsberg *erecta* ecotypes of *Arabidopsis*. This DNA was partially digested with *Sau*3A, size fractionated by sucrose gradient centrifugation, ligated to the vector pCIT30 and packaged. Plasmid pCIT30 was derived from the plasmid pMON200 (ref. 26) by replacing the kanamycin resistance gene with a fragment that encodes hygromycin resistance. Plasmid rescue was achieved by digesting DNA from homozygous *ag-2* mutant plants with *Sal*I, followed by ligation and transformation into *Escherichia coli*. The digest with *Sal*I produces a fragment containing an *E. coli* origin of replication and the ampicillin resistance (Amp^r) gene encoded by the T-DNA, as well as plant sequences flanking the insertion[28]. Plasmid pCIT505 was placed on the RFLP map of Chang *et al.*[14] using an *Eco*RI RFLP and found to reside at about the same position as *ag-1*. To map directly pCIT505 relative to *ag-1* recombinants derived from a cross between Landsberg *erecta* (Ler) and Niederzenz (Nd-O) ecotypes that included the *ag-1* mutation were analysed. Plants heterozygous for the *ag-1* mutation (*ag-1* (Ler)/+(Ler)) were crossed to plants that were wild-type for *AG* in the Nd-O background (+(Nd-O)/+(Nd-O)). The resulting F1 progeny that were heterozygous for the *ag-1* mutation (*ag-1* (Ler)/+(Nd-O)) were allowed to self-fertilize and DNA was isolated from the F2 plants that displayed the *ag* phenotype (*ag-1*/*ag-1*). DNA blots were prepared and hybridized with probes revealing *Eco*RI and *Bgl*II RFLPs. The cDNA library was prepared from 2 μg poly(A)$^+$RNA isolated from immature floral buds[3,20] before stage 12 using the Stratagene kit. After size-selection of the cDNA (>400 bp) *Eco*RI linkers were added before ligating to the vector λgt10 and packaging using Gigapack gold (Stratagene). Many cDNA clones (3×10^6) were obtained, a portion of which were screened with a probe derived from the region flanking the T-DNA insertion site in *ag-2* mutant plants. Twenty seven of the longest independently isolated cDNAs were subcloned and fully or partially sequenced. DNA sequence analysis of one of these clones, pCIT559, demonstrated that it contained the fourth (138 base) intron. All of the other cDNAs seemed to represent the same fully processed RNA. Sequence analysis was performed on double-stranded plasmids using the USB Sequenase Kit. Both strands of cDNAs and the corresponding genomic region were sequenced.

sequences in pCIT505 was determined by restriction fragment length polymorphism (RFLP) analysis[14]. No crossovers were observed between a pCIT505-linked polymorphism (Fig. 2*a*) and the *ag-1* mutation in 118 meiotic products scored, indicating that this cloned segment maps less than two centimorgans from the *AG* gene. Taken together, these results suggest that the T-DNA is inserted in or near the *AG* gene and that we have recovered DNA sequences from this region. The pCIT505 plasmid was then used as a probe to screen a cosmid library of wild-type *Arabidopsis* sequences and several clones were isolated and characterized. One of these cosmids, pCIT540 (Fig. 2*a*), when introduced into the genome of homozygous *ag-2* mutant plants, complemented the mutation and the resulting flowers appeared to be of wild type, with stamens and carpels (Fig. 1*b*). As the T-DNA insertion has been stably maintained through eight generations, and the introduced sequences restore a wild-type flower phenotype to this stable strain, it seems that this region indeed contains the wild-type *AG* gene.

Nucleotide sequence

Restriction fragments spanning the T-DNA insertion site were used to probe a complementary DNA library prepared from RNA isolated from wild-type *Arabidopsis* flowers. Only one class of cDNA clones spanned the T-DNA insertion site. The nucleotide sequence of several overlapping cDNA clones and the corresponding genomic region was determined (Fig. 2*c*). These results indicate that this gene contains at least nine exons ranging in size from 42 to 235 bases, and eight introns ranging in size from 84 to 2,985 bases (Fig. 2*b*). Sequence analysis of the T-DNA-plant junction fragment indicates that the foreign DNA in the *ag-2* allele is inserted in the 2,985-base intron (Fig. 2*b*).

To confirm that these sequences represent the *AG* gene, genomic fragments were isolated and sequenced from the EMS-generated mutant *ag-1*. The *ag-1* mutation was induced in the same genetic background, Landsberg *erecta*, from which we have isolated and characterized the wild-type sequences. This analysis revealed a single nucleotide change that is presumably responsible for the *ag-1* mutant phenotype. This nucleotide substitution changes the AG dinucleotide to AA at the acceptor site of the fourth (138-base) intron shown in Fig. 2*b*.

The deduced protein product contains at least 285 amino-acid residues, although from analysis of the sequences it seems that none of the cDNA clones are of full length. As the deduced open reading frame continues to the 5′ end of the longest cDNA, and a translational initiation codon has not been identified, the full-length AG protein could be slightly longer. But many attempts with primer extension, anchored polymerase chain reaction and the screening of two different cDNA libraries have been unsuccessful at obtaining longer clones. Nearly all of the isolated cDNAs end within a region of a few nucleotides, suggesting that the RNA may have a secondary structure that prevents extension beyond this region. As the longest cDNAs are of 1,043 base pairs (bp) and the polyadenylated messenger RNA is close to 1.1 kilobases (kb) in length (see below), these cDNAs are of nearly full length. We have recently isolated the two presumed *AG* homologues from the allotetraploid mustard *Brassica napus* and each of these genes contains a translational initiation codon corresponding to the TCG codon at nucleotides 112–114 of the *AG* cDNA sequence (M.F.Y., H.M. and E.M.M., unpublished results). This indicates that all the evolutionarily conserved sequences of the *Arabidopsis* gene have been identified.

The deduced *AG* amino-acid sequence was used to search the database of protein sequences using the program of Lipman and Pearson[15]. The AG protein shares significant sequence similarity to a class of transcription factors from humans[16] and yeast[17,18], and to the product of a recently isolated *Antirrhinum* homeotic gene[19] (see Fig. 3).

```
                    10        20        30        40        50
                    |         |         |         |         |
AG          52 RGKIEIKRIENTTNRQVTFCKRRNGLLKKAYELSVLCDAEVALIVFSSRGRLYEYS
DEF A        3 RGKIQIKRIENQTNRQVTYSKRRNGLFKKAHELSVLCDAKVSIIMISSTQKLHEYI
SRF        144 RVKIKMEFIDNKLRRYTTFSKRKTGIMKKAYELSTLTGTQVLLVASEKGWVYTFA
MCM1        18 RRKIEIKFIENKTRRHVTFTKRKNGIMKKAFELSVLTGTDVLLLVVSETGLVYTFS
ARG80       80 RRKQPIKYIENKTRRHVTFSKRRHGIMKKAYELSVLTGANILLLIANSGLVYTFT

AG/DEF A        RGKI IKRIEN TNRQVT  KRRNGL KKA ELSVLCDA V IV SS  L EY   41/56
AG/MCM1         R KIEIK IEN T R VTF KR  G  KKA ELSVL    V L V S  G Y  S  32/56
AG/SRF          R KI    I N  R  TF KR  G  KKAYELS L    V L V S  G Y     25/56
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FIG. 3 Amino-acid sequence comparison (single-letter code). An alignment is shown for the deduced amino-acid sequences for the gene products from *Arabidopsis thaliana* (AG), *Antirrhinum majus* (DEF A), humans (SRF) and yeast (MCM1, ARG80). Identical residues between AG and each of DEF A, MCM1 and SRF are shown below, as are the fractions of identical amino acids shared by these proteins. Numbers to the left of each sequence indicate the position of the first amino acid shown for each protein.

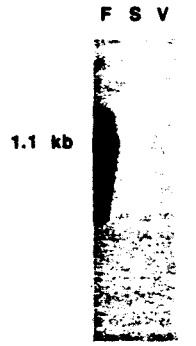

FIG. 4 RNA blot. An autoradiogram is shown for an RNA blot hybridized with ^{32}P-labelled *AG* cDNA. RNA was from immature floral buds (lane F), floral stems (lane S) and from vegetative tissue (lane V).
METHODS. Poly(A)$^+$RNA was isolated from *Arabidopsis thaliana* Landsberg (*er*) as previously described[29] and 2 μg each RNA type was size-fractionated on a 1.5% formaldehyde agarose gel, transferred to Hybond N nylon membrane (Amersham), and hybridized with a radiolabelled gel-isolated DNA fragment from the *AG* cDNA, pCIT565. Hybridization was in 50% formamide, 5 × SSC buffer, 10 × Denhardt's buffer, 100 μg ml^{-1} single-stranded salmon sperm DNA and 10% Dextran sulphate. The filter was washed three times in 2 × SSC, 1% SDS at room temperature and then four times in 0.2 × SSC, 1% SDS at 58 °C. The flower RNA was prepared from a collection of young floral buds before stage 12 (before the flowers open)[3,20]. Vegetative RNA was obtained by growing plants in a liquid culture for ~18 days before collection; the resulting tissue consisted of roots, stems and leaves. Floral stem RNA was prepared from floral stems with cauline leaves. The vegetative and floral stem RNA preparations also contain a small amount of floral RNA as these tissues contain immature stage-3 flowers[20]. To prove that RNA was present in the floral stem and vegetative lanes, this RNA blot was also hybridized with a cDNA specific for the *Arabidopsis* G-protein gene *GPA1* (ref. 30); this clone hybridized more strongly to the stem and vegetative RNA (lanes S and V) than to flower RNA.

RNA analysis

To study the pattern of *AG* gene expression, poly(A)$^+$ RNA was isolated from several different tissue types and hybridized with an *AG* cDNA. An RNA from flowers (length ~1.1 kb) hybridized with the *AG* cDNA probe (Fig. 4, lane F). After a much longer exposure, a similarly sized transcript was detected in RNA from inflorescence stems and whole plants before the appearance of the primary inflorescence stem (Fig. 4, lanes S and V), although the intensity of this signal was about 1% of that in flowers. The plant tissue used for these RNA isolations did contain a small number of early developing flowers[20], and

it is possible that this low level of signal is the result of RNA in these developing flowers. We estimate the level of *AG* RNA to be $\sim 10^{-4}$ in poly(A)$^+$ RNA of flowering apices, on the basis of both reconstruction experiments and the frequency of cDNA isolation.

To determine if *AG* expression is organ-specific, *in situ* hybridizations were performed. Sections of wild-type *Arabidopsis* flowers were prepared and probed with ^{35}S-labelled RNA synthesized from the *AG* cDNA template (Fig. 5). These results indicate that *AG* is expressed in stamens and carpels and not (or at much lower levels) in sepals or petals.

Discussion

We have isolated and characterized a flower-specific *Arabidopsis* homeotic gene, *AG*. The insertion mutant allele, *ag-2*, and the EMS-induced mutant allele, *ag-1*, both display alterations in the genomic region from which the presumed *AG* RNA is transcribed. The changes in the mutant alleles seem to interfere with normal transcription or splicing.

The deduced sequence of the AG protein shows striking similarity to the sequences of transcription factors from both humans (SRF, ref. 16) and yeast (encoded by *MCM1*, ref. 17, and *ARG80*, ref. 18), and to the deduced amino-acid sequence of a recently isolated homeotic gene (*defA*) from the flowering plant *Antirrhinum majus*[19]. In humans, the serum response factor (SRF) is thought to be required for the serum-inducible transcriptional activation of genes such as c-*fos*, a nuclear proto-oncogene of mammals[16]. In yeast, the product of the *MCM1* gene (GRM/PRTF) is a transcriptional regulator of mating-type-specific genes[21]. The region shared by these proteins spans ~56 amino-acid residues and, allowing for conservative substitutions, *AG* is ~80% similar in this region to all of these proteins (Fig. 3). This same region of SRF encodes the DNA-binding and dimerization domains[16,22]. On the basis of this sequence similarity and the *ag* phenotype we propose that the *AG* protein is a transcription factor involved in regulating genes that determine stamen and carpel development in wild-type flowers.

Like those of *Arabidopsis*, flowers of *Antirrhinum* consist of four concentric whorls of organs (sepals, petals, stamens and carpels). Mutations in the *Antirrhinum defA* homeotic gene cause the conversion of petals into sepals and stamens into carpels. This phenotype does not resemble that of *ag*, but closely resembles the phenotype of the *ap3* and *pi* mutants of *Arabidopsis*[3], suggesting that the *Antirrhinum defA* gene could be the homologue of either the *AP3* or *PI* gene of *Arabidopsis*. The *AG* gene product displays a high degree of similarity to DEF A in the DNA-binding region (Fig. 3), but not elsewhere. As the phenotypes of *Arabidopsis ag* and *Antirrhinum defA* mutants are very different, it seems likely that these genes function in completely different regulatory steps in flower development. The AG and DEF A proteins are more similar to each other than either is to the SRF, MCM1 and ARG80 proteins (Fig. 3).

We have recently isolated a family of other genes from *Arabidopsis*, some of which are preferentially expressed in flowers, that all share extensive sequence similarity to the DNA-binding regions of the SRF-MCM1 class of transcription factors (H.M., M.F.Y. and E.M.M., unpublished results). This indicates that there is a family of related genes that may act to control many of the steps in organ development in *Arabidopsis* flowers. This situation might be analogous to animal development where specific DNA-binding motifs have been adopted for different regulatory circuits. For example, *Drosophila* homeotic selector genes share the homeobox motif, the *Drosophila* gap genes all encode zinc-finger-type proteins, and genes involved in neurogenesis share the helix-loop-helix motif[23,24]. The MCM1[21] and SRF[25] gene products interact with other factors in regulating gene expression, and it is therefore possible that both AG and DEF A also interact with other factors to exert their regulatory control; some of these factors may be encoded by other homeotic genes known to exist in *Arabidopsis* and *Antirrhinum*.

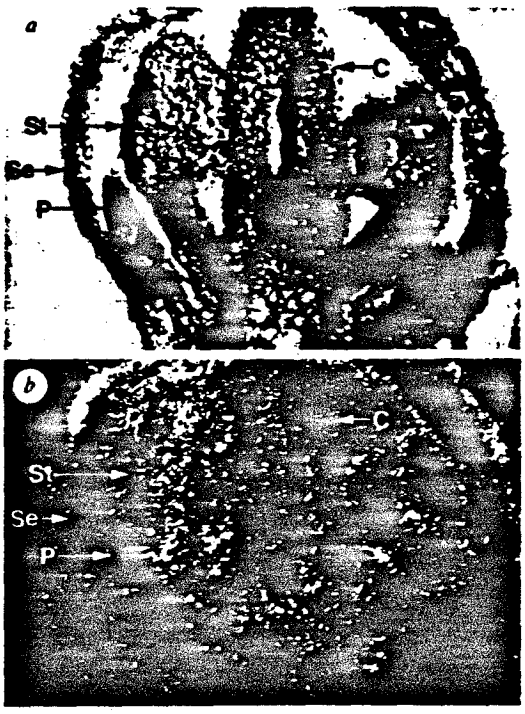

FIG. 5 *In situ* hybridization of *AG*. To determine the pattern of *AG* expression, ^{35}S-labelled antisense RNA was synthesized from an *AG* cDNA template (pCIT565), and hybridized to 8 μm longitudinal sections of stage-9 *Arabidopsis* flowers[3,20] as previously described[31]. *a*, Bright field and *b*, dark field. Arrows indicate the position of floral organs including Se (sepals), P (petals), St (stamens) and C (carpels). Hybridization of antisense RNA to the *AG* mRNA is localized to the positions of stamens and carpels and not to sepals and petals, indicating that it is in these organs that the *AG* gene is preferentially expressed. The signal over the upper portion of the sepals is observed both with sense and antisense RNA probes and therefore does not represent specific hybridization.

In addition, we used RNA blot analysis to identify the 1.1-kb *AG* mRNA and found it to be preferentially expressed in flowers. *In situ* hybridization experiments show that this RNA is localized to the stamens and carpels of wild-type flowers, the same organs that are lacking in *ag* mutants. It will be interesting to determine if the pattern of *AG* gene expression is altered in any of the other *Arabidopsis* homeotic flower mutants. □

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CHAPTER FIVE

NEGATIVE REGULATION OF THE ARABIDOPSIS HOMEOTIC GENE *AGAMOUS* BY THE *APETALA2* PRODUCT

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Summary

We used in situ hybridization to characterize the distribution of *AGAMOUS* (*AG*) RNA during early flower development in Arabidopsis. Mutations in this homeotic gene cause the transformation of stamens to petals in floral whorl 3, and of carpels to another *ag* flower in floral whorl 4. We found that *AG* RNA is present in the stamen and carpel primordia but is undetectable in sepal and petal primordia throughout early wild-type flower development, consistent with the mutant phenotype. We also analyzed the distribution of *AG* RNA in *ap2* mutant flowers. *AP2* is a floral homeotic gene that is necessary for the normal development of sepals and petals in floral whorls 1 and 2. In *ap2* mutant flowers, *AG* RNA is present in the organ primordia of all floral whorls. Thus, the wild-type *AP2* product appears to be a negative regulator of *AG* RNA accumulation in whorls 1 and 2 of wild-type flowers. This observation shows that the expression patterns of the Arabidopsis floral homeotic genes is in part established by regulatory interactions between these genes.

Introduction

Very little is known about the molecular processes governing pattern formation and cellular differentiation in plants. The flower provides a good system to address these problems because flowers consist of an invariant pattern of several organ types. Furthermore, mutations affecting the pattern of floral organs have been identified in several plants (reviewed by Meyerowitz et al., 1989), indicating that flower pattern is genetically controlled. We have been using the flower of Arabidopsis thaliana as a system to study the genetic control of flower pattern. The organs of this flower are arranged in four concentric whorls designated whorls 1 (outermost whorl) through 4 (innermost whorl). As shown in Figure 1, whorls 1 through 4 contain four sepals, four petals, six stamens, and two carpels, respectively. Within each whorl, the organs are found at specific positions relative to each other, to the organs in the other whorls, and to the apical inflorescence meristem. To achieve this pattern, the cells of the developing flower primordium must somehow assess their position relative to others, and subsequently differentiate appropriately. The molecular mechanisms by which this positional information is specified are largely unknown.

To understand the processes by which the fates of the organ primordia are specified, our laboratory and others have been characterizing the phenotypes of the homeotic mutations *agamous* (*ag*), *apetala1* (*ap1*), *apetala2* (*ap2*), *apetala3* (*ap3*), and *pistillata* (*pi*) (Pruitt et al., 1987; Komaki et al., 1988; Bowman et al., 1988, 1989, 1991; Meyerowitz et al., 1989; Hill and Lord, 1989; Kunst et al., 1989; Irish and Sussex, 1990). All of these mutations cause the cells of specific organs to misinterpret their position, and consequently, to develop into types inappropriate for their position (Table 1). The phenotypes of the homeotic mutants indicate that the wild-type products of the corresponding genes are essential for the specification of organ fate during flower development. Genetic experiments have led to a model by which four of the floral

homeotic genes (*AG*, *AP2*, *AP3*, and *PI*) act alone and in combination to specify the identities of the floral organs (Bowman et al., 1991).

One of the Arabidopsis homeotic genes, *AG*, has recently been cloned (Yanofsky et al., 1990). Several mutations (*ag-1*, *-2*, *-3*) in this gene cause whorls 3 and 4 to develop abnormally (Table 1). In whorl 3, six petals develop in the positions normally occupied by stamens. In whorl 4, the cells that would normally give rise to the gynoecium instead form another *ag* flower. This process repeats itself indeterminately, resulting in the formation of >70 floral organs in the pattern $(\text{sepals, petals, petals})_n$ (Pruitt et al., 1987; Bowman et al., 1989; Bowman et al., 1991). Therefore, *AG* appears to be involved in the processes that establish the identities of organs in whorls 3 and 4. These genetic experiments indicate that the realm of action of *AG* is whorls 3 and 4 in wild-type flowers. However, when combined with an *ap2* mutation, *ag* mutants exhibit phenotypic effects in the first and second whorls that are not observed in *ap2* single mutants (Bowman et al., 1991). Thus, in *ap2* mutants, the realm of action of *AG* appears to be all four floral whorls. To explain this observation, it has been proposed that *AP2* is a negative spatial regulator of *AG* activity in whorls 1 and 2, such that *AG* activity is confined to whorls 3 and 4 in wild-type flowers, but that *AG* activity is present in all floral whorls in *ap2* mutants.

Hybridization experiments with an *AG* probe have shown that *AG* RNA is flower-specific, being >100 fold enriched in floral tissue as compared to vegetative tissue, and that *AG* RNA is confined to stamens and carpels in relatively mature flowers (Yanofsky et al., 1990). Although this result is consistent with *AG* being expressed in the organs that are affected when this gene is mutated, the question of where and when *AG* is expressed early in development was left unanswered, as was the question of whether the appropriate expression of *AG* depends on the action of the other homeotic genes.

In this paper, we describe the expression of *AG* throughout flower development, in wild type, *ap2*, *ap3* and *pi* genetic backgrounds. We show that in wild type, *AG* RNA is localized to whorls 3 and 4, the regions of the flower that are affected when this gene is

mutated, and that the expression of *AG* RNA begins before the primordia of the third and fourth whorl organs appear. We also show that in *ap2* mutant flowers, *AG* RNA is not restricted to whorls 3 and 4, but is present in all floral whorls. Thus, the *AP2* gene product is a negative spatial regulator of *AG* RNA accumulation, supporting the contention that the *ap2* mutant phenotype is due to ectopic expression of the *AG* product (Bowman et al., 1991). Both of these results confirm and extend predictions of an organ-specification model proposed to explain the homeotic mutant phenotypes (Bowman et al., 1991; Bowman and Meyerowitz, 1991; Meyerowitz et al., 1991), and show that organ specification in flowers depends on regulatory interactions between different homeotic genes.

Results

We performed in situ hybridization with a single-stranded RNA probe derived from sequences within one of the *AG* cDNA clones. *AG* contains a putative DNA-binding region, called the MADS box (Schwarz-Sommer et al., 1990), which is present in at least 15 copies within the Arabidopsis genome (Drews and Meyerowitz, unpublished results). At least eight of the copies are in genes (Ma et al., 1991; Jack, Brockman, and Meyerowitz, unpublished results). Therefore, to avoid the possibility of cross-hybridization with other RNAs in the in situ hybridization experiments, we removed the MADS box sequences from the *AG* probe. We call this probe *AG* anti-mRNA. Control experiments showed that this probe hybridizes only with *AG* RNA, and not with other mRNAs of the *AG* gene family (see Experimental Procedures).

AG RNA is First Detectable Very Early in Flower Development

Figures 2A and 2B show the development of the Arabidopsis flower up to the time when the sepal primordia emerge. Floral primordia at stages 1-4 are labeled in these figures (Table 2). During stages 1 and 2, floral primordia arise (stage 1) and become distinct (stage 2) from the inflorescence meristem. Stage 3 is marked by the appearance of sepal primordia at the flanks of the floral primordium (Table 2; Smyth et al., 1990).

A dark field micrograph of the in situ hybridization experiment is shown in Figure 2C. To more easily orient the signal to the tissue, we prepared the bright field/dark field double exposure shown in Figure 2D. In this figure, and the double exposures that follow, the silver grains were made to appear red. Hybridization signal is not present above background in the stage 1 and stage 2 floral primordia (Figure 2C), but is evident in the stage 3 primordium (Figure 2C and 2D). The stage 2 flower primordium shown in Figures 2F and 2G is the oldest stage 2 floral primordium that we obtained in our tissue sections for the present study. It is late in the development of this stage, and no *AG* RNA is detectable. The youngest stage 3 floral primordium obtained is shown in Figure 2H and 2I; in this flower, the sepal primordia have just emerged. This flower contains a low but detectable amount of *AG* RNA. Thus, *AG* RNA is first detectable between late stage 2 and early stage 3. This corresponds to the time when the organ primordia of whorl 1 emerge, but is well before the appearance of the organ primordia whose fate is affected by *ag* mutations (Table 2).

At stage 3, *AG* RNA is localized to the floral meristem of the developing flower, and is not present above background in the sepal primordia (Figure 2E). Within the floral meristem, hybridization signal is seen over all cellular regions, including L1, the outermost cell layer , L2, the cell layer just inside of L1, and L3, the several cell layers immediately inside of L2 (Satina et al., 1940). The flower section in Figures 2B-2E is oriented 90° relative to the section in Figures 2J and 2K (see Figure 2A). These figures show that the distribution of *AG* RNA in the floral meristem is the same regardless of the orientation of

the section, and thus that *AG* RNA is distributed over a hemisphere of cells in the developing flower.

We hybridized flower sections with [^{3}H]-poly(U) to characterize the distribution of poly(A) RNA during early flower development. The poly(U) probe produced an even distribution of silver grains in the stage 3 flower section (Figures 2L and 2M). This pattern is in contrast to the distribution of silver grains produced by the *AG* anti-mRNA probe (Figures 2B-2K). This control shows that *AG* RNA is specific to the cells of the floral meristem in stage 3 flowers.

AG RNA is Specific to Whorls 3 and 4 of the Floral Meristem

The organ primordia of whorls 2-4 do not form until stages 5-7 (Table 2). Stage 5 begins with the emergence of the petal and stamen primordia (Figure 3A). Because this is a relatively transient stage (stage 5 lasts six hours at 25°C compared to 12-60 hours for the other stages), we obtained very few tissue sections through stage 5 flowers. Of those few that we did obtain, none had easily-observed petal tissue in them, primarily because the petal primordia are very small (5-10 μm) at this stage (Smyth et al., 1990).

During stage 6, the petal primordia enlarge only slightly, but become somewhat distinct from the floral meristem (Figure 3B). This stage is also characterized by a large increase in the size of the stamen primordia (Figure 3B). In stage 6 flowers, the sepal and petal primordia have only background hybridization signal (Figures 3E-G). By contrast, the *AG* probe hybridizes strongly with RNA present in the stamen primordia. As observed earlier in development (Figures 2E and 2J), *AG* RNA is distributed throughout the floral meristem.

Stage 7 is marked by the emergence of the carpels (Figures 3C and 3D). During this stage, the stamens grow rapidly to become stalked, but the petals remain relatively

small (<25 μm; Smyth et al., 1990; Figures 3C and 3D). Figures 3H-3M are micrographs of two serial sections through a stage 7 flower. As with all earlier stages of development, hybridization signal is not present above background in the cells of the sepal and petal primordia. By contrast, the *AG* anti-mRNA probe hybridized strongly with RNA present in the stamen and carpel primordia. Stage 7 flower sections that are hybridized with [^{3}H]-poly(U) have a uniform distribution of silver grains throughout each organ primordium (data not shown).

Expression of *AG* in *apetala2* Mutant Flowers

The *AP2* locus is represented by a large allelic series (Pruitt et al., 1987; Komaki et al., 1988; Bowman et al., 1989; Meyerowitz et al., 1989; Kunst et al., 1989; Bowman et al., 1991). Most mutations at the *AP2* locus reduce the number of organs in the first three whorls, and cause the homeotic transformation of whorls 1 and 2 into carpels and stamens, respectively. Carpels and stamens are organs whose development, in wild-type flowers, depends on *AG* activity. Furthermore, while *ag* mutations affect only the organs of whorls 3 and 4 in an otherwise wild-type flower, *ag* mutations have distinct effects in the first and second whorls when crossed into an *ap2* mutant background (Bowman et al., 1991). It thus seems possible that one important function of the wild-type *AP2* product is to repress the activity of *AG* in the first and second whorls, and that much of the *ap2* mutant phenotype is due to ectopic expression of *AG*. To determine whether mutations in the *AP2* gene have an effect on the domain of *AG* gene expression, we hybridized the *AG* anti-mRNA probe with tissue sections of the strongest known allele of the *AP2* locus, *ap2-2* (Bowman et al., 1991).

ap2-2

ap2-2 flowers consist of a medial first whorl of carpels, a reduced number of stamens in the third whorl and a relatively normal gynoecium in the fourth whorl. Many lateral first whorl, all second whorl, and most third whorl organs are absent (Figure 4D; Table 1). Until the emergence of the outer whorl organ primordia in early stage 3 (Figure 4K), *ap2-2* flower development is morphologically indistinguishable from wild-type flower development (compare Figures 2A and 4A). *AG* RNA is not present above background in *ap2-2* flowers from the initial stages of development, through early stage 2 (Figures 4E-4G). In late stage 2 floral primordia, however, silver grains are dense (Figures 4H-4J). This establishes the time of development when *AG* RNA is first detectable in *ap2-2* flowers as between early stage 2 and late stage 2. This is in contrast to *AG* expression in a wild-type background, where *AG* RNA is not detectable in late stage 2 flowers (Figures 2F and 2G).

During stage 3, the first whorl organ primordia emerge at the flanks of the flower primordium (Figures 4A and 4K). At this stage, hybridization signal is present in the cells of the floral meristem, and a lower but significant signal is present in the the medial outer whorl organ primordia (Figure 4K-4M). These data indicate that, unlike wild-type flowers, the medial outer whorl organ primordia of ap2-2 flowers contain *AG* RNA at the earliest stages of their formation.

Subsequent development of *ap2-2* flowers is markedly distinct from wild type; the medial outer whorl organs become more elongate relative to wild type (compare Figure 2A with Figure 4A), and begin to develop cell files characteristic of carpel development (Figures 4B, 4C, and 4N). Figures 4N-4P are micrographs of a flower that has undergone many of these developmental changes. The pattern of hybridization signal in this flower is similar to that seen earlier in development (stage 2; Figures 4K-4M); silver grains are present over the cells of both the floral meristem and the medial first whorl organ primordia. Unlike earlier in development, however, the hybridization signal in the first whorl organ primordia is about equal in intensity to that in the floral meristem. This

suggests that the concentration of *AG* RNA increases in the cells of the medial first whorl organ primordia as development progresses. The data presented here indicate that *AG* RNA is present in the medial first whorl organ primordia from the time that they emerge in stage 3 until after they begin to differentiate into carpels.

In contrast to the medial positions, the lateral first whorl primordia do not have any signal above background at stage three (Figure 4Q-4S). The subsequent development of these primordia is variable; some develop into leaf-like organs while others fail to develop altogether (Bowman et al., 1991). Due to the variable nature of these organs, stages beyond stage 4, at which time no signal was observed (data not shown), were not analyzed.

ap2-1

The phenotype of *ap2-1* is unlike that of most other *ap2* alleles; all of the first whorl organs are leaves, or slightly carpelloid leaves, and the second whorl organs are present and have characteristics of both petals and stamens (Bowman et al., 1989). The third and fourth whorls are normal (Figure 5D). *ap2-1* seems thus to represent a partial loss of function. Figure 5A depicts the initial stages of *ap2-1* flower development. As in wild type, the floral primordia arise at the flanks of the inflorescence meristem (stage 1), become distinct from the inflorescence meristem by lateral growth (stage 2), and give rise to the first whorl organ primordia at its flanks (stage 3, Figures 5A and 5E). Figures 5E-5G show that *AG* RNA is present in *ap2-1* flowers shortly after emergence of the outer whorl organ primordia (early stage 3). A signal is present in the cells of the floral meristem, but *AG* RNA is not present above background in the cells of the first whorl organ primordia. Thus, in contrast to *ap2-2* flowers, *AG* RNA is not detectable in the outer whorl of *ap2-1* flowers at the earliest stages of their development.

The first whorl organ primordia of an *ap2-1* flower develop the unique qualities of leaves during stage 3; as the outer whorl organ primordia begin to elongate, wild-type sepals enclose the bud tightly (Figures 2A, 2B, and 2E), whereas *ap2-1* first whorl organs grow more upright (Figure 5A). Shortly thereafter, the *ap2-1* outer whorl organs develop structures that are characteristic of leaves, such as stipules (Figure 5C) and stellate trichomes (Figure 5D). Figures 5H-5J show the pattern of hybridization of the *AG* anti-mRNA probe with stage 5 *ap2-1* flowers. As observed earlier in development (Figure 5E-5G), the *AG* probe hybridized with RNA present in the floral meristem. Hybridization signal also appears to be present in the emerging third whorl organ primordia. By contrast, the *AG* probe did not produce silver grains above background over the cells of the first whorl organ primordia. Thus, *AG* RNA is not present above background in the first whorl organ primordia during the time when these organs have already begun to develop morphological features of leaves.

Figures 5K-5M are micrographs of an ap2-1 flower in which the fourth whorl carpels have begun to differentiate (approximately stage 8; Figure 5C). As with wild type, this flower contains a very high concentration of silver grains over the cells of the third whorl stamens and fourth whorl carpels. Unlike wild type, hybridization signal is also present in the first whorl organ primordia. The tissue section in Figures 5K-5M does not contain *AG* RNA above background in the second whorl organ primordia. However, this result is variable; of ten second whorl primordia observed between stages 5 and 8, seven had a very low signal, one had a high signal, and two appeared to have a patchy signal (some cells with a high signal and other cells with a much lower signal). Thus, later in development (>stage 6), the cells of whorls 3 and 4 contain very high concentrations of *AG* RNA, the cells of whorl 1 contain a lower but detectable amount of *AG* RNA, and the cells of whorl 2 have variable expression of *AG* RNA. Taken together, these data show that the strong *ap2-2* allele has a dramatic effect on ectopic *AG* mRNA accumulation, while the weaker *ap2-1* allele has a distinct but lesser effect.

Expression of *AG* in *apetala3-1* and *pistillata-1* Mutant Flowers

apetala3 and *pistillata* are homeotic mutants that affect the development of whorls 2 and 3. In contrast to *ap2*, the mutant phenotypes of *ap3* and *pi*, either alone, or in combination with *ag* mutations, give no reason to suspect that these genes influence the expression of *AG* (Bowman et al., 1989; Bowman et al., 1991). To determine if this is true, we hybridized the *AG* anti-mRNA probe in situ with longitudinal sections of *ap3-1* and *pi-1* mutant flowers. In these flowers, *AG* RNA was localized to the organ primordia and mature organ of whorls three and four (data not shown). In the third whorl carpels of *ap3-1* flowers, the distribution of silver grains was exactly as occurs in the fourth whorl carpels of wild-type flowers. Thus, mutations in these genes do not appear to affect the spatial distribution of *AG* RNA.

Discussion

AG RNA Accumulates in the Organs Affected by *ag* Mutations When Their Fates are Specified

The question of when the cells of the floral primordium become specified to differentiate into particular organ types is largely unanswered. However, temperature-shift experiments with temperature-sensitive alleles of two of the homeotic genes have been carried out to determine the times when the fates of a whorl can be changed. The temperature-sensitive period (tsp) of the *ap3-1* allele is stages 5-7 for whorl 2 and stages 5-

6 for whorl 3, and the tsp of the *ap2-1* allele in whorl 2 is stages 2-4 (Bowman et al., 1989). Since the organ primordia of these whorls arise at stage 5 and begin to morphologically differentiate at about stage 7 (Smyth et al.,1990), these data suggest that the specification process begins prior to the emergence of the organ primordia, but is not complete until the time when they begin to differentiate. Stages 2-7 are thus when at least part of the specification of floral organs takes place.

AG RNA is first detectable between late stage 2 and early stage 3 (Figures 2F-2I). This corresponds to the time when the first whorl primordia arise, but is prior to the formation of second, third and fourth whorl primordia. In stage 3 flowers, *AG* RNA is found only in the cells that will later form the inner whorls. This distribution of *AG* RNA is also observed with stage 4 flowers (data not shown). When the second, third and fourth whorl organ primordia arise (stage 5), *AG* mRNA is confined to the primordia of the inner two whorls and this pattern of expression persists through stage 7 (Figures 3E-3M). We previously showed that *AG* RNA is specific to stamens and carpels in stage 9 flowers (Yanofsky et al., 1990), and we have also found the same to be true of mature flowers (stage 12) (Bowman, Drews, and Meyerowitz, unpublished results). *AG* RNA is thus localized to the regions of the flower that are affected by *ag* mutations, and it is present throughout the developmental period when the identity of those organs is thought to be specified. In this regard, *AG* expression resembles that of the *Drosophila* homeotic genes, which also are regulated at the RNA level, and are expressed primarily in the areas of the embryo that are perturbed when mutated (e.g. Levine et al., 1983; Akam 1983; Akam and Martinez-Arias, 1985; reviewed by Akam, 1987).

AG Expression is Not Confined to Clonally-Derived Regions

Work with a variety of plants has shown that individual organs are not derived from distinct cell lineages. Rather, three different cell lineages present in the inflorescence meristem and in the floral meristem contribute cells to the floral organs (Satina and Blakeslee, 1941; Derman, 1947; Blaser and Einset, 1950; Derman and Stewart , 1973). These cell lineages form three layers in the floral meristem, designated L1, L2, and L3. L1 is the outermost layer of the floral meristem, L2 is the single layer of cells immediately inside of L1, and L3 consists of several cell layers immediately inside of L2. Each germ layer gives rise to particular structures in the floral organs (Satina and Blakeslee, 1941; Satina and Blakeslee, 1943; Satina, 1944; Satina, 1945; Derman, 1947; Blaser and Einset, 1950; Derman and Stewart, 1973). For example, the epidermis of each floral organ comes from L1, the gametes are products of L2, and the internal structures of the floral organs are derived from L2 and L3.

Since each floral organ contains cells from L1, L2, and L3, some mechanism must exist to specify the appropriate organ identity of cells from clonally distinct lineages. We found that *AG* is expressed in all three lineages of the floral meristem (Figures 2E and 2J). This suggests that the fates of the cells of the third and fourth whorls are conferred by *AG* being expressed in all three germ layers, and that *AG* expression results from some earlier process of communication between clonally distinct groups of cells. It must be stressed, however, that we do not yet know whether *AG* activity is present in all three cell layers. For example, it is possible that, although *AG* RNA is present in all three cell layers, the *AG* product functions autonomously in only one or two of these layers. Examples of genes being expressed in cells where they have no apparent function have been found previously (Banerjee et al., 1987; Casanova and Struhl, 1989; Riggleman et al., 1990). Future experiments with genetic mosaics, in which *AG* expression is restricted to one or two cell layers, will tell us whether *AG* expression is necessary in all three cell layers, and in which cell layer(s) *AG* has its primary effects.

AG RNA Level is Negatively Regulated by the *AP2* Product

AP2, like *AG*, is a homeotic gene that affects the specification of floral organ fate (Pruitt et al., 1987; Komaki et al., 1988; Bowman et al., 1988, Bowman et al.,1989; Kunst et al., 1989; Meyerowitz et al., 1989; Bowman et al., 1991; Meyerowitz et al., 1991). In contrast to *AG*, the realm of action of the *AP2* gene is whorls 1 and 2; most *ap2* mutations cause carpels and stamens to form where sepals and petals are formed in wild type. The realms of action of the *AP2* and *AG* products thus appear to be mutually exclusive. However, in *ap2-2 ag-1* double mutant combinations, the *ag-1* mutation has phenotypic effects in the first and second whorls (they develop leaves and organs intermediate between stamens and petals) that are not seen in *ag-1* or *ap2-2* single mutant flowers (Bowman et al., 1991). To explain the appearance of an *ag* phenotype in the outer whorls when the wild-type *AP2* product is absent, it has been postulated that *AP2* prevents the expression of *AG* in whorls 1 and 2 of wild-type flowers, and that *AG* is expressed in all floral whorls in *ap2* mutants; therefore, the *ap2* phenotype is thought to result in part from *AG* derepression (Bowman et al., 1991). This hypothesis accounts for the apparently paradoxical formation of carpels and stamens in the outer whorls in *ap2* mutants, which are organs that require wild-type *AG* function to develop. If *ap2* mutations allow *AG* to express ectopically in whorls 1 and 2, then it is not surprising that organs characteristic of the normal domain of *AG* action can form in the first and second whorls in these mutants. In agreement with this hypothesis, we found that *AG* RNA is present in the cells of the medial first whorl at all stages of *ap2-2* flower development (Figure 4K-4P). These data demonstrate that the *AP2* product is indeed a negative spatial regulator of *AG* activity, as suggested by the genetic evidence, and support the contention that the *ap2-2* phenotype is due in part to the ectopic expression of *AG*. The in situ hybridization data also go beyond

the genetic model, showing that *AP2* represses *AG* activity by preventing the accumulation of *AG* RNA.

The *ap2-1* phenotype presents an apparent contradiction to the genetic model for organ specification. If one function of the wild-type *AP2* product is to repress *AG* function in whorl 1, and another function is to activate genes necessary for sepal development in whorl 1, then *ap2* mutants should have *AG* active and *AP2* inactive in this whorl, and the organs that form there should be carpels (Bowman et al., 1991; Bowman and Meyerowitz, 1991; Meyerowitz et al., 1991). In *ap2-1* homozygotes, the first whorl organs are leaves, or leaves with a slight carpelloid character (stigmatic tissue at their tips, Bowman et al., 1989). Previous experiments in which triple mutants were made, lacking all three classes of homeotic products, show that leaves or slightly carpelloid leaves are the organs that form when the *AP2*, *AG* and *PI/AP3* products are absent. This implies that *ap2-1* is a partial loss-of-function allele, in which the gene product cannot activate the genes necessary for sepal development, but still continues to repress *AG* activity in the first whorl organs. In *ap2-1* flowers, *AG* RNA is not present above background in the outer whorl organs during stages 1-6 (Figures 5E-5J, and data not shown), the period when these organs begin to develop morphological features of leaves. In contrast to this, *AG* RNA is present in the first whorl organs late in development (stage 8; Figures 5K-5M); however, AG expression late in development in not expected to influence the identity of the outer whorl organ because, at this time of development, the identity of these organs has already been establised and they have already begun to differentiate into leaves. Rather, the late expression of AG might have a role in causing specific cells within the outer whorl leaves to develop carpel character (e.g., causing the cells at the tips of the leaves to develop into stigmatic tissue).The phenotype conferred by the *ap2-1* allele is thus consistent with the current model of action of the homeotic genes, and it is directly demonstrated that the two postulated functions of the wild-type *AP2* product are separable by mutation.

In early *ap2-2* flowers, *AG* RNA is less concentrated in the medial first whorl organ primordia than the floral meristem (Figures 4K-4M, and data not shown). It is not until later in development that the signal in the medial first whorl organ primordia becomes equivalent to that in the floral meristem (Figures 4N-4P). This indicates that early in development, the wild-type pattern of *AG* expression is not totally dependent upon the *AP2* product, suggesting either that *ap2-2* is not a completely null allele, or that other gene products in addition to *AP2* are involved in modulating *AG* expression early in development. One candidate is *AP1*, which also is a floral homeotic gene with phenotypic effects in the outer two whorls when mutated (Irish and Sussex, 1990). One role of *AP2* on *AG* expression, then, might be to maintain the spatially-restricted domain of *AG* expression later in development. A similar mechanism is found with the homeotic genes of *Drosophila*. The pattern of expression of the *Drosophila* homeotic genes is established by the coordinate and segmentation genes early in development (e.g., Ingham and Martinez-Arias, 1986; White and Lehman, 1986; Irish et al., 1989; Jack and McGinnis, 1990; reviewed by Akam, 1987). Later in development, however, cross-regulatory interactions between the Drosophila homeotic genes play a role in maintaining their spatially-restricted patterns of expression (e.g., Hafen et al., 1984; Harding et al., 1985; Carroll et al., 1986; reviewed by Akam, 1987). The in situ hybridization experiments thus indicate that the present genetic model for organ specification an over-simplification, and reveal a level of temporal control hitherto unsuspected.

Experimental Procedures

Plant Material

The wild-type variety used in this study was Landsberg *erecta*. The *ap2-2*, *ap2-1*, *ap3-1*, and *pi-1* mutations were in the Landsberg *erecta* genetic background. All plants were grown at 25°C. 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, and mutant alleles in lower case italics.

Preparation of Plant Material for In Situ Hybridization

Individual flowers or a cluster of flower buds at stages 1-12 of development were dissected and fixed in 3.7% formaldehyde, 5% acetic acid, 50% ethanol. Fixed tissue was dehydrated with ethanol, cleared with xylene, and embedded in paraffin (Paraplast Plus). Embedded tissue was sliced into serial 8 or 10 μm sections with a Sorvall JB-4 microtome and attached to microscope slides that were coated with poly-L-lysine (Sigma). For comparison, we also fixed tissue with 1% glutaraldehyde; we observed no signal above background in tissue fixed in this way.

In Situ Hybridization Probes

Three probes were utilized in these experiments. The *AG* anti-mRNA probe and control probes were single-stranded ^{35}S-RNA probes derived from sequences within the cDNA clone pCIT565. The *AG* anti-mRNA probe contained nucleotides 240 to 977 of the *AG* sequence published in Yanofsky et al. (1990). The control probe contained nucleotides 9 to 977 of the *AG* sequence published in Yanofsky et al. (1990). This probe was synthesized in the opposite orientation of the *AG* anti-mRNA probe and thus contained sequences identical to *AG* mRNA. The control probe was used to measure background hybridization. The third probe was [^{3}H]-poly(U), which was purchased from Amersham

(TRK.481). The poly(U) probe was used to describe the distribution of total poly(A) RNA in floral tissue. This probe also allowed us to assess whether the RNA within a cell was accessible to the in situ hybridization probes.

Our laboratory has cloned several genes that are similar to *AG* in regions both including and outside of the MADS box (Ma et al., 1991). The gene that is most similar is *AGL1*. We hybridized the *AG* anti-mRNA probe with a dilution series of in vitro synthesized *AG* mRNA and *AGL1* mRNA sequences to determine the extent to which this probe cross-hybridizes with other mRNAs of the *AG* gene family. We found that the *AG* anti-mRNA probe hybridized about 10,000 times less strongly with *AGL1* RNA than *AG* RNA. Thus, the *AG* anti-mRNA probe hybridizes specifically with *AG* RNA, and not to a significant extent with other RNAs of the *AG* gene family.

In Situ Hybridization

The in situ hybridizations were carried out as described by Cox and Goldberg (1988). Prior to hybridization, the flower sections were soaked in xylene to remove the paraffin, hydrated with an ethanol series, incubated with 1 μg/ml proteinase K for 30 minutes at 37°C, acetylated with acetic anhydride, dehydrated with an ethanol series, and allowed to air dry. The *AG* anti-mRNA probe and the control probes were hydrolyzed to an average size of 0.15 kb, and hybridized with flower sections for 12-16 hours at 42°C in a solution containing 50% formamide, 0.3 M NaCl, 10 mM Tris (pH 7.5), 1 mM EDTA, 70 mM DTT, 1X Denhardt's reagent, 10% dextran sulfate, 150 μg/ml tRNA, and with 200-300 ng/ml of probe per kilobase of probe complexity. The [^{3}H]-poly(U) probe (1 ng/ml) was hybridized with flower sections for 12-16 hours at 42°C in 0.3 M NaCl, 10 mM Tris (pH 7.5), 1 mM EDTA, 1X Denhardt's reagent, and 150 μg/ml tRNA. Following hybridization, the flower sections were rinsed in 0.6 M NaCl, incubated with 50

μg/ml ribonuclease A for 20 minutes at 37°C, and washed at room temperature for one hour in 0.3 M NaCl. The flower sections that were hybridized with the *AG* anti-mRNA probe and the control probe were given an additional wash of 1 hour at 57°C in 0.015 M NaCl.

The *AG* anti-mRNA probe and control probe produced a background signal in the outer edge of the sepals (Yanofsky, et al., 1990). This background signal was present only in flowers that had already initiated carpel development (>stage 7). The following prehybridization treatments were unsuccessful in eliminating this background signal: soaking floral sections in 1% BSA; doubling the acetic anhydride treatment; prehybridizing in a solution containing 50% formamide, 0.3M NaCl, 10X Denhardt's reagent, and 500 μg/ml tRNA; varying the probe concentration in the hybridization solution; increasing the concentration of tRNA to 500 μg/ml, and of Denhardt's reagent to 10X in the hybridization solution.

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Table 1. Summary of Phenotypes of Homeotic Flower Mutants

	First Whorl		Second Whorl	Third Whorl	Fourth Whorl
	Medial	Lateral			
Wild type	Sepals	Sepals	Petals	Stamens	Carpels
agamous-1	wt	wt	wt	Petals	Another *ag-1* flower
apetala2-2	Carpels	Leaves or absent	Absent	Stamens or absent	wt
apetala2-1	Leaves	Leaves	Petaloid stamens	Stamens	wt
apetala3-1	wt	wt	Sepals	Carpelloid stamens	wt
pistillata-1	wt	wt	Sepals	Absent	wt

wt = wild-type

Table 2. Stages of Flower Development in A. thaliana

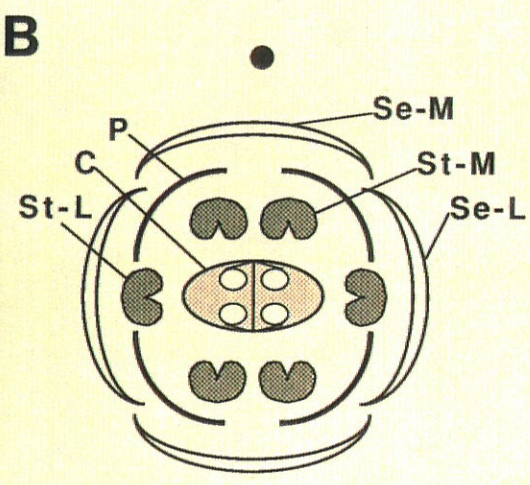
Stage	Description
1	Flower buttress arises on flank of apical meristem
2	Flower pimordium becomes distinct from main axis by a groove
3	Sepal primordia arise at flanks of floral primordium; pedicel develops
4	Medial sepal primordia elongate to overlie floral primordium
5	Petal and stamen primordia arise; petal primordia barely visible; medial sepals almost cover bud
6	Medial and lateral sepals enclose bud; long stamens become distinct from central dome; petal primordia very small; no visible differentiation of carpels
7	Stamens become stalked at base; petal primordia hemispherical; Gynoecium begins to differentiate and to form cylinder
8	Stamens become lobed; petal growth accelerates but petals remain relatively small
9	Petals become stalked at base and form flattened blades; gynoecial cylinder becomes constricted at top
10	Petals reach top of short stamens; gynoecial cylinder closed at top
11	Gynoecium develops stigmatic papillae
12	Petals level with long stamens

SEM observations described by Smyth et al. (1990)

Figure 1. Wild-type flower of Arabidopsis thaliana.

(A) Photograph of an Arabidopsis flower. (B) Diagram of the Arabidopsis flower. The mature Arabidopsis flower consists of four whorls of organs. The first whorl contains four sepals in positions medial and lateral to the inflorescence meristem. The second whorl has four petals that are at positions alternate to the sepals. The third whorl has six stamens, four long in the medial positions, and two short in the lateral positions. The fourth whorl is occupied by a gynoecium of two fused carpels topped by a short style and stigmatic papillae. Flower structures are given the following abbreviations: C, gynoecium of two fused carpels; P, petals; Se-L, lateral sepals; Se-M, medial sepals; St-L, lateral stamens; St-M, medial stamens. The filled circle above the flower diagram in (B) represents the position of the inflorescence meristem.


A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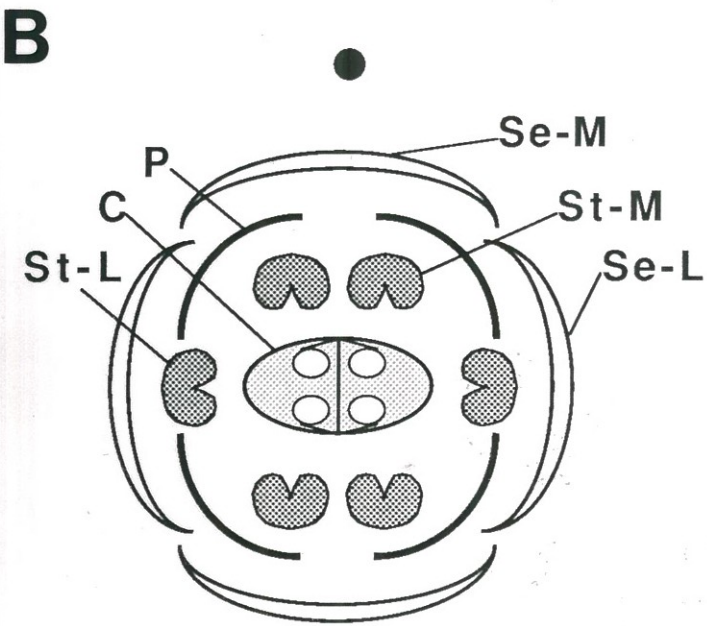
Se-M
P
C
St-M
St-L
Se-L


A


B
Se-M
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C
St-M
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Se-L

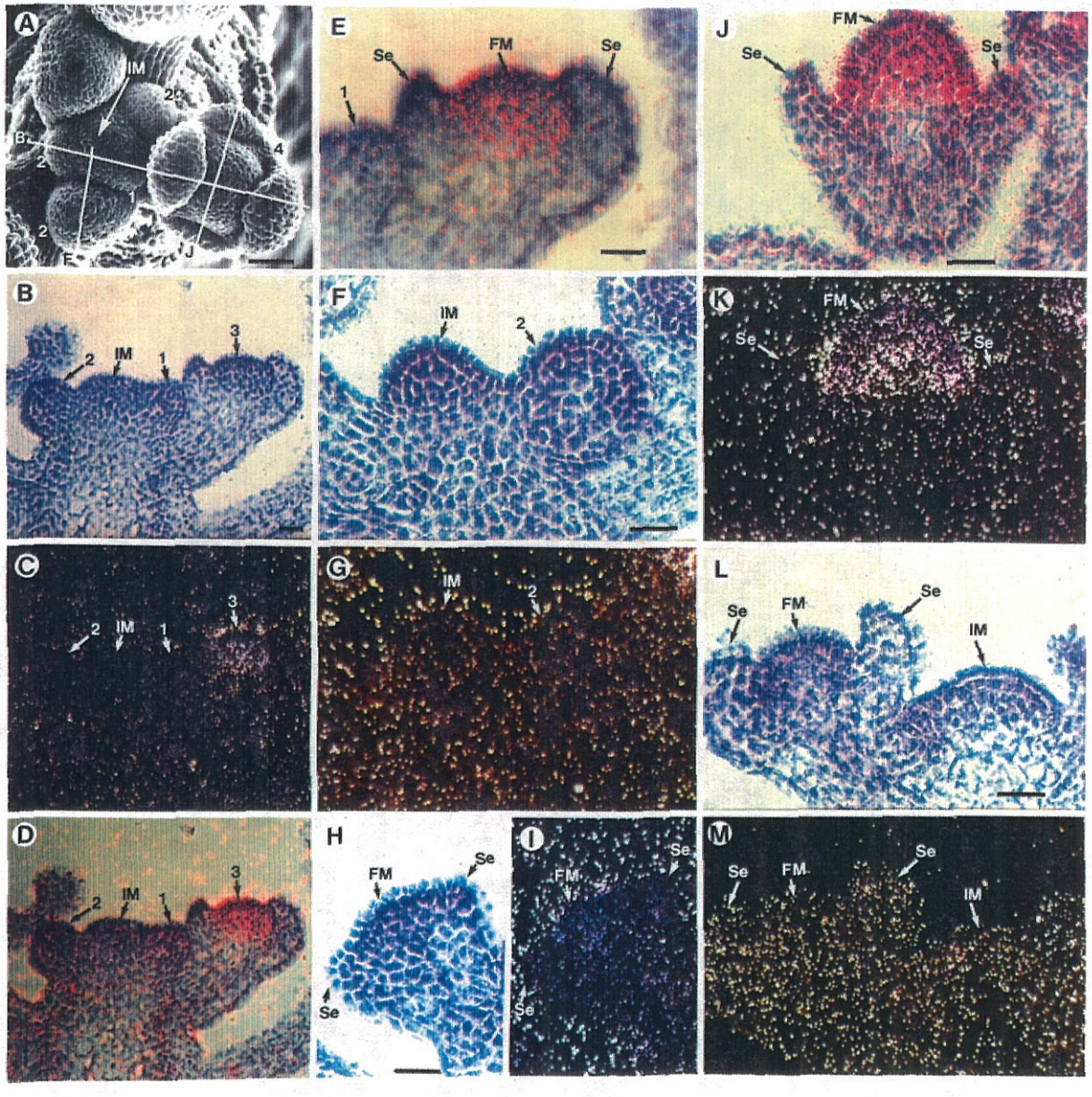
Figure 2. Distribution of *AG* RNA during early wild-type flower development.

(A) SEM micrograph of several floral primordia at stages 1 through 4 of development.

(B) to (K) In situ hybridization of an *AG* anti-mRNA probe with wild-type flower sections. (B) to (E) Longitudinal section through the inflorescence meristem and floral primordium at stages 1, 2, and 3 of development. The orientation of this section is indicated by the white line labeled "B" in (A). The sepals in this section are in the medial positions. (E) 2X magnification of the late stage 3 floral primordia in (D). (F) and (G) Longitudinal section through the inflorescence meristem and a late stage 2 floral primordium. The orientation of this section is indicated by the white line labeled "F" in (A). (H) and (I) Longitudinal section through an early stage 3 floral primordium. The orientation of this section is the same as in (B)-(E). The sepals in this section are in the medial positions. (J) and (K) Longitudinal section of a floral primordium at mid stage 3 of flower development. The orientation of this section is indicated by the white line labeled "J" in (A). The sepals in this section are in the lateral positions. (B), (F), and (H) Bright-field micrographs. (C), (G), (I), and (K) Dark field micrographs. (D), (E), and (J) Bright-field/dark-field double exposures; the dark-field exposures were taken with a red filter, causing the silver grains to appear red.

(L) and (M) In situ hybridization of [^{3}H]-poly(U) with a stage 3 floral primordium. The orientation of this section is the same as in (B)-(E). The sepals in this section are in the medial positions. (L) Bright-field micrograph. (M) Dark-field micrograph.

In this figure, the numbers indicate the stage of development of the respective floral primordia (Table 2). Flower structures are given the following abbreviations: FM, floral meristem; IM, inflorescence meristem; Se, sepals; . Bar = 20 μm.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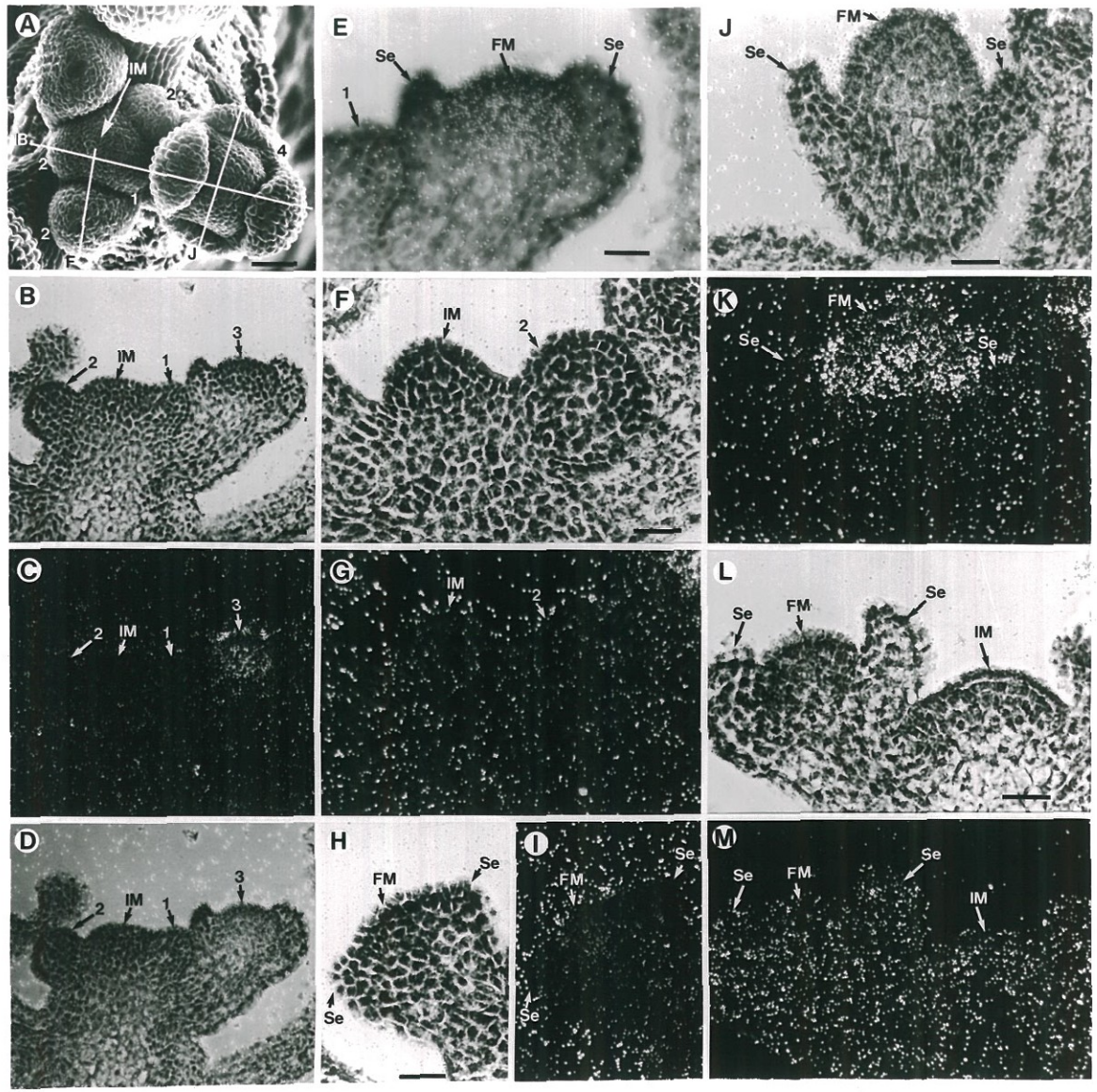
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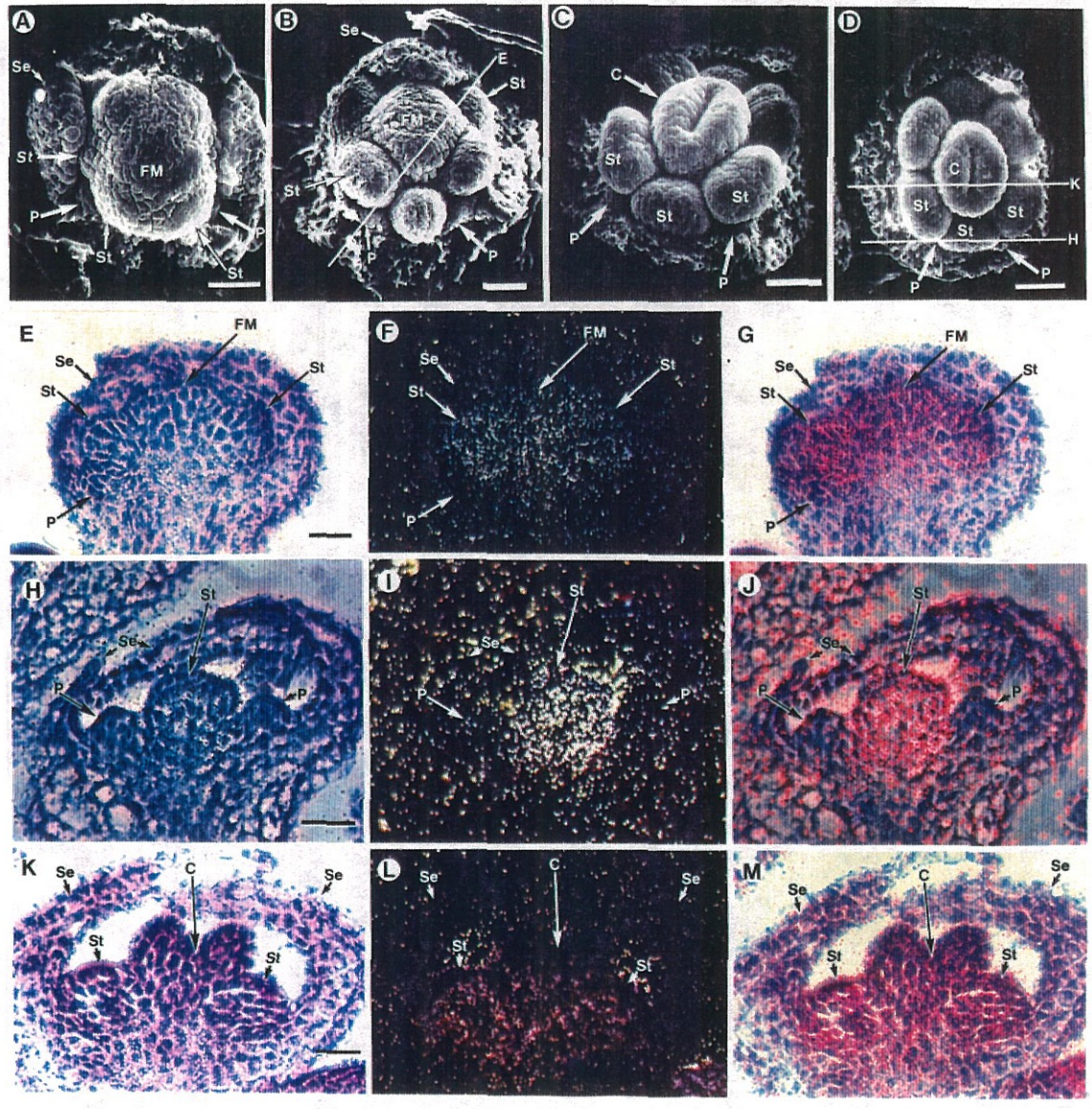
Figure 3. Distribution of *AG* RNA during stages 5-7.

(A) to (D) SEM micrographs of floral primordia at stages 5 through 7 of flower development. (A) Stage 5. (B) Stage 6. (C) and (D) Different views of a stage 7 floral primordium. In these preparations, three or all four sepals were removed so that the inner structures could be viewed.

(E) to (M) In situ hybridization of an *AG* anti-mRNA probe with tissue sections of flowers at stages 6 and 7 of development. (E) to (G) Longitudinal section through a stage 6 floral primordium. The orientation of this section is indicated by the white line labeled "E" in (B). The sepals and stamens in this section are in the medial positions. (H) to (M) Micrographs of two serial sections of a stage 7 floral primordium. The orientation of these sections is indicated by the white lines labeled "H" and "K" in (D). In (H) to (J), the sepals are in the medial positions and the stamen is in the lateral position. In (K) to (M), the sepals and stamens are in the medial positions. (E), (H), and (K) Bright-field micrographs. (F), (I), and (L) Dark-field micrographs. (G), (J), and (M) Bright-field/dark-field double exposures in which the dark-field exposure was taken with a red filter, causing the silver grains to appear red.

Flower structures are given the following abbreviations: C, gynoecium composed of two carpels; FM, floral meristem; P, petals; Se, sepals; St, stamens. Bar = 20 μm.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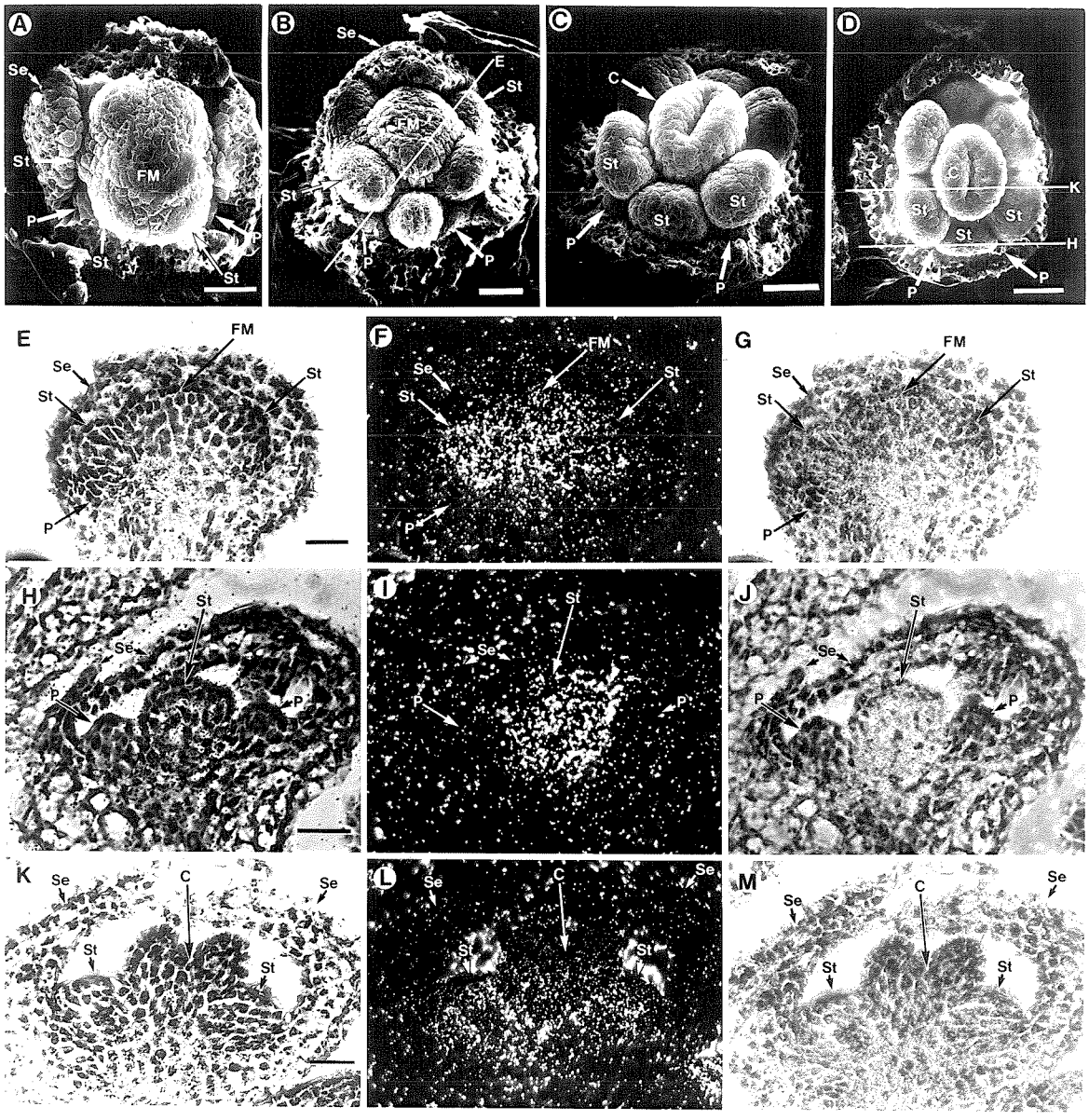
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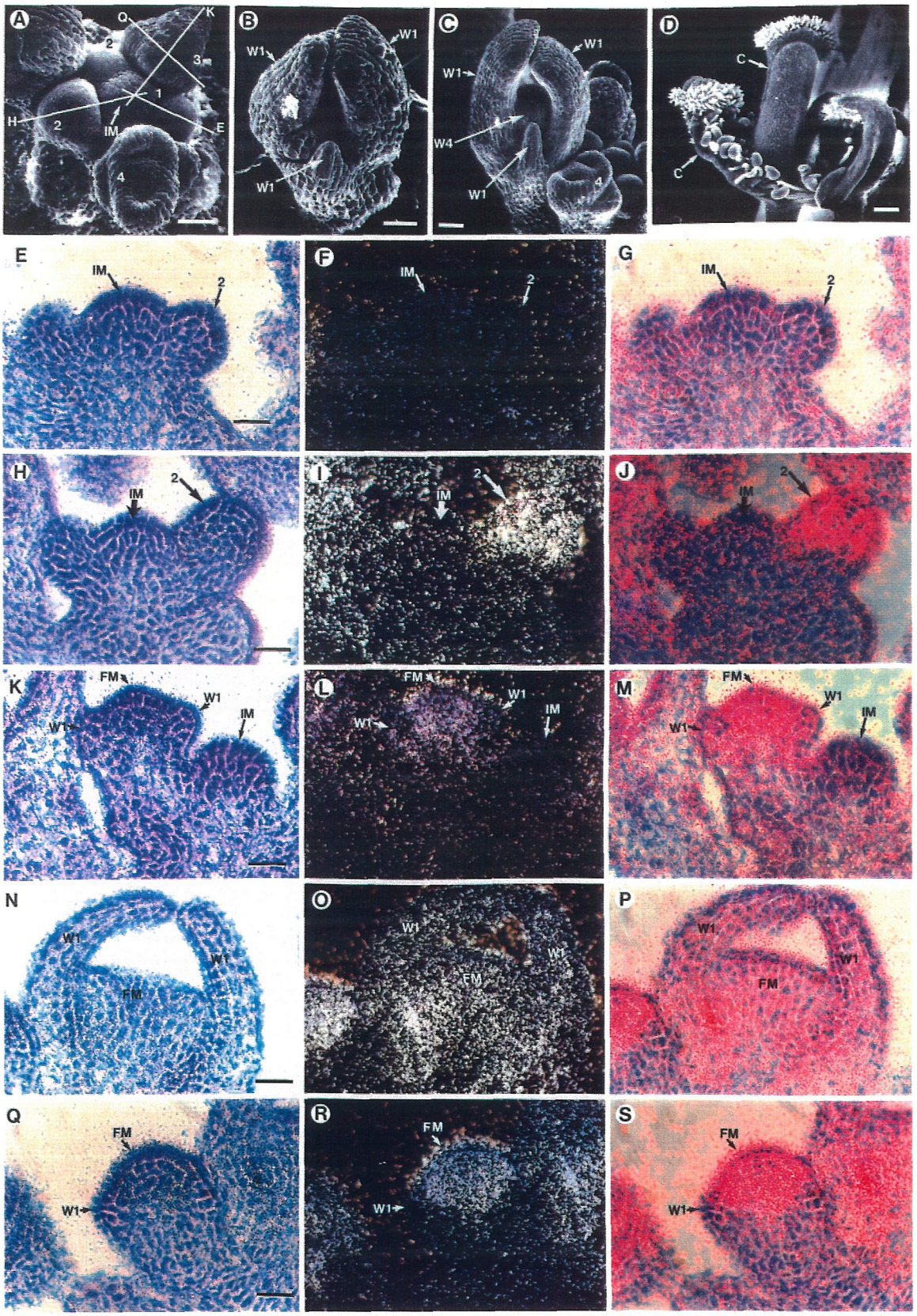
Figure 4. Distribution of *AG* RNA in *ap2-2* flowers.

(A) to (D) SEM micrographs of developing *ap2-2* flowers. (A) An *ap2-2* inflorescence with floral primordia at stages 1-4 of development. (B) Prior to differentiation of the fourth whorl, approximately equivalent to stage 5 of wild-type flower development. (C) After differentiation of the fourth whorl has begun, approximately equivalent to stage 7 or 8 of wild-type flower development. (D) Mature ap2-2 flower.

(E) to (S) In situ hybridization of an *AG* anti-mRNA probe with sections of *ap2-2* flowers at various stages of development. (E) to (G) Longitudinal section through the inflorescence meristem and a floral primordium at early stage 2 of development. The orientation of this section is indicated by the white line labeled "E" in (A). (H) to (J) Longitudinal section through the inflorescence meristem and a floral primordium at late stage 2 of development. The orientation of this section is indicated by the white line labeled "H" in (A). (K) to (M) Longitudinal section through the inflorescence meristem and a floral primordium at mid stage 3 of development. The orientation of this section is indicated by the white line labeled "K" in (A); this section is through the medial first whorl organs. (N) to (P) Longitudinal section through a floral primordium just prior to the differentiation of the fourth whorl carpels, which is approximately equivalent to stage 6 of wild-type flower development. This flower is between the stages of development shown in (B) and (C). This section is through the medial first whorl organs. The section shown in (N) is the adjacent serial section to that shown in (O) and (P). (Q) to (S) Longitudinal section through the lateral first whorl organs of an early stage 3 floral primordium. The orientation of this section is indicated by the white line labeled "Q" in (A); this section is through the lateral first whorl organs. (E), (H), (K), (N) and (Q) Bright-field micrographs. (F), (I), (L), (O) and (R) Dark-field micrographs. (G), (J), (M), (P), and (S) Bright-field/dark-field double exposures, in which the dark-field exposure was taken with a red filter, causing the silver grains to appear red.

In this figure, the numbers indicate the stage of development of the respective floral primordia. Flower structures are given the following abbreviations: FM, floral meristem; IM, inflorescence meristem; and W1, W2, W3, and W4 refer to the organ primordia of whorls 1 through 4, respectively. Bar = 20 μm except in (D) where it is 200 μm.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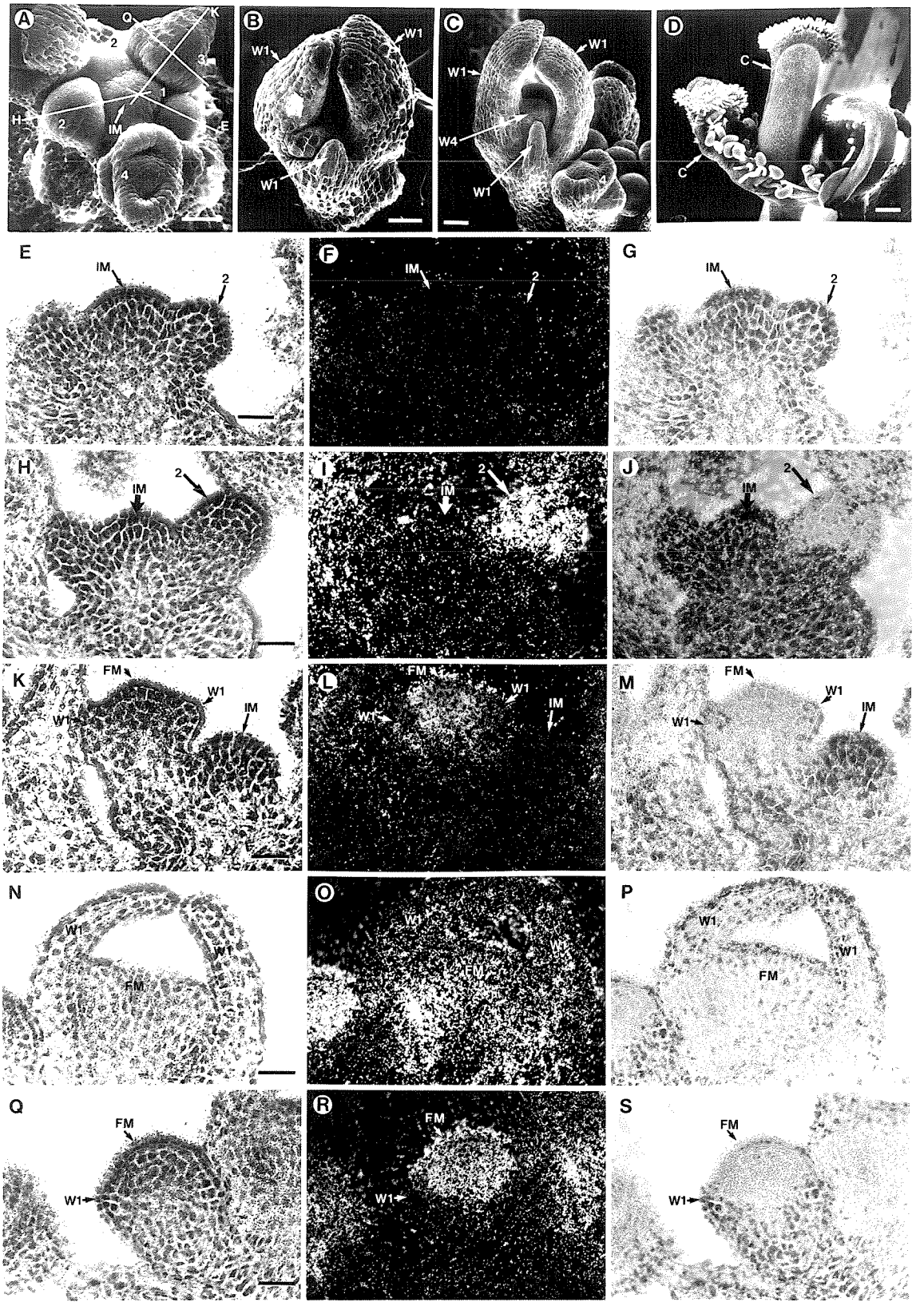
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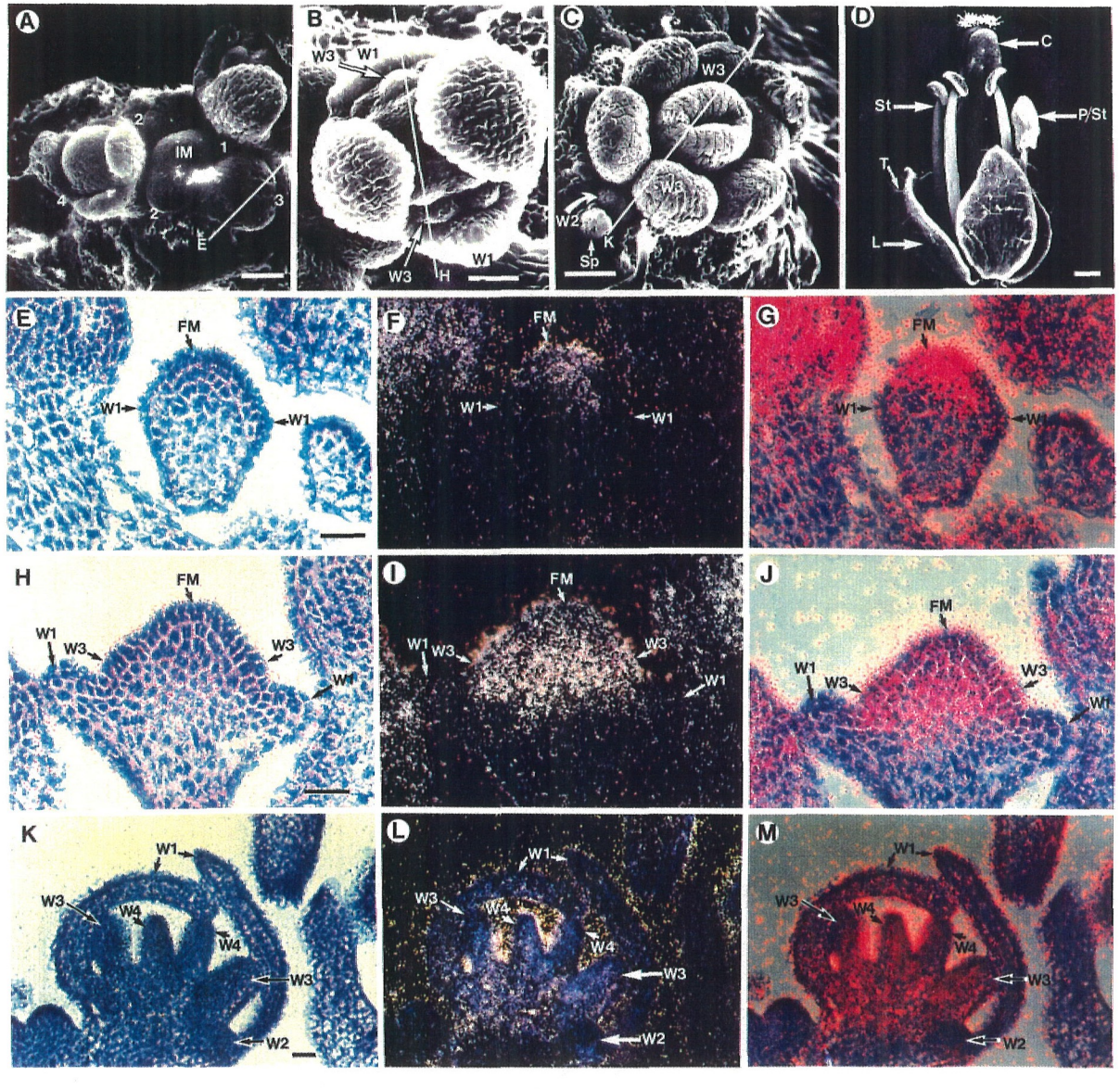
Figure 5. Distribution of *AG* RNA in *ap2-1* flowers.

(A) to (D) SEM micrographs of *ap2-1* flowers at various stages of development. (A) An ap2-1 inflorescence with several floral primordia at stages 1-4 of development. (B) Stage 5 (C) Stage 7. In this flower, all four first whorl organs were removed to reveal the inner structures. (D) Mature ap2-1 flower.

(E) to (M) In situ hybridization of an *AG* anti-mRNA probe with flower sections of *ap2-1* flowers at various stages of development. (E) to (G) Longitudinal section through a stage 3 floral primordium. The orientation of this section is indicated by the white line labeled "E" in (A); this section is through the lateral first whorl organ primordia. (H) to (J) Longitudinal section through a stage 5 floral primordium. The orientation of this section is indicated by the white line labeled "H" in (B), except that the flower in (B) is slightly older than the flower in (H). This section is through the lateral first whorl organs. (K) to (M) Longitudinal section through a stage 7 floral primordium. The orientation of this section is indicated by the white line labeled "K" in (C); this section is through the medial organs of the first and third whorls. (E), (H), and (K) Bright-field micrographs. (F), (I), and (L) Dark-field micrographs. (G), (J), and (M) Bright-field/dark-field double exposures in which the dark field exposure was taken with a red filter, causing the silver grains to appear red.

In this figure, the numbers indicate the stage of development of the respective floral primordia. Flower structures are given the following abbreviations: C, carpels; FM, floral meristem; IM, inflorescence meristem; L, first whorl leaf; P/St, petal-stamen hybrid organ; Sp, stipule; St, stamen; T, stellate trichome; and W1, W2, W3, and W4 refer to the organ primordia of whorls 1 through 4, respectively. Bar = 20 μm except in (D) where it is 200 μm.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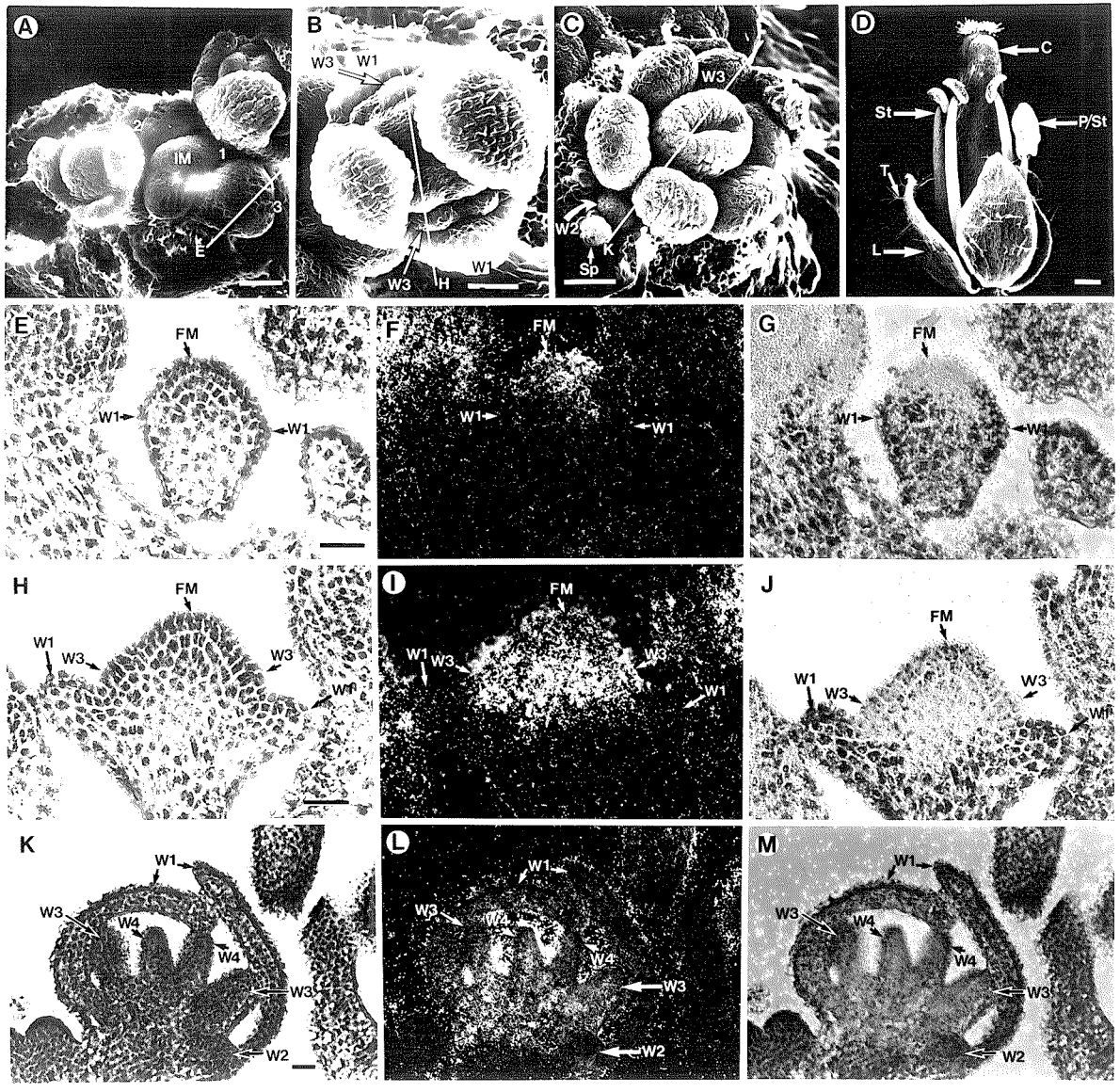
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CHAPTER SIX

EXPRESSION OF THE *ARABIDOPSIS* FLORAL HOMEOTIC GENE *AGAMOUS* IS RESTRICTED TO SPECIFIC CELL TYPES LATE IN FLOWER DEVELOPMENT

John L. Bowman, Gary N. Drews, and Elliot M. Meyerowitz

(This chapter was prepared for submission)

Summary

Mutations in the *AGAMOUS* gene cause transformations in two adjacent whorls of the *Arabidopsis thaliana* flower. Petals develop in the third floral whorl rather than the normal stamens, and the cells which would normally develop into the fourth whorl gynoecium behave as if they constituted an *agamous* flower primordium. Early in flower development, *AGAMOUS* RNA is evenly distributed throughout third and fourth whorl organ primordia but is not present in the organ primordia of whorls one and two (Drews et al., 1991). In contrast to the early expression pattern, later in flower development, *AGAMOUS* RNA is restricted to specific cell types within the stamens and carpels as cellular differentiation occurs in those organs. Ectopic *AGAMOUS* expression patterns in other floral homeotic mutants suggest that the late expression patterns are established independently of the early expression.

Introduction

Just as each flower consists of a precise pattern of organ types, each individual floral organ consists of several cell types in stereotyped positions. The organs of the flower begin their development as small outgrowths of cells from the floral meristem. At about the time these floral organ primordia arise, their identity is thought to be determined in accordance with their position within the flower, causing them to follow organ-type specific developmental programs. Subsequently, the cells of each of the determined organ primordia must then assess or know their relative position within the primordia and differentiate into the appropriate cell types. It is this precise pattern of cellular differentiation that results in the different morphological characteristics of each floral organ type. Thus, during flower development, cells must assess or know their position relative to others not only during the specification of organ primordium identity, but also later during the cellular differentiation of individual floral organs.

Several homeotic mutations have been isolated in *Arabidopsis thaliana* that cause cells in the developing flower to misinterpret their position and thus differentiate into inappropriate cell types (Pruitt et al., 1987; Komaki et al., 1988; Bowman et al., 1988, 1989, 1991; Meyerowitz et al., 1989; Hill and Lord, 1989; Kunst et al., 1989; Irish and Sussex, 1990). The result is a flower with morphologically normal organ types found in abnormal positions. A model has been proposed, based on a series of genetic experiments, to explain how four homeotic genes (*AGAMOUS*, *APETALA2*, *APETALA3*, and *PISTILLATA*) specify the identity of the floral organs (Bowman et al., 1991). However, genes involved in specifying positional information within the floral organs have not yet been identified. In *Drosophila*, the expression of many homeotic genes that are expressed early, and are responsible for the specification of segmental identity during embryogenesis, is required, again later in development for proper cellular differentiation within segments (Jürgens, 1985; Gibson and Gehring, 1988). For example, misexpression of the homeotic

selector gene *Antennapedia* during the larval stages of development, after segmental identity has been established, results in homeotic transformations of some tissues (Gibson and Gehring, 1988).

To investigate whether the *Arabidopsis* floral homeotic gene *AGAMOUS* (Yanofsky et al., 1990) could have a role in specifying cell fate late in flower development, in addition to its early role in specifying organ identity (Bowman et al., 1991; Drews et al., 1991), we examined the RNA expression pattern of *AGAMOUS* during the process of cellular differentiation in the flower. Mutations in *AGAMOUS* (*AG*) cause alterations in the third and fourth whorl of the flower. In whorl 3, six petals develop in the positions normally occupied by stamens and in the fourth whorl, the cells that would normally give rise to the gynoecium behave as if they constituted a flower primordium. This process repeats itself indeterminately, resulting in the formation of >70 floral organs in the pattern $(Sepal, Petal, Petal)_n$ (Bowman et al., 1989). Therefore, *ag* mutations have both homeotic (the establishment of the identities of organs in the third and fourth whorls) and heterochronic (the cells that would usually give rise to the fourth whorl behave as their ancestors did) effects. The predicted amino acid sequence of *AG* protein contains a domain that is similar to the DNA binding sequence of the mammalian transcription factor SRF (Norman et al., 1988; Yanofsky et al., 1990). Among the other proteins containing this domain are MCM1 of yeast (Passmore et al., 1988) and DEFICIENS of *Antirrhinum majus* (Sommer et al., 1990). Hybridization experiments showed that *AG* RNA is flower-specific, being >100 fold enriched in floral tissue as compared to vegetative tissue (Yanofsky et al., 1990).

During the stages of flower development that the third and fourth whorl organ primordia are thought to be specified to become stamens and carpels, *AG* RNA is detected only in the third and fourth whorls, a pattern that is consistent with *AG* having a role in the specification of organ identity (Drews et al., 1991). *AG* RNA is detected uniformly throughout the third and fourth whorl organ primordia and is detected even earlier in

development in the region of the floral meristem that will alter give rise to the third and fourth whorl organ primordia. Uniform expression in the primordia continues until after morphological differentiation of the organ primordia has started. Another of the floral homeotic genes, *APETALA2* (*AP2*), was found to be a negative regulator of this early *AG* expression. In an *ap2* mutant background, *AG* RNA is present in all whorls of the flower during the time that floral organ identity is specified (Drews et al., 1991). This confirms a prediction based on observed genetic interactions between *AG* and *AP2* in which *ag* mutations have phenotypic effects in the outer two whorls when in an *ap2* mutant background (Bowman et al., 1991).

In this paper, we show that the expression of *AG* is dynamic during flower development. In contrast to the uniform expression pattern of *AG* throughout the third and fourth whorl organ primordia during the early stages of flower development (when the identity of the organ primordia is being specified), *AG* RNA is restricted to certain cell types in stamens and carpels at later stages of flower development (after morphological differentiation of the floral organs has started). Examination of late *AG* expression in *ap2* mutant flowers suggests that, in contrast to early *AG* expression, *AP2* does not appear to regulate late *AG* expression and, in addition, that the late *AG* expression patterns seem to be set up independently of early *AG* expression. Although no conditional *ag* alleles have been isolated at present to provide clues the the function of late expression patterns, these data suggest that *AG* has a heretofore unsuspected role late in flower development.

Results

Flower development in *Arabidopsis thaliana* has been described in detail and broken down into a series of stages, the first 14 of which are described in Table 1 (Smyth et al., 1990; Müller, 1961). During stages 1-6 the organ primordia form and the specification of

their identity is thought to take place. We refer to these stages as early flower development. During stages 8-12, which we refer to as late flower development, much of the cellular differentiation takes place. Stage 7 is the stage at which morphological differentiation begins to occur in the third and fourth whorls. (The second whorl organ primordia are still morphologically undifferentiated until stage 8 and the first whorl primordia are morphologically differentiated as early as stage 5.) We have summarized some of the landmark events in the development of the ovule and pollen sac (Misra, 1962; Webb and Gunning, 1990) in Table 1.

AG RNA Becomes Concentrated Within Specific Cell Types of the Stamens and Carpels Late in Flower Development

We hybridized an *AG* anti-mRNA probe with tissue sections of stage 8-12 flowers to determine if *AG* RNA is present in stamens and carpels while cellular differentiation of the structures within these organs is taking place. As with early flower development (Drews et al., 1991), hybridization signal is not present above background in sepals and petals (data not shown). As is shown below, however, *AG* RNA is readily detectable in a limited number of cell types of the stamens and carpels as the maturation of these organs progresses.

Stamens

Stamens begin their development when the floral meristem produces six undifferentiated third whorl primordia (stage 5). By stage 7, the regions which will give rise to the filament and the anther become morphologically distinct (Smyth et al., 1990). During this time, a uniform signal is observed throughout the entire organ primordium (Drews et al., 1991). Subsequently, the anther region becomes lobed on its adaxial side as

the hypodermal cell layer divides, giving rise to the primary parietal cells (pre-anther wall cells) and the sporogenous cells (pre-pollen cells; stage 8). The primary parietal cells then undergo anticlinal and periclinal divisions producing the anther wall layers (tapetum, middle layer, and endothecium) and the primary sporogenous cells develop into microspore mother cells which become separated from the anther walls (stage 9). The diploid microspore mother cells undergo meiosis to form a tetrad of microspores (also in stage 9). Figure 1A is a bright field micrograph of a stamen section at the stage of development at about the time the microspore mother cells undergo meiosis (early stage 9; Table 1). This section contains two pollen sacs, each having a pocket of microspore mother cells surrounded by the anther wall layers. A signal is present in the cells of the filament, connective, and anther walls, but the microspore mother cells exhibit only a background level of hybridization grains (Figure 1B). By contrast, [^{3}H]-poly(U) produced a much stronger signal in the sporogenous tissue than the remainder of the anther (Figures 1C,D).

Each microspore, a meiotic product of the microspore mother cells, develops into a microgametophyte (pollen grain; stage 10). Figure 1E is a bright-field micrograph of a longitudinal stamen section from a stage 11 flower by which time pollen grains have matured (Table 1). The connective and two pollen sacs, each composed of a locule with pollen grains and surrounded by the anther wall layers, are visible. A nectary is present at the base of the filament. Figure 1F shows that the hybridization signal is concentrated over the connective of the anther. In addition, the anther walls and filament contain a lower but significant signal above background. By contrast, the pollen grains have no hybridization signal above background.

Prior to dehiscence (stages 11-12), two of the cell layers of the anther wall (the middle layer and the tapetum) and the epidermis degenerate leaving only the endothecial layer surrounding the mature pollen grains (Misra, 1962). Figure 1G is a bright field micrograph of an anther during stage 13, after most of the anther wall has degenerated but prior to dehiscence. A signal is associated with the endothecium (Figure 1H) but not the

mature pollen grains. Thus, *AG* RNA is not expressed at detectable levels in the microspore cell lineage at any stage of their development and is present in fully differentiated cell types, such as the endothecium, in the stamen.

Nectaries

Nectaries arise as small outgrowths of cells at the base of the lateral stamens toward the end of stage 9. By stage 11 they are a dome-like structure with stomata at their apices. The occurrence of nectaries at the base of medial stamens is variable; when they do form they are much smaller than those in lateral positions. Nectary tissue is visible on both sides at the base of the filament in the stage 11 lateral stamen shown in Figure 1E. Figure 1F shows that a strong hybridization signal is associated with the nectary tissue.

Carpels

The first morphological characteristic of the developing gynoecium is the formation of a cylinder during stages 7-8 (Table 1; Smyth et al., 1990). Throughout this time a uniform signal is associated with the gynoecial tissue (Drews et al., 1991). Shortly thereafter (stage 9), ovule primordia arise in four rows from placental tissue on the internal ovary walls of the gynoecial cylinder. At this time, a low uniform signal is associated with the tissue of the ovary walls while a higher signal is associated with the entire ovule primordia (Figure 2A,B).

During stages 9-11, the ovule primordia become stalked, and the two integuments (of the bitegmic ovules) are initiated by periclinal divisions in the epidermis, delimiting the small nucellus (tenuinucellate). As the integuments develop, the ovule curves to assume its anatropous form (stage 12). The hypodermal single-celled archesporium functions directly as the megaspore mother cell which undergoes meiosis to form a multiplanar tetrad of

megaspores (stage 11; Misra, 1962; Webb and Gunning, 1990). The signal associated with the developing ovule remains spatially uniform throughout the ovule from stages 9 to early stage 12 and increases in intensity through stage 12 (data not shown) . In contrast, the signal associated with the ovary walls diminishes during this time. Figure 2C shows an early stage 12 gynoecium with several morphologically differentiated tissues such as the stigma and style, and the ovary. The stigmatic tissue and ovules show high signals whereas the style, the ovary walls, and the placenta of the ovary exhibit little or no detectable signal over background (Figure 2D). Stigmatic papillae are first visible during stage 11 (Smyth et al., 1990). At this time a signal is observed in the developing stigmatic tissue at the apex of the gynoecium (data not shown). In contrast, [^{3}H]-poly(U) produces a signal that is relatively uniform, except the signal over the developing ovule is slightly more intense (Figure 2E,F).

During stages 12-14, the functional megaspore (chalazal megaspore) expands, displacing the three degenerative megaspores, and becomes highly vacuolate, forming the embryo sac. Its nucleus undergoes three successive rounds of mitosis resulting in an eight-nucleate embryo sac. The mature embryo sac contains three chalazal antipodals, an egg apparatus of three cells at the micropylar end, and two central polar nuclei. By this time the integuments have enclosed the nucellus, except for the micropyle. The micropylar nucellar cells degenerate causing the micropylar end of the embryo sac to come into contact with the inner integument (Misra, 1962; Webb and Gunning, 1990). Figure 2G is a bright-field micrograph of an ovule at about the time of development when fertilization takes place (stage 14 of Smyth et al., 1990). This tissue section contains a multinucleate embryo sac that is surrounded by a single layer nucellus, and enclosed by the integuments. Figure 2H shows that the *AG* anti-mRNA probe hybridizes strongly with RNA present within the nucellus. By contrast, the embryo sac, and the cells of the integuments have little or no signal above background. Thus, in fully differentiated ovules, *AG* RNA is localized to the nucellus. We also found that *AG* RNA is present in the stigma of flowers at this stage of

development (data not shown). The distribution of *AG* RNA in the stigma of fertilized flowers is exactly the same as the pattern shown in Figure 2H. Taken together, these data show that *AG* RNA is present in fully differentiated cells, such as stigmatic papillae and the nucellus in the carpels, and that as development proceeds, *AG* RNA becomes progressively restricted to specific cell types.

Expression of *AG* in *apetala2* Mutant Flowers

apetala2-2

In *apetala2-2* flowers (Meyerowitz et al., 1989; Bowman et al., 1991) the medial first whorl organs develop into solitary carpels whose development parallels that of fourth whorl carpels in wild-type flowers. *AG* RNA is present in the medial first whorl organ primordia during the early stages of flower development, suggesting that the *ap2* phenotype is due in part to the ectopic expression of *AG* in the outer whorl of *ap2* flowers (Drews et al., 1991). The expression of *AG* RNA late in flower development, in the carpels that arise ectopically in the medial first whorl positions of *ap2-2* flowers, is in the same pattern as that observed in fourth whorl carpels of wild-type flowers. A relatively uniform expression pattern is seen during stage 8 (data not shown), and then expression of *AG* RNA becomes restricted to developing ovules and stigmatic papillae (Figure 3A,B). Figure 3C shows an ovule originating from a first whorl carpel. This tissue section contains an embryo sac surrounded by a nucellus and enclosed by integuments. The hybridization signal is primarily localized to the nucellus (Figure 3D) while the embryo sac has little or no signal above background. A lower signal is also observed in the surrounding integument tissue. At a later stage of ovule development than that shown, no signal above background is detected in the integuments (data not shown). Thus, the pattern of expression *AG* RNA in ectopic carpels is similar to the progression seen in wild-type carpels, a high uniform

signal early becoming restricted to the stigmatic tissue and to the nucellus as the ovules mature.

apetala2-1

In *apetala2-1* flowers all first whorl positions are occupied by cauline leaf-like organs with stipules at their bases and stellate trichomes on the abaxial surfaces. Late in flower development (stages 8-12) these organs develop carpelloid features, frequently having stigmatic papillae at their tips and occasional outgrowths of cells along their lower margins. The marginal outgrowths appear to be rudimentary ovules because they have structures resembling the nucellar epidermis, integuments, and funiculus (Bowman et al., 1989).

Figure 3E shows a longitudinal section through stage 5, 7, and 10 *ap2-1* flowers. The first whorl organs of the stage 5 flower have no signal above background (Figure 3F; Drews et al., 1991). By stage 7, a hybridization signal is observed in the first whorl organs (Figure 3F). The signal is not uniform over the entire organ but is higher in some areas than others. In both the stage 5 and stage 7 flowers a high uniform signal is observed in the third and fourth whorls (Figure 3F). The first whorl organs of the stage 10 flower in Figure 3E exhibit some carpelloid characteristics. The hybridization signal in these organs is primarily localized at the tip where stigmatic papillae are evident and along the margin over what appears to be a rudimentary ovule (Figure 3F). In general, the hybridization signal observed in mature first whorl organs of *ap2-1* flowers is variable with the carpelloid regions (identified by morphological criteria) displaying higher signal, as well as other regions with no apparent carpelloid morphological characteristics.

Mature second whorl organs of *ap2-1* flowers range in phenotype from deformed stamens to slightly staminoid petals. The deformed stamens have *AG* anti-RNA probe signal in the same cell types that it is associated with in wild-type stamens (data not shown). Signal over staminoid petals is either uniformly low (or not above background) or

mosaic with sectors of high and low signal distributed throughout the organ (data not shown).

Expression of *AG* in *apetala3-1* and *pistillata-1* Mutant Flowers

In *ap3-1* and *pi-1* flowers, hybridization signal is restricted to the third and fourth whorls (data not shown). In the mature fourth whorl carpels of both *pi-1* and *ap3-1* flowers the hybridization signal is the same as seen in wild type fourth whorl carpels (data not shown). The third whorl organs of *ap3-1* flowers have features of both stamens and carpels. The pattern of hybridization grains in these organs is restricted to the same cell types as occurs in the third whorl stamens and fourth whorl carpels of wild-type flowers (data not shown). Thus, mutations in these genes do not appear to affect the spatial distribution of *AG* RNA.

Discussion

Mutations in the floral homeotic gene *AGAMOUS* cause cells in whorls three and four of the developing *Arabidopsis* flower to misinterpret their position and thus to differentiate inappropriately (Bowman et al., 1989; 1991). Consistent with *AG* having a role in floral organ specification, we have previously shown that *AG* RNA is evenly distributed throughout those cells which will give rise to the third and fourth whorl organs, during the time when the fates of the floral organ primordia are thought to be specified (Drews et al., 1991). We show here that the expression of *AG* is not limited to early flower development, but continues in fully differentiated floral organs. Furthermore, the expression pattern is dynamic, evolving from a uniform distribution throughout stamen and

carpel primordia early in development, to a restricted pattern with expression in a small number of specific cell types late in flower development.

AG RNA is Present in Mature Stamens and Carpels

We found that *AG* RNA is present in mature stamens and carpels, after cellular differentiation has taken place. In these organs, *AG* RNA is more concentrated in some cells than in others. In the stamens, *AG* RNA is present at highest levels in the connective of the anther, and at lower levels in the anther walls and the filament. In the carpels, *AG* RNA is present at its highest levels in stigmatic papillae and developing ovules. As the ovules mature *AG* RNA becomes further restricted to just the nucellus. In addition, a high level of *AG* RNA expression is observed in the nectaries.

AG RNA is not detectable in the pollen grains nor in the sporogenous tissue that gives rise to the pollen grains. Clonal analysis (Satina and Blakeslee, 1941; Derman and Stewart, 1973) and morphological studies (Misra, 1962; Bhandari, 1984) of anther development indicate that the pollen grains are ontogenetically related to the anther wall layers, which, as described above, do contain *AG* RNA. Since *AG* RNA appears to be present in all stamen cells early in development (Drews et al., 1991), one feature of the pollen grain cell lineage is a diminution of *AG* RNA concentration. *AG* RNA appears to be present in all cells of the ovule early in their development. At a later stage of development, when the ovules are fully differentiated, *AG* RNA is localized to the nucellus, which is the cell layer surrounding the embryo sac. We did not detect *AG* RNA in the embryo sac nor the integuments of mature ovules. Thus, a decline in *AG* RNA concentration is characteristic of both megagametophyte and microgametophyte development. These data show that as carpel and stamen development proceeds, *AG* RNA becomes progressively restricted to particular cell types and that *AG* RNA is present in fully differentiated cell types.

APETALA2 Does Not Negatively Regulate Late *AGAMOUS* RNA Expression as It Does Early *AGAMOUS* RNA Expression

Based on genetic studies, we previously proposed that *AP2* and *AG* negatively regulate each others' activity, such that *AP2* activity is present in all floral whorls in *ag* mutant flowers, and conversely, *AG* activity is present in all floral whorls in *ap2* mutant flowers (Bowman et al., 1991). Drews et al. (1991) showed that *AP2* is a negative regulator of *AG* RNA accumulation in the outer two whorls of the flower; *AG* expression expands to include all floral whorls in an *ap2-2* mutant background. This negative regulation occurs in the early stages of flower development, during the time in which the floral organ primordia are specified.

In *ap2-2* flowers, carpels arise in the medial first whorl positions, which is proposed to be due, at least in part, to the ectopic expression of *AG* RNA in those organ primordia early in development (Bowman et al., 1991). Later in development, the *AG* RNA expression pattern observed in these ectopic carpels is the same as that observed in the fourth whorl carpels of wild-type flowers; *AG* RNA is expressed at a high level only in specific cell types. The correct temporal and spatial late expression pattern of *AG* RNA observed in the ectopic first whorl carpels of *ap2-2* flowers suggests that the factors which regulate the late *AG* expression pattern in wild-type fourth whorl carpels must also be present in the ectopic first whorl carpels of *ap2-2* flowers. Thus, the data indicate that the *ap2-2* mutation does not perturb the late expression patterns of *AG*, suggesting strongly that *AP2* does not have a role in regulating the pattern of cell type-specific expression of *AG* RNA late in flower development.

AG RNA is not detectable in the first whorl organ primordia of *ap2-1* flowers early in development, during the time in which the organs develop characteristics of leaves, such as stipules and stellate trichomes (Drews et al., 1991). However, *AG* RNA is present in

the first whorl organs of *ap2-1* flowers later in development after they have already begun to develop leaf-like characters (>stage 7; Drews et al., 1991). This suggests that the *AG* RNA detected here is equivalent to the *AG* RNA present in specific cells of wild-type stamens and carpels late in development. In agreement with this, *AG* RNA is not distributed evenly throughout the outer whorl organs of *ap2-1* flowers. Rather, it is present in patches of cells (Figure 3E,F). This late expression of *AG* could be related to the appearance of carpelloid features of the first whorl organs, such as stigmatic tissue at the tips and rudimentary ovules at the margins, which arise late in development (Bowman et al., 1989). That *AG* RNA is present in carpelloid cell types in the first whorl organs of *ap2-1* flowers late in development, but is not detected in these organs early in flower development suggests that the late expression patterns of *AG* are independent of the early expression of *AG*.

Similarity of *AG* Expression Patterns with Expression Patterns of Drosophila Developmental Genes

Early in flower development, *AG* expression is uniform and is localized to those regions of the flower which are altered in *ag* mutants. In this regard, *AG* expression resembles the *Drosophila* homeotic genes involved in pattern formation during embryogenesis. Many of these genes are regulated at the RNA level, and are expressed primarily in the areas of the embryo that are perturbed when mutated (see Ingham, 1988 for review). A general feature of the early expression patterns of the *Drosophila* homeotic genes is that their domain of expression is in part determined by negative regulatory interactions between the homeotic genes (see Ingham, 1988 for review). In this respect, the mutually antagonistic interactions observed for *AP2* and *AG* are reminiscent of interactions seen between *Drospophila* homeotic genes. The early expression patterns of the *Drosophila* homeotic genes are primarily responsible for the specification of segmental

identity, a role similar to that proposed for the early expression of *AG* of in defining organ identity in developing flowers.

Many of the *Drosophila* developmental control genes continue to be expressed throughout embryonic and larval development, their late expression patterns usually involving a subset of cells that exhibited early expression (Beachy et al., 1985; Carroll et al., 1986; Mahaffey and Kaufman, 1987; Riley et al., 1987; Jack et al., 1988; LeMotte et al., 1989). The function of the late expression patterns of at least some of these *Drosophila* genes is necessary to maintain cell fate specification (Lewis, 1978; Jürgens, 1985; Struhl and Akam, 1985; Wedeen et al., 1986; Gibson and Gehring, 1988; Doe and Scott, 1988). For example, transient and ectopic late expression of the *Antennapedia* gene using inducible heterologous promoters leads to different homeotic transformations at different times throughout development, with early misexpression having a more severe effect than misexpression later during larval development (Gibson and Gehring, 1988). In addition, the *Polycomb* group genes, which are not homeotic selector genes themselves, are necessary for the proper maintenance of homeotic selector gene expression after their initial patterns have been established (Struhl and Akam, 1985; Wedeen et al., 1986). Mutations in the *Polycomb* group genes result in indiscriminate late expression of the homeotic genes due to derepression of the homeotic genes (Lewis, 1978; Jürgens, 1985). This indiscriminate late expression causes homeotic transformations similar to, but less severe than those observed for mutations in the homeotic genes themselves. The 'derepression' of *AG* in *ap2-1* flowers late in development resulting in the formation of carpelloid features in the first whorl organs seems to be an analogous situation. In this case, *AG* is ectopically expressed late in flower development in some cells of the first whorl organs despite its apparently wild-type pattern of expression early in flower development, when interaction with another homeotic gene, *AP2*, is in part defining the initial pattern of expression of *AG*.

What is the Function of Late *AG* Expression?

The expression of *AG* in mature stamens and carpels raises the question of whether *AG* has a function late in development. That the pattern of late expression is restricted to only a few cell types argues against *AG* having a general role in the maintenance of carpel and stamen fates as the organ primordia differentiate. Rather, a role in the specification of certain cell types of stamens and carpels seems more likely. The existing *ag* alleles cannot shed light on the possible late functions of *AG* because, in these alleles, neither stamens nor carpels develop. Future experiments in which *AG* is expressed early in development but not late in development will better define the late functions of *AG*. The isolation of weak, conditional alleles of *ag* and the construction of transgenic plants with the *AG* coding region under control of a heterologous inducible promotor will be useful in this regard. Another floral homeotic gene, *DEFICIENS* of *Antirrhinum majus*, is also expressed throughout the development of the organs affected by *deficiens* mutations (Sommer et al., 1990; Schwarz-Sommer et al., 1990). Furthermore, by mosaic analysis, *DEFICIENS* activity has been shown to be present late in development, where it appears to control the fates of at least some cells (Coen et al., 1990).

If *AG* is involved in the specification of cell fate late in development within stamens and carpels, it is likely that it would exert its control on cell type specification by regulating the expression of other genes, since *AG* encodes a putative transcription factor. These downstream genes through which *AG* mediates its effects may be of two classes, those involved directly in cellular differentiation and those that are regulatory factors themselves, representing another level of hierarchy in the control of the specification of cell fate. Two candidates in this latter class are *AGL1* and *AGL2* (Ma et al., 1991). Remarkably, these two genes belong to the same gene family as *AG*, in which there are at least eight members, (Ma et al., 1991; Jack, Brockman, and Meyerowitz, in prep.), with each member possessing a conserved DNA-binding domain originally identified in the mammalian transcription factor, SRF. In addition, the temporal and spatial patterns of *AGL1* and

AGL2 expression are consistent for them to act with or after *AG* in the process of cellular differentiation. The expression of *AGL1* is limited to carpels, with a high level observed in the ovules, while *AGL2* expression is detected only in stamens and carpels, again with a high level of expression detected in ovules. The expression of both *AGL1* and *AGL2* begins in about stage 10 and continues until well after fertilization (Ma et al., 1991). Therefore, these genes could act in combination with or subsequent to *AG* to control cellular differentiation in developing ovules.

Methods

Plant Material

The wild-type variety used in this study was Landsberg *erecta*. The *ap2-2*, *ap2-1*, *ap3-1*, and *pi-1* mutations were in the Landsberg *erecta* genetic background. All plants were grown at 25°C. 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, and mutant alleles in lower case italics.

In Situ Hybridization

Individual flowers or a cluster of flower buds at stages 1-14 of development were dissected and fixed in 3.7% formaldehyde, 5% acetic acid, 50% ethanol. Fixed tissue was dehydrated with ethanol, cleared with xylene, and embedded in paraffin (Paraplast Plus). Embedded tissue was sliced into serial 8 or 10 μm sections with a Sorvall JB-4 microtome

and attached to microscope slides that were coated with poly-L-lysine (Sigma). The in situ hybridizations were carried out as described by Cox and Goldberg (1988).

Three probes were utilized in these experiments. The *AG* anti-mRNA probe and control probes were single-stranded ^{35}S-RNA probes derived from sequences within the cDNA clone pCIT565. The *AG* anti-mRNA probe contained nucleotides 240 to 977 of the *AG* sequence published in Yanofsky et al. (1990). This probe did not cross-hybridize with any of the other cloned genes that have significant sequence similarity with *AG* (Yanofsky et al., 1990; Ma et al., 1991; Drews et al., 1991). The control probe contained nucleotides 9 to 977 of the *AG* sequence published in Yanofsky et al. (1990). This probe was synthesized in the opposite orientation of the *AG* anti-mRNA probe and thus contained sequences identical to *AG* mRNA. The control probe was used to measure background hybridization. The third probe was [^{3}H]-poly(U), which was purchased from Amersham (TRK.481). The poly(U) probe was used to describe the distribution of total poly(A) RNA in floral tissue. This probe also allowed us to assess whether the RNA within a cell was accessible to the in situ hybridization probes.

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Table 1. Stages of Flower Development in *Arabidopsis thaliana*

Stage	Description
1	Flower buttress arises on flank of apical meristem
2	Flower pimordium becomes distinct from main axis by a groove
3	Sepal primordia arise at flanks of floral primordium; pedicle develops
4	Medial sepal primordia elongate to overlie floral primordium
5	Petal and stamen primordia arise; petal primordia barely visible; medial sepals almost cover bud
6	Medial and lateral sepals enclose bud; long stamens become distinct from central dome; petal primordia very small; no visible differentiation of carpels
7	Stamens become stalked at base; petal primordia hemispherical; Gynoecium begins to differentiate and to form cylinder
8	Stamens become lobed; petal growth accelerates but petals remain relatively small; pockets of sporogenous tissue evident in each pollen sac
9	Petals become stalked at base and form flattened blades; gynoecial cylinder becomes constricted at top; microspore mother cells become separated from each other and the tapetum; microspore mother cells undergo meiosis to form tetrads (isobilateral and tetrahedral) of microspores; ovule primordia arise; ovules grow to become elongate
10	Petals reach top of short stamen; gynoecial cylinder closed at top; microspores separate from each other to lie freely in pollen sac; microspores round up; ovules becomes stalked
11	Gynoecium develops stigmatic papillae; tapetum of pollen sac degenerates; integuments grow around ovule; megaspore mother cell undergoes meiosis to form multiplanar tetrad of megaspores
12	Petals level with long stamen; endothecium of pollen sac develops fibrous wall thickenings; tapetum of pollen sac gone; epidermis of pollen sac withering; functional megaspore enlarges to become very expanded and highly vacuolated; embryo sac becomes multinucleate; nuclei are visible within embryo sac; ovule begins to show anatropous orientation
13	Bud opens; petals visible; anthesis
14	Long stamens extend above stigma; fertilization occurs

[a] SEM observations described by Smyth et al. (1990); ovule and pollen sac development described by Misra (1962) and Webb and Gunning (1990).

[b] Stages after 12 described by Müller (1961).

Figure 1. Distribution of *AG* RNA in developing stamens.

(A) and **(B)** In situ hybridization of an *AG* anti-mRNA probe with a stage 9 anther. (A) Bright-field micrograph. (B) Dark-field micrograph.

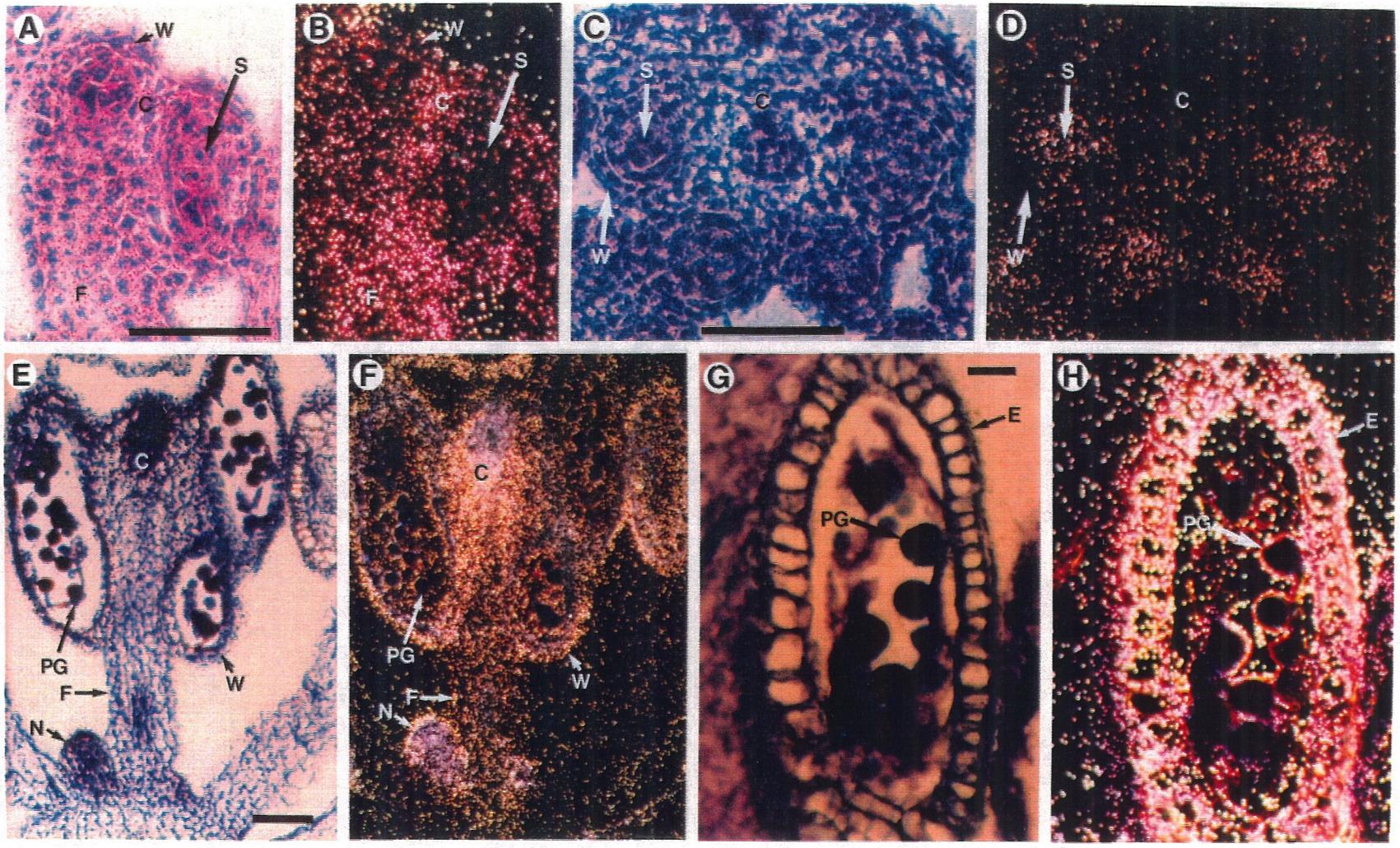
(C) and **(D)** In situ hybridization of ^{3}H Poly-U probe with a stage 9 anther. (C) Bright-field micrograph. (D) Dark-field micrograph.

(E) and **(F)** In situ hybridization of an *AG* anti-mRNA probe with a stage 11 stamen. (E) Bright-field micrograph. (F) Dark-field micrograph.

(G) and **(H)** In situ hybridization with AG anti-mRNA probe with a stage 13 anther. (G) bright-field micrograph. (H) Dark-field micrograph.

Flower structures are given the following abbreviations: C, connective; F, filament; S, sporogenous tissue; N, nectary; PG, pollen grains; W, anther wall layers.

Bar = (A-F) 50 μm and (G-H) 10 μm.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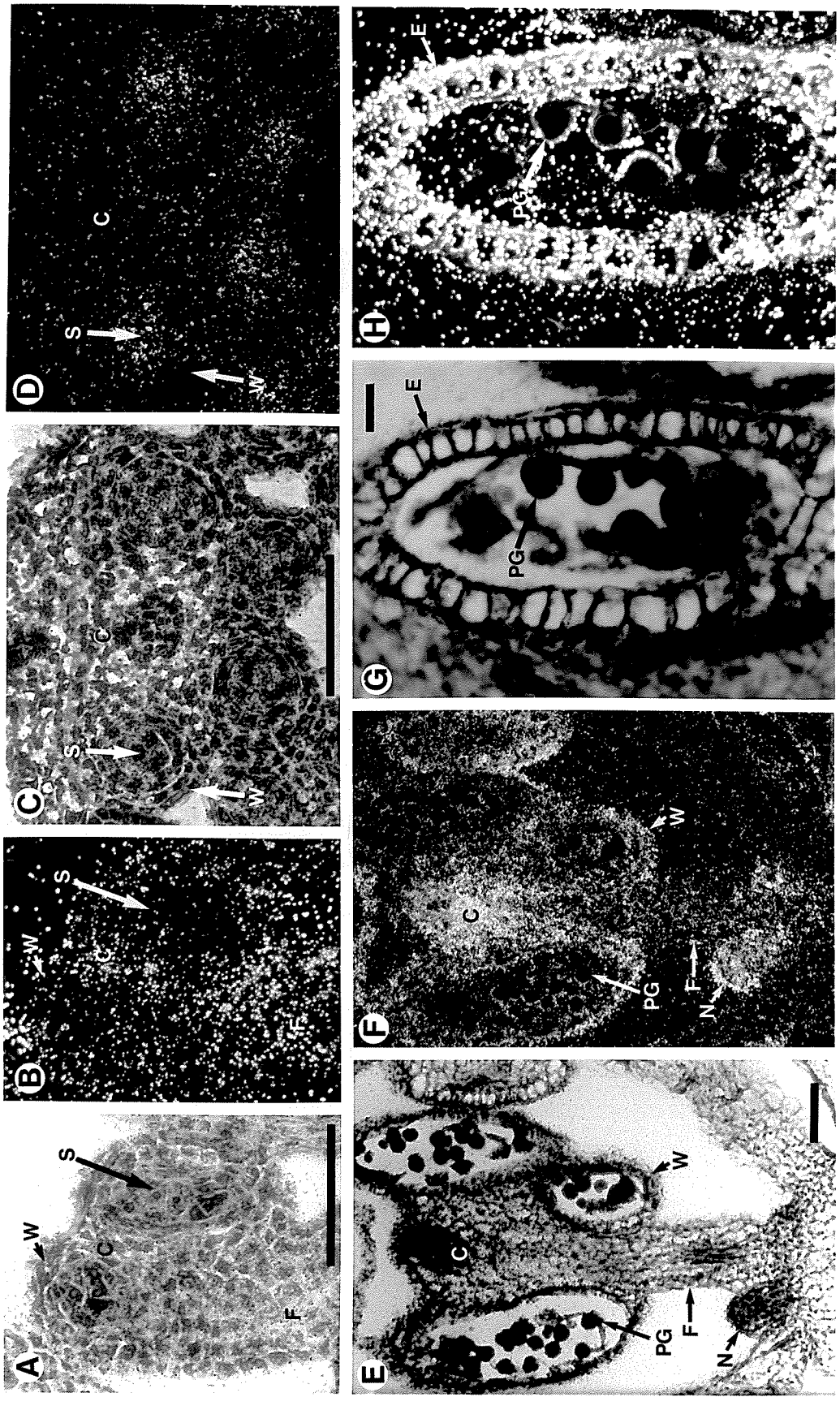
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Figure 2. Distribution of *AG* RNA in developing carpels.

(A) and **(B)** In situ hybridization of an *AG* anti-mRNA probe with a stage 9 gynoecium.

(A) Bright-field micrograph. (B) Dark-field micrograph.

(C) and **(D)** In situ hybridization of an *AG* anti-mRNA probe with a stage 12 gynoecium.

(C) Bright-field micrograph. (D) Dark-field micrograph.

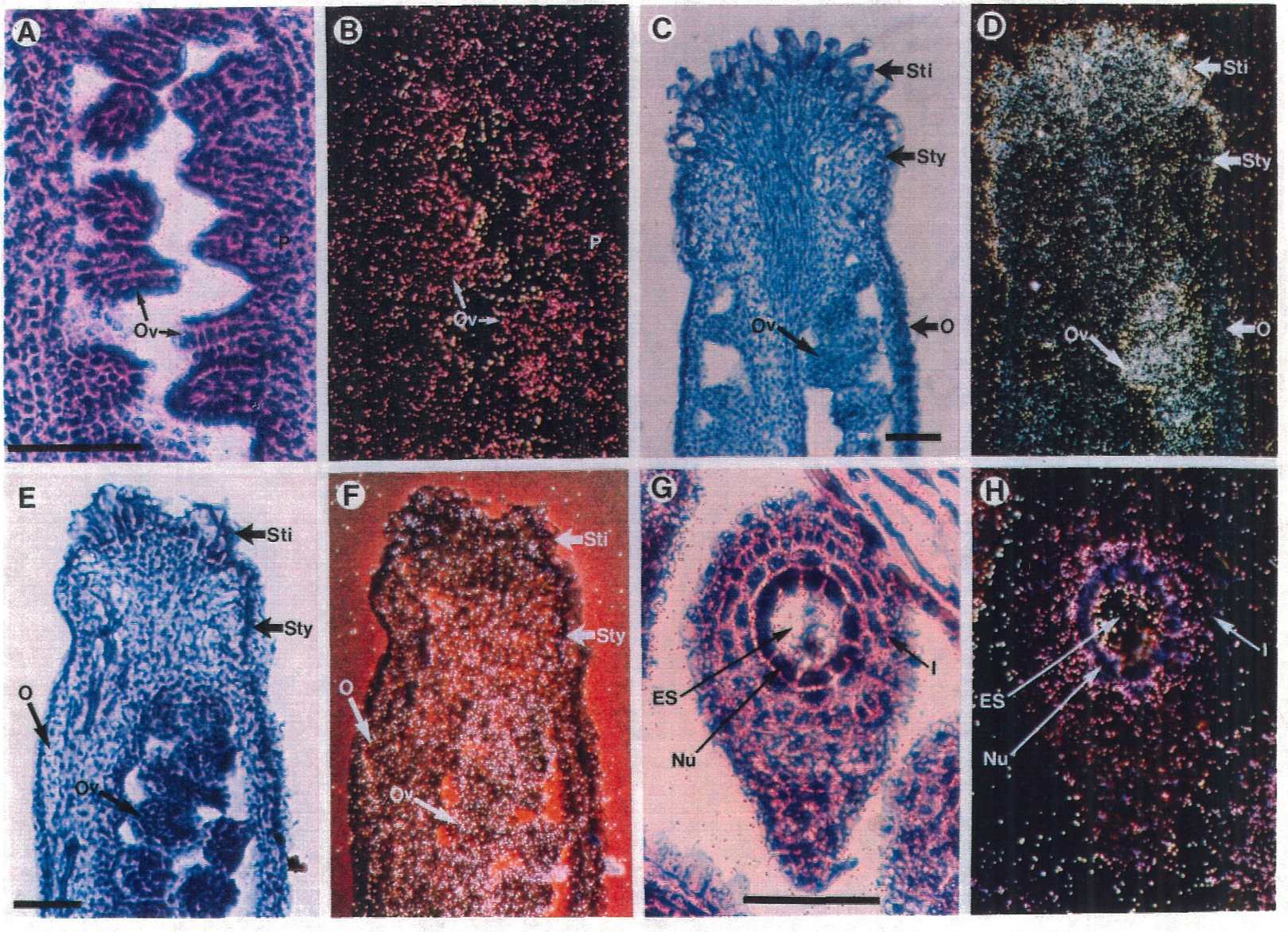
(E) and **(F)** In situ hybridization of ^{3}H Poly-U probe with a stage 12 gynoecium. (E) Bright-field micrograph. (F) Dark-field micrograph.

(G) and **(H)** In situ hybridization of an *AG* anti-mRNA probe with a differentiated ovule.

(G) Bright-field micrograph. (H) Dark-field micrograph.

Flower structures are given the following abbreviations: ES, embryo sac; I, integuments; Nu, nucellus; O, ovary; Ov, ovule; P, placenta; Sti, stigma; Sty, style.

Bar = 50 μm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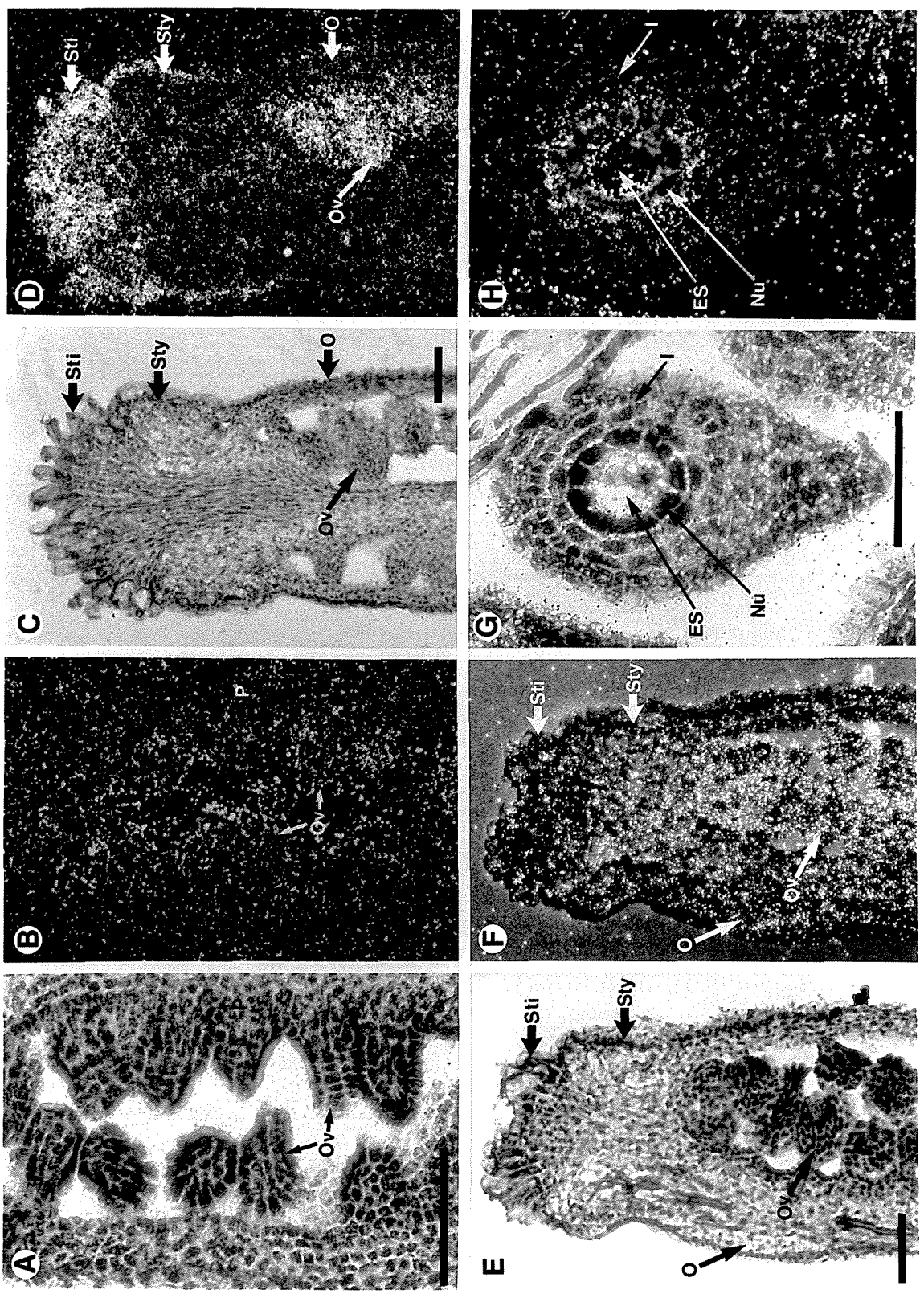
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Figure 3. Distribution of *AG* RNA in *apetala2* flowers.

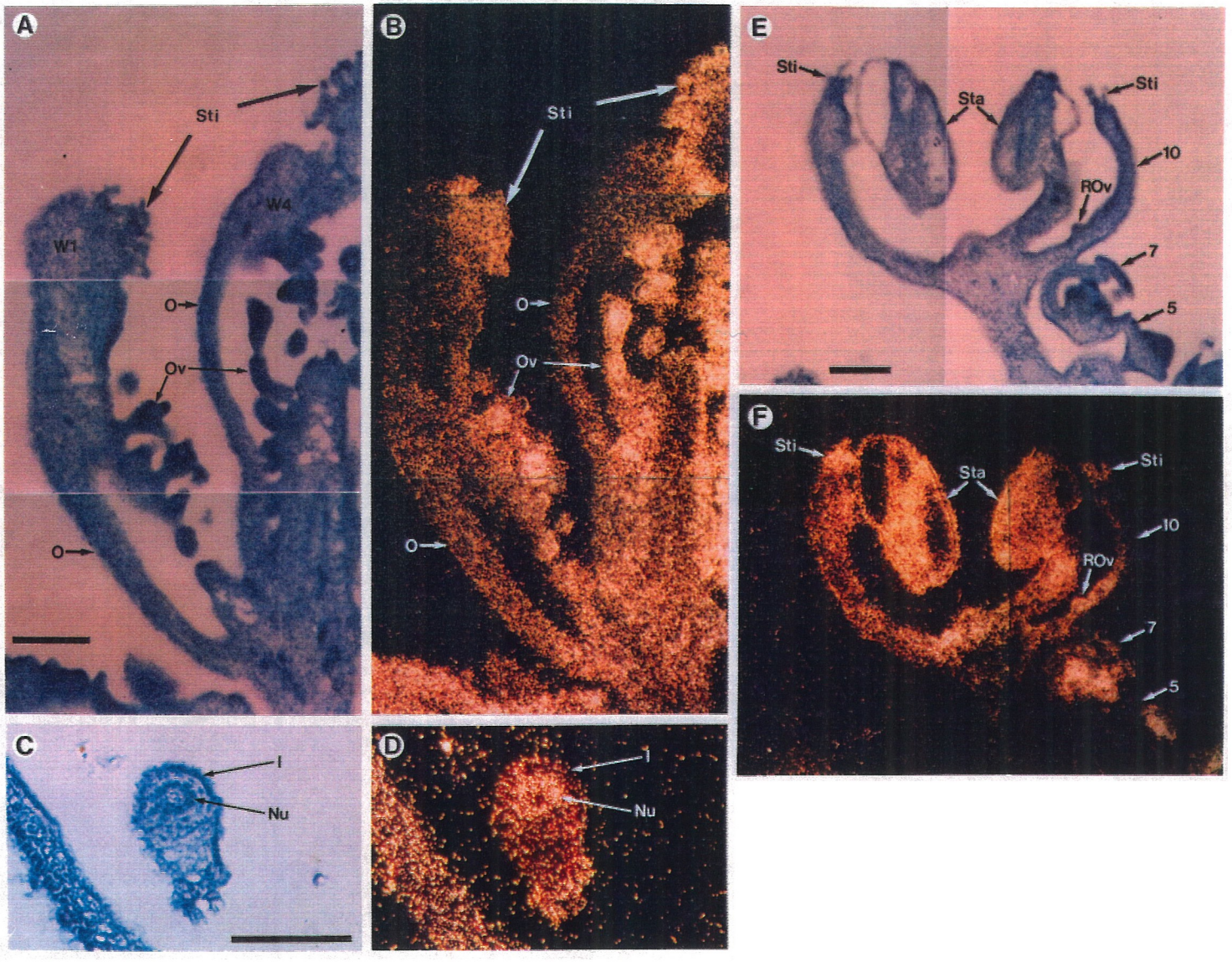
(A) and **(B)** In situ hybridization of an *AG* anti-mRNA probe with first and fourth whorl carpels of an *ap2-2* flower. (A) Bright-field micrograph. (B) Dark-field micrograph.

(C) and **(D)** In situ hybridization of an *AG* anti-mRNA probe with a differentiating ovule on a first whorl carpel of an *ap2-2* flower. (C) Bright-field micrograph. (D) Dark-field micrograph.

(E) and **(F)** In situ hybridization of an *AG* anti-mRNA probe with three *ap2-1* flowers of different developmental stages. Two medial first whorl organs of a stage 10 flower (10) are visible, as well as a stage 7 flower (7) and a stage 5 flower (5). The arrows identifying the flowers of different stages point to a first whorl organ of each. (E) Bright-field micrograph. (F) Dark-field micrograph.

Flower structures are given the following abbreviations: I, integuments; Nu, nucellus; O, ovary; Ov, ovule; ROv, rudimentary ovule; Sta, stamen; Sti, stigma.

Bar = 100 μm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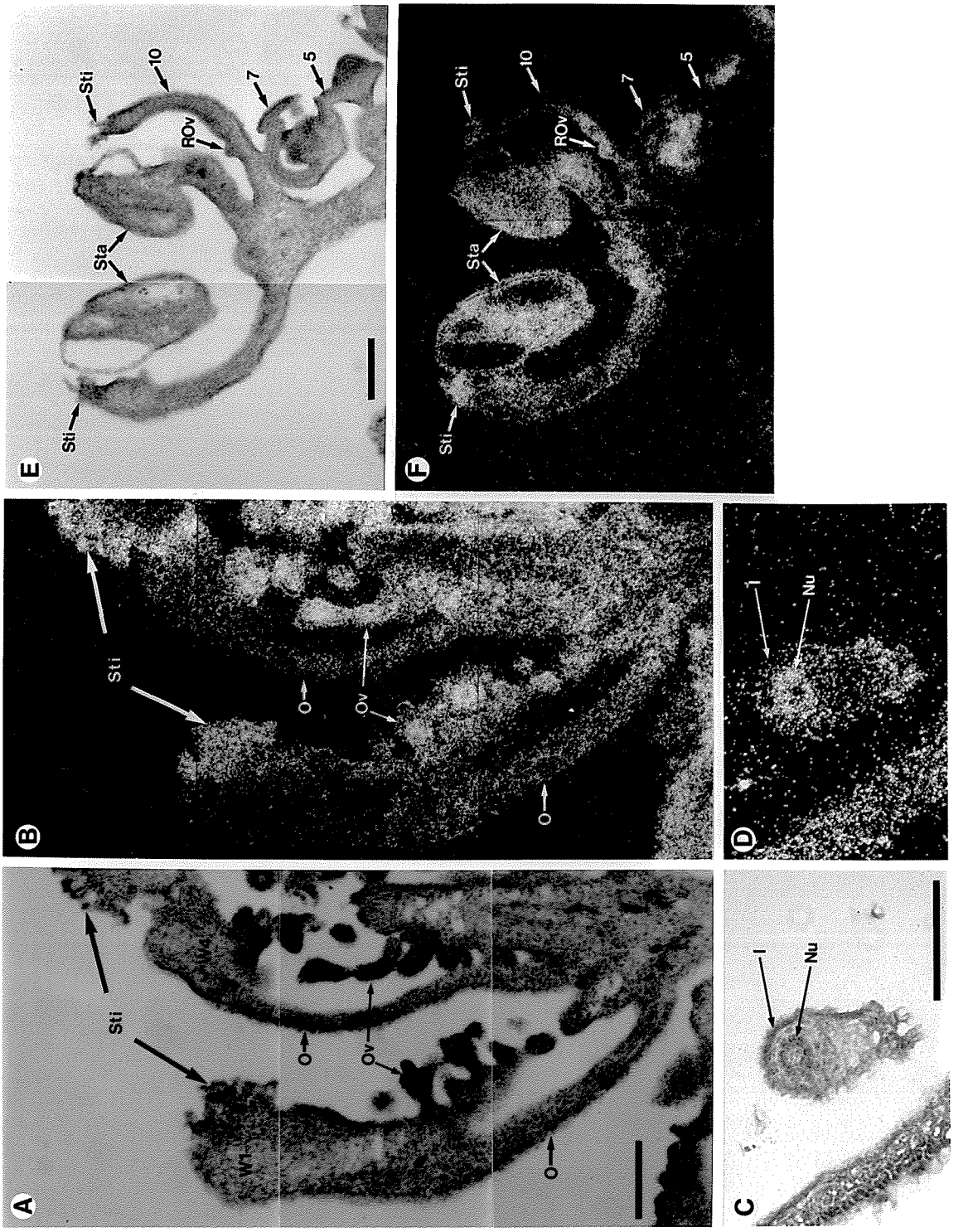
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CHAPTER SEVEN

SUPERMAN AND *APETALA1*, CANDIDATES FOR GENES REGULATING THE FLORAL HOMEOTIC GENES

Introduction

Thus far the genetic and morphological analyses (Chapters 2 and 3) have concentrated on several homeotic mutations. These were chosen because each appears to cause the cells in the developing flower to misinterpret their position and thus differentiate into inappropriate cell types. The result is morphologically normal organ types in ectopic positions within the flower. Each mutation affects the identity of organs in two adjacent whorls, and has specific homeotic effects in each of the affected whorls. The four genes identified by these homeotic mutations are *AGAMOUS*, *APETALA2*, *APETALA3*, and *PISTILLATA*. The model presented in Chapter 3 (Bowman et al., 1991), in which different combinations of homeotic genes specify the identity of floral organs, was based on a series of genetic experiments. It is clear that cross regulatory interactions between the homeotic genes help define the spatial domains of activity of the homeotic genes (for example, the mutual antagonistic interactions between *AG* and *AP2*). However, this does not thoroughly explain the proposed localization of activity in specific whorls. That at least two of the genes, *AG* and *AP3*, are expressed in a well defined spatial pattern early in flower development during stages 3-4 (Chapter 5, Drews et al., 1991; Tom Jack, unpublished) suggests that the homeotic genes are responding to a prepattern.

Several other mutations have been isolated which affect pattern formation in developing flowers. None of these has effects that are strictly homeotic transformations, so they were not included in the previous analyses (Chapters 2-3, Bowman et al., 1989; Bowman et al., 1991). The genes identified by these mutations are candidates for those responsible for setting up the prepattern to which the homeotic genes respond. The first to be discussed, *SUPERMAN*, alters the pattern of organ primordia, both positions and identities, that form inside of the second whorl of the flower (Introduction, Bowman and

Meyerowitz, 1991). By contrast, *APETALA1* is involved in the same developmental processes in the outer two whorls (Koornneef et al., 1983; Irish and Sussex, 1990). Both of the genes have some effect on the determinacy of the floral meristem, although in very different manners. Possible roles for these genes in pattern formation in the developing flower will be discussed in context of the model for specification of organ identity presented in Chapter 3 (Bowman et al., 1991).

Materials and Methods

Both *sup-1* and *ap1-1* are recessive and were isolated in the Landsberg ecotype, homozygous for the *erecta* mutation, and were generated by mutagenesis of seeds with ethylmethane sulfonate (EMS). *sup-1* was a gift from Uli Mayer and Gerd Jürgens (U. of Munich, Munich, Germany). *ap1-1* was a gift from Maarten Koornneef (Department of Genetics,Wageningen Agricultural University, The Netherlands). All other strains have been previously described (Bowman et al., 1991). 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. Doubly and triply mutant strains were constructed by manual cross-pollination, using as parents strains homozygous for individual mutations, except in the strains involving *agamous* alleles, which are sterile when homozygous, thus necessitating the use of heterozygotes as parents. The resulting F_1 plants were allowed to self-pollinate, and double and triple mutants were selected from the F_2 plants. Seeds were planted on a peat moss/potting soil/sand (3:3:1, v:v:v) mixture. The plants were grown in incubators under constant cool-white fluorescent light at 25°C (unless otherwise stated) and 70% relative humidity.

For scanning electron microscopy (SEM), young primary inflorescences were fixed in 4% glutaraldehyde in 0.025 M sodium phosphate (pH 7.0) at 4°C overnight, and then transferred to 1% osmium tetroxide in the same buffer at 4°C overnight. They were rinsed

in the same buffer 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 using glass needles. The mounted specimens were coated with gold and palladium (4:1) in a Technics Hummer V sputter coater following each dissection. SEM was performed on an ETEC Autoscan scanning electron microscope at an accelerating voltage of 20 kV, and the images recorded on Kodak 4127 or Polaroid 55 film.

Results

superman-1

Flowers of plants homozygous for the *superman-1* (*sup-1*) mutation have pattern defects in the third and fourth whorls (Figures 1B, 2E-P). The outer two whorls are phenotypically normal with four sepals and four petals respectively. Interior to the petals however, where in wild-type flowers six stamens and the gynoecium occupy the third and fourth whorls respectively, from 8 to 26 stamens are present (average 14.6; 876 stamens in 60 flowers; Figures 2I-M). The number of stamens produced decreases acropetally such that the first few flowers often have more than twenty stamens while the later ones may have only 6-10 stamens. The innermost stamens may be carpelloid. The extra stamens appear to form at the expense of the gynoecium which is usually greatly reduced in *sup-1* flowers. Nectaries may be found at the base of the outermost as well as the inner stamens. Sometimes no carpel tissue forms interior to the extra stamens (13/60 flowers) such that the flower meristem terminates during the production of stamens. When present, gynoecia consist of one (26/60 flowers) or two (10/60 flowers) unfused carpelloid organs or of single fused filamentous carpels lacking ovules and capped with stigmatic papillae (10/60 flowers; Figure 2L). Filamentous organs with cellular morphology similar to stamen

filaments but capped with stigmatic papillae are also observed (Figure 2N). Occasionally, phenotypically nearly-normal gynoecia will develop (1/60 flowers), thus allowing for self-fertilization. The carpelloid organs that develop interior to the stamens are usually mosaics of carpel and stamen tissue and may be fused with the innermost stamens. In these organs the sectors of stamen tissue and carpel tissue are often in large distinct sectors (Figure 2O), with only two longitudinal sectors (one of each type) in the organ suggesting an early fusion of organ primordia or organ primordia overlapping the third and fourth whorl boundary. Alternatively, small sectors of stamen tissue flank a central carpelloid sector. In this case ovules are produced not from the margin of the carpel tissue but in a column in the center of the carpel tissue. Occasionally, the sectors of stamen and carpel tissue are smaller and interspersed (Figure 2P), similar to the third whorl organs of *ap3-1* flowers (Bowman et al., 1989). More than one of these mosaic organs can occur in the same flower.

Observations on developing *sup-1* flowers (Figures 2E-P) show that the development of whorls one and two is normal; four sepals and four petals form in their wild-type positions. The third whorl organ primordia usually, but not necessarily, arise in their normal positions (Figures 2F-I). They may be altered in number (5-7) as well as position. In addition, the third whorl primordia may vary in size with some primordia larger than normal and others smaller than normal. Following the initiation of the third whorl organ primordia, several more primordia are produced in a whorled fashion (although the pattern is somewhat irregular) interior to the third whorl primordia (Figures 2H-I). Each of these 3-6 primordia per whorl also develops into a stamen. This process continues for a variable period of time; for example as few as a single whorl or as many as five to six whorls of organ primordia, all of which develop into stamens, may be produced. Following the production of primordia which develop into stamens, the remaining floral meristem, if there is any, develops into carpel tissue. Often the carpel primordium is irregularly shaped and is congenitally fused to one of the innermost presumptive stamen

primordia, resulting in the formation of a mosaic organ (Figure 2O). These mosaic organs have a well defined boundary between the stamen and carpel tissue.

A mutation with a similar phenotype has been described in an abstract by Schultz and Haughn (1990); allelism tests between this mutation and *superman-1* are in progress.

superman-1 apetala2-2

As in the other double mutant strain involving *sup* and *ap2* alleles, flowers of plants homozygous for both *sup-1* and *ap2-2* exhibit a nearly additive phenotype (Figures 1C, 3A-E). The medial first whorl organs are solitary carpels (52/78 positions counted in 39 flowers), phylloid carpels (2/78), or mosaic organs with sectors of carpel and stamen tissue (24/78). The stamen sectors of the mosaic organs always occupy the margins while the carpel sectors occupy the central regions of the organs, just as is observed in *ap2-2* flowers. The lateral first whorl organs are most often absent (43/78), but may be cauline leaf-like organs (15/78), filamentous structures (17/78) or carpelloid leaves (3/78); again this similar to that observed in *ap2-2* flowers. No second whorl organs are present. The central region of the flower is again highly variable in phenotype. On average 3 organs (118 organs/39 flowers) occupy this region of the flower but from 0 to 6 organs were observed. The phenotypes of these organs were stamens (60/118 organs), carpelloid stamens (12/118), staminoid carpels (20/118), solitary carpels (14/118), and filamentous fused carpels lacking all internal structures and capped with stigmatic papillae (12/118). Normal, fully fused gynoecia were not observed. The outer organs tend to be stamens while the organs arising from the region that would ordinarily develop into the gynoecium are mosaics of both stamen and carpel tissue. The mosaic organs resemble those observed in *sup-1* flowers. If more than one carpelloid organ is present the carpelloid organs are usually fused to each other along the carpel tissue. Whether the observation that every *ap2-2 sup-1* flower had some carpel tissue while many *sup-1* flowers do not have any, is of

some significance or alternatively reflects statistical variation due to the small number of flowers examined is unknown.

The development of the outer whorl of organs in *sup-1 ap2-2* flowers parallels that observed for *ap2-2* flowers (Figures 3A-E; Chapter 3). The development of the central organs, the third and fourth whorls, is variable. The organ primordia are not produced in a consistent pattern and they are often fused congenitally (Figures 3B-D). The more abaxial organ primordia develop into staminoid organs while those more central develop into carpelloid organs. The development of these organs is similar to that observed for the innermost stamens and stamen-carpel mosaic organs of *sup-1* flowers (see above). Occasionally, when no second, third, or fourth whorl primordia are produced, the medial first whorl carpels fuse congenitally resulting in a flower that consists only of a two-carpelled gynoecium (Figure 3E). This has also been observed in *ap2-2* flowers.

superman-1 apetala2-1

sup-1 ap2-1 homozygotes produce flowers whose phenotype is essentially an addition of the effects of the two single mutants (Figures 1D, 3F-J). The outer whorl consists of four cauline leaf-like organs that may develop carpelloid features such as stigmatic papillae at their tips and rudimentary ovules along their margins. The second whorl is occupied by organs with features of both stamens and petals. The phenotypes of the first two whorls of organs are the same as observed for the outer two whorls in *ap2-1* flowers (Chapter 2). Interior to the outer two whorls, 4 to 9 stamens develop (average 6.8; 204 stamens/30 flowers), a number smaller than that observed in *sup-1* flowers. This is probably due to the reduced number of stamens (4.9 per flower) produced in *ap2-1* flowers as compared to wild type. The innermost stamens may have stigmatic tissue at their tips. The remainder of the flower consists of a variable amount carpelloid tissue similar to that

observed in *sup-1* flowers. This tissue is usually a mosaic between carpel and stamen tissue (42 staminoid carpels/30 flowers). In only one of 30 flowers scored was there no carpelloid organ present.

Development of *sup-1 ap2-1* flowers parallels that of *ap2-1* and *sup-1* flowers (Figures 3F-J). The outer two whorls develop as has been described for *ap2-1* flowers (Chapter 2). Third whorl primordia develop in a similar fashion to that observed in *ap2-1* flowers; there may be fewer than six stamens and they may arise in ectopic positions (Figure 3G). Each of these primordia develops into a stamen. Interior to the third whorl, a fourth whorl generally consisting of 3 or 4 organ primordia arises (Figures 3I-J). Each of these primordia develops into a stamen although these organs often develop carpelloid characteristics such as stigmatic tissue at their tips. Development interior to the fourth whorl is variable. In most cases a staminoid carpel or filamentous structure capped with stigmatic papillae is produced, although occasionally the floral meristem ceases to exist following the development of the fourth whorl (Figure 3J).

superman-1 agamous-1

Flowers of plants homozygous for both *sup-1* and *ag-1* consist of an first whorl of sepals followed by an indeterminate number of petals (Figure 1E). Observations of developing flowers elucidates the developmental basis of this phenotype (Figure 4A-H). The first and second whorl organ primordia arise in the correct positions and numbers as in *sup-1* or *ag-1* single mutants (Figures 4A-B). These organ primordia subsequently differentiate normally into wild-type sepals and petals, respectively. The production of third whorl organ primordia, although usually normal, may be altered in a manner similar to the third whorl primordia of *sup-1* flowers. Their position as well as their number and size may be altered. Each of these primordia develops into a petal as in *ag-1* flowers. As

in *ag-1* flowers, the organ primordia that arise in the second and third whorls are approximately the same size (Figure 4B). The remaining floral meristem, rather than behaving like another flower meristem as occurs in *ag-1* flowers, continues to produce whorls of organ primordia that subsequently develop into petals (Figures 4C-E). The organ primordia of each of these later whorls are variable in number (4-8) and position (Figures 4C-E). This process continues indeterminately resulting in a double flower phenotype, and the ultimate *Arabidopsis* ornamental flower. Both single mutations, *sup-1* and *ag-1*, cause indeterminate growth to varying extents. In *sup-1 ag-1* flowers, after several whorls are produced, fasciation of the floral meristem may occur with the meristem becoming enlarged (over 100 μm in length) and elongate in shape. Organ primordia, all of which develop into petals, are produced along the entire circumference of the fasciated meristem (Figure 4F-G). Thus, the two mutations interact synergistically in causing increased indeterminate growth. Close examination of older flowers reveals that some of the inner organs, while being primarily petaloid in character, may have some sepaloid characteristics such as stomata, and some sepaloid epidermal cells (Figure 4H).

The double mutants, *sup-1 ag-1*, are easily distinguished from *ag-1* flowers due to the differences in growth rates of sepals and petals. In *ag-1* flowers the sepals that arise in the fourth whorl rapidly dwarf the adjacent developing petals and grow to cover the floral meristem forming a structure resembling an internal flower bud. In contrast, since all of the primordia (except the first whorl) of *sup-1 ag-1* flowers develop into petals, all have the same slow growth rate characteristic of petals. The result is that the floral meristem is not covered by developing organs. Thus, it is exposed even in relatively old flowers (Figure 4F).

superman-1 agamous-1 apetala2-1

sup-1 ag-1 ap2-1 flowers have a similar architecture, with minor differences, in terms of the pattern of organ primordia formation as *sup-1 ag-1* flowers (Figure 4I-L). The minor differences are that the second whorl primordia do not always form in the triply mutant flowers, probably due to the *ap2-1* mutation since loss of second whorl organs also occurs in *ap2-1* flowers. Additionally, the pattern of third (and subsequent whorls) whorl organ primordia formation is more often irregular (Figure 4J; altered third whorl positions are also observed in *ap2-1* flowers).

The identity of the organs resembles those of *ag-1 ap2-1* flowers except that the leaf-like organs which occur in inner whorls of *ag-1 ap2-1* flowers are not present in the triply mutant flowers (Figure 1F). The outer whorl organs of *sup-1 ag-1 ap2-1* flowers are cauline leaves with some carpelloid characteristics, just as is observed in the first whorl of *ag-1 ap2-1* flowers (Figures 4F,L). All organs interior to the first whorl are staminoid petals, like those occupying the second and third whorls of *ag-1 ap2-1* flowers (Figures 4F,L). Thus, in the triple mutant, the pattern of organs is similar to that seen in *sup-1 ag-1* flowers, while the identity of the organs is like that observed in *ag-1 ap2-1* flowers.

superman-1 pistillata-1

The phenotype of *sup-1 pi-1* flowers is the same as that of *pi-1* flowers. The first whorl contains four wild-type sepals while the second whorl is occupied by four smaller sepals. No third whorl organs are formed. The remaining floral meristem gives rise to an enlarged gynoecium composed of 2 to 4 carpels (3.3, n=14). Filamentous structures like those observed associated with *pi-1* gynoecia may be present. Since no plants in the F2 generation from this cross produced flowers with a phenotype different from *sup-1* and *pi-1* flowers, the double mutant plants were identified by crossing putative double mutants

(which were thought to look like *pi-1*) with plants homozygous for *superman-1*. Five plants with a phenotype indistinguishable from *pi-1* single mutants were crossed to *sup-1* homozygotes. One cross generated 100% (8/8) progeny with the *sup-1* phenotype indicating that this particular plant was homozygous for both *pi-1* and *sup-1*. To prove this conclusively, plants of the genotype

sup-1 pi-1

sup-1 + were allowed to self-fertilize.

One-fourth of the progeny should be a double homozygote and have a *pi-1* phenotype. This cross should also help clarify the segregation data from the original cross (Appendix Four). This experiment is now in progress. Thus, *pi-1* is epistatic to *sup-1*.

apetala1-1

There are three major aspects of the *ap1-1* phenotype, each of which will be discussed separately. Firstly, there are alterations in the pattern and identity of floral organs primarily in the outer two whorls although all four whorls may be affected (Figure 1G; Irish and Sussex, 1990). Secondly, axillary buds may be produced in the axils of the first whorl organs (Figures 1G, 5A-J; Irish and Sussex, 1990). These buds also display the *ap1-1* phenotype. And thirdly, homeotic conversions of floral meristems to inflorescence meristems occasionally occur in the first few primordia produced by the inflorescence meristem (Figures 5A-G). These transformed meristems behave in a similar manner to the primary apical inflorescence meristem from which they developed.

Plants homozygous for the recessive *ap1-1* mutation primarily display pattern defects in the outer two whorls of their flowers (Figure 1G). Organs occupying the first

whorl positions (Table 1) have characteristics of both leaves and sepals and have been referred to as bract-like as they resemble bracts that subtend flowers in other species of plants (Irish and Sussex, 1990). These organs have a cellular morphology similar to that of leaves but lack the large number of stellate trichomes associated with leaves. Stipules may develop at their bases (Figure 5D). The presence of long abaxial epidermal cells characteristic of sepals is variable. The pedicel may elongate between the first whorl organs themselves and between the first whorl organs and the inner whorls of the flower resulting in conspicuous internodes (Figure 1G). Thin filamentous structures called squamules (Arber, 1931a, 1931b) may arise on the pedicels of *ap1-1* flowers. In the first whorl positions lacking well-developed organs, small outgrowths of cells are usually present. Filamentous structures flank the small outgrowths and a group of cells resembling a meristem is present in the axil of the outgrowths (Figure 5I). The filamentous structures may be either stipules flanking an aborted leaf primordium or squamules. Most of the second whorl positions are unoccupied. When present, second whorl organs are either petals, mosaic organs with characteristics of both petals and the leaf-like organs that occur in the first whorl positions, or leaf-like organs like those that appear in the first whorl (Figure 1G). The third whorl is occupied by morphologically normal stamens although fewer than six are usually present and they may occupy ectopic positions and be reduced in size relative to wildtype. The central gynoecium found in the fourth whorl is morphologically wild type. Later flowers exaggerate these phenotypic characteristics; typically these flowers have with no first or second whorl organs, 4-6 third whorl stamens, and an unfused fourth whorl gynoecium (Figure 5H).

In the following description, the flower that develops from a primordium produced directly by the primary inflorescence meristem is referred to as the primary flower. Flowers that develop in the axils of the primary flower's first whorl organs or on its pedicel are referred to as secondary, and those that develop from the analogous positions on the

secondary flower are termed tertiary flowers. The term axillary flowers is used to refer to secondary, tertiary, or higher order flowers in general. In the axils of the first whorl organs, secondary floral buds may develop (Figure 5C). These secondary flowers can occur in the axils of all four first whorl organs, although this is rarely the case. In the first few primary flowers produced by the inflorescence meristem, 'axillary' flowers can arise from the pedicel of the primary flower in positions that are not well-defined with respect to the primary flower. Secondary flowers may also arise in well-defined lateral and medial positions that do not have a fully developed first whorl organ (Figure 5D). In this case, small outgrowths of cells with associated filamentous structures accompany the axillary flower. Thus, the formation of an 'axillary' bud is not dependent on the presence of a fully developed first whorl organ. The lateral positions are more frequently occupied by axillary buds than the medial. This process is reiterated up to four times resulting in a structure which can be composed of greater than 15 flowers. The basic structure of the secondary and tertiary flowers is the same as that of the primary, except that more organs tend to be missing; the adaxial first whorl position is seldom occupied by an organ (Figure 5J). The secondary flowers are oriented with respect to the primary flower, tertiary flowers with respect to the secondary flower from which it developed and so on. The frequency of secondary and tertiary flowers decreases acropetally such that tertiary flowers are no longer formed by about the fifth flower and secondary flowers are infrequent by about the tenth flower although these boundaries vary from plant to plant (Table 2). The first whorl organs of the inner flowers are often carpelloid, and their central gynoecium may be unfused. Mosaic organs between petal and bract-like tissue and between stamen and bract-like tissue are observed at a low frequency in all (primary, secondary, and tertiary) flowers. These mosaic organs consist of large longitudinal sectors with little hybrid tissue. Squamules also develop on the pedicels of secondary and tertiary flowers. Occasionally, groups of apparently undifferentiated cells are observed between the first and fourth whorl positions (Figure 5I). The origin and fate of these cells are unknown. In summary, the

effects on floral organ formation and identity increase acropetally while the number of axillary buds produced decreases.

Following the production of cauline leaves, whose development in number and position is not altered in *ap1-1* plants, primordia are produced on the flanks of the inflorescence meristem in a phyllotactic spiral. The first few of these primordia may behave like inflorescence meristems rather than floral meristems (though, like floral meristems they are not subtended by a cauline leaf) as they would in wild type (Figure 5A-B). These transformed inflorescence meristems do not produce cauline leaves but rather produce floral primordia in a phyllotactic spiral (Figure 5E-F). The flower first primordium produced in such a secondary inflorescence may also behave as a tertiary inflorescence meristem (Figure 5G). The later floral primordia produced by both the primary inflorescence and the transformed inflorescence meristems develop as described for *ap1-1* flowers above. Thus, the *ap1-1* mutation can cause a homeotic (and heterochronic) transformation of floral meristems to inflorescence meristems.

The development of *ap1-1* flowers diverges from that of wild type at stage four when the first whorl organs of *ap1-1* flowers fail to curve around the remaining floral meristem. This may be due to the failure to develop the long abaxial epidermal cells that characterize wild-type sepals. In some of those positions lacking fully developed first whorl organs, structures that may be aborted organ primordia are present (see above) since these structures arise at the time the first whorl primordia are produced (Figure 5D). Some of these structures have what appear to be stipules flanking an aborted leaf-like primordium (Figure 5D,I). These structures may have an associated secondary flower, or what appears to be an aborted meristem. Second whorl primordia fail to arise in most cases. The third whorl primordia are positioned correctly when six are formed, but may occur in ectopic positions when fewer than six develop. Secondary buds are evident as early as stage 2-3 and their development parallels that of primary flowers (Figure 5A), except that their

developmental age is younger than the primary flower. Similarly, the tertiary buds arise when the secondary buds are in stage 2-3. The elongation of the pedicel to produce prominent internodes between the first whorl organs occurs later in development (after stage 9). The primordia which behave as inflorescence meristems develop in the same time course as the primary inflorescence meristem.

Enhancer of ap1-1

In the process of constructing an *ap1-1 ag-2* double mutant strain, the *ap1-1* mutation was crossed out of the Landsberg *erecta* background into the Ws background. In many of the families segregating from this cross, plants were identified in which the phenotype of *ap1-1* was enhanced. This occurred in families that did not segregate *ag-2* and is thus not an effect of the *ag-2* mutation. The third phenotypic aspect of *ap1-1* discussed above, the homeotic transformation of cells that would ordinarily constitute floral meristems in wild-type plants, to inflorescence meristems, is enhanced in the Ws genetic background (Figures 1H-I, 5K-T). The cells in every primordium produced on the flank of the apical meristem behave as if they constituted an inflorescence meristem rather than a flower meristem (Figure 1I, 5K). These inflorescence meristems produce primordia on their flanks which also behave as if they were inflorescence primordia. Thus, the enhanced phenotype resembles a homeotic (and heterochronic) conversion of the flower meristem to an inflorescence meristem. The transformed meristems do not produce cauline leaves but rather produce primordia in a phyllotactic spiral, a pattern reminiscent of flower primordia on the flanks of a wild-type inflorescence meristem (Figures 5N-R). Eventually, after two to three repeats of this cycle, flower primordia are formed and they develop into flowers with a phenotype similar to the more apical flowers produced on *ap1-1* plants (Figures 5S-T); first whorl organs are either leaf-like or missing, second whorl organs are absent, and

the third and fourth whorl organ are nearly wild type although there may be fewer stamens than normal and the gynoecium often fails to fuse properly. Only a small proportion of the primordia complete development to produce a flower (Figure 1I, 5S). Most of the primordia remain in a relatively undifferentiated state resembling stage two flower primordia (Figure 1H, 5T); only 2-10 flowers develop from structures composed of over one hundred primordia. This enhancement of the *ap1-1* phenotype has been observed in an independent cross between W100, a marker strain in the Landsberg *erecta* background (Koornneef et al., 1987) and the wild-type Ws strain in which *ag-2* was induced (Maarten Koornneef, personal communication).

To facilitate discussion, the primordia produced by the apical inflorescence meristem will be called first order primordia. The primordia produced by the first order meristems will be called second order, and so on. As one proceeds apically in such a plant, the frequency of third and higher order inflorescence meristems decreases, with flowers forming from second or third order primordia (Figure 5R). Lower on the stem, second and third order primordia are often inflorescence meristems, with flowers appearing as fourth or fifth order structures (Figures 5N-O). In these later inflorescences, some structures are produced that resemble squamules since they arise from the pedicel of the inflorescence.

The enhancer of *ap1-1* appears to segregate independently of *ap1-1*. It does not appear to have a floral phenotype when *ap1-1* is not also homozygous in the genetic background, although it is not clear that the *erecta* mutation is not involved. In addition, the *ag-2* and *ap2-1* mutations do not appear to significantly alter the enhanced phenotype other than the homeotic conversions of floral organ identity associated with these mutations. Further genetic experiments are required to determine if the enhancer is a single locus, and to characterize its nature and interaction with *ap1-1*.

apetala1-1 apetala3-1, *apetala1-1 pistillata-1*, and *apetala1-1 superman-1*

Additive interactions are observed in *ap1-1 ap3-1* and *ap1-1 pi-1* flowers (Figures 1K-M, 6A-H; Irish and Sussex, 1990). In both genotypes the first whorl organs are initiated and subsequently differentiate like the first whorl organs of *ap1-1* flowers. Second whorl organs are usually absent in both doubly mutant genotypes, however, in those cases that they are present (about 5% of the time), they are leaf-like organs like those found in the first whorl positions. Third whorl organs are carpelloid stamens in *ap1-1 ap3-1* flowers, their phenotype the same as the third whorl organs of *ap3-1* flowers and the central gynoecium is normal in *ap1-1 ap3-1* flowers (Figures 1M, 6B-D). Third whorl organ primordia do not form in *ap1-1 pi-1* flowers, rather the cells that would normally develop into the third whorl organs are incorporated into the central gynoecium (Figures 1K, 6F-H). This results in an abnormal gynoecium like that observed in *pi-1* flowers. Secondary and tertiary flowers arising in the same manner as in *ap1-1* flowers are produced that have the developmental pattern as the primary doubly mutant flower. In one *ap1-1 pi-1* flower analyzed, an axillary meristem produced in the the axil of a first whorl organ of a secondary flower behaved as if it was an inflorescence meristem (Figure 6H).

Additive effects are also observed in *ap1-1 sup-1* double mutants (Figures 1L, 6I-L). The outer two whorls develop as they do in *ap1-1* flowers while the inner whorls develop as in *sup-1* flowers. The production of axillary buds is similar to that observed in *ap1-1* flowers and these axillary flowers display the same phenotype as the primary flower.

apetala1-1 agamous-2

Flowers on plants homozygous for both *ap1-1* and *ag-2* exhibit indeterminate growth in two respects (Figures 1J, 7A-F). Firstly, secondary and tertiary flowers are

produced in the axils of floral organs as well as on the pedicels of *ap1-1 ag-2* flowers, a characteristic of *ap1-1* development. Secondly, *ap1-1 ag-2* flowers display the indeterminate growth associated with *ag* mutants; the cells which would ordinarily give rise to the fourth whorl organs behave instead as if they constituted a new flower primordium. The outer whorl of *ap1-1 ag-2* flowers is occupied by 1-4 bract-like organs similar to those observed in the first whorl of *ap1-1* flowers. Following this, 6-15 organs with characteristics of both petals and the first whorl bract-like organs are produced. The mosaicism of these organs is variable with some nearly wild-type petals and others mostly bract-like. Mosaic organs with sectors of petal tissue and bract-like tissue may also develop; the boundary between the sectors is usually well-defined and abrupt (Figure 7F). This process repeats indeterminately except that the numbers and positions of the internal organs becomes more irregular and the distinction between the bract-like and petal-like organs becomes obscured (Figure 1J, 7E). Many filamentous squamules may be present arising from near the base of the flower (Figure 7E). Secondary flowers can develop in the axils of any of the organs as well as from the pedicel. These axillary buds develop in the same manner as the primary flower (figures 7B-E). The frequency of axillary bud formation decreases acropetally.

Observations of developing *ap1-1 ag-2* flowers shows that the positions of the organs interior to the first whorl organs are not in a well-defined pattern (Figures 7A-D). Their positions are not the stereotypical positions of either second or third whorl organs. In addition, in some flowers the organs do not appear to arise in well-defined whorls, with organs of different ages occupying positions in the same whorl (Figures 7B-D). Perhaps the differences in size reflects the difference in growth rate between petals and leaf-like organs. Thus, these organs are variable in number, position, and identity.

apetala1-1 apetala2-9

With respect to the number and identity of floral organs, *ap2-9* is almost entirely epistatic to *ap1-1*; *ap1-1 ap2-9* flowers have essentially the same phenotype as *ap2-9* flowers (Figures 1O, 8A-F; see Chapter 3, Bowman et al., 1991). In addition, the number of axillary flowers produced is reduced in *ap1-1 ap2-9* flowers as compared to *ap1-1* flowers.

ap1-1 ap2-9 flowers have solitary carpels in the medial first whorl positions. These organs may be slightly phylloid or may be mosaic organs with stamen sectors along their margin. The lateral first whorl organs, when present, are solitary carpels or carpelloid leaves. The first whorl organs are often fused to each other with ovules developing along the margins of fusion. Squamules may be present originating from the pedicel or the region around the first whorl positions. Second whorl organs do not develop. The third whorl positions are variable in number, 0 to 4 organs, as well as phenotype. The third whorl organs are usually morphologically wild-type stamens but the organs may also have carpelloid characteristics such as stigmatic papillae and rudimentary ovules (Figure 8E). These stamen-carpel mosaic organs have been described for *ap2-9* flowers (Bowman et al., 1991). The central gynoecium, in the fourth whorl, is often unfused (Figure 8E). The phenotype increases in severity acropetally such that the apical flowers may consist of only a two-carpelled gynoecium as has been described for *ap2-2* flowers (Bowman et al., 1991). One phenotypic aspect seen on *ap1-1 ap2-9* flowers that is not observed in *ap2-9* flowers is the presence of small outgrowths of cells on the pedicels of *ap1-1 ap2-9* flowers. These outgrowths do not appear to be aborted first whorl primordia since they are observed in flowers in which all four first whorl organs develop.

Secondary flowers may develop, nearly always in lateral positions (21/22 secondary flowers), irrespective of the identity or presence of first whorl organs (Figures

8E-F). Secondary flowers often develop in flowers in which all four first whorl organs are carpelloid organs and these secondary flowers originate below the first whorl organs on the pedicel. Thus they are not developing in the axil of any first whorl organ. Secondary flowers on the first flowers (flowers 1-2) produced are usually similar to the primary flower in phenotype (Figure 8F). However, secondary flowers of more apical flowers (flowers 3-10) consist merely of carpel tissue, often of a two-carpelled gynoecium with no other structures present (Figure 8F; 9/12 secondary flowers; 6/9 failed to fuse properly). Tertiary flowers are observed only on the first 1-2 flowers produced. All 6 tertiary flowers examined consisted of a single gynoecium composed of one or two carpels. The axillary flowers may be associated with the small outgrowths of cells on the pedicels described above but this is not always the case. Occasionally, the first primordia produced by the primary inflorescence meristem that would in wild type develop into a flower behaves instead like an inflorescence meristem (although no cauline leaves are produced).

The development of *ap1-1 ap2-9* flowers is similar to that of *ap2-9* flowers (Figures 8A-F). The outer whorl organs develop into carpels or phylloid carpels and often fuse congenitally (Figure 8A). No second whorl primordia are formed. A variable number of third whorl primordia form in ectopic positions and subsequently develop into stamens (Figure 8B). The cells that would ordinarily give rise to the gynoecial cylinder have a variable fate in *ap1-1 ap2-9* flowers. They may develop normally or alternatively give rise to one or two primordia that develop into solitary carpels or mosaic organs composed of stamen and carpel tissue (Figures 8B-E). The development of secondary flowers is similar to that observed in *ap2-2* flowers in which the entire floral meristem develops as a gynoecial cylinder (Bowman et al., 1991).

apetala1-1 apetala2-1

A synergistic phenotypic interaction is seen in *ap1-1 ap2-1* flowers (Figures 1N, 8G-L; Irish and Sussex, 1990). The phenotype of *ap1-1 ap2-1* flowers varies considerably acropetally. The most basal *ap1-1 ap2-1* flowers resemble *ap1-1* flowers except that the frequency of secondary, tertiary, and quaternary flowers is increased with upwards of twenty flowers frequently developing from each primary flower (Figures 1N, 8L). An average of 16 (n = 5) axillary flowers in addition to the primary flower was observed on the first five primary flowers produced by the inflorescence meristem. As is also the case with *ap1-1* flowers, the frequency of axillary flowers decreases acropetally in *ap1-1 ap2-1* plants, with only an average of 5.4 (n =10) axillary flowers associated with primary flowers 6 through 10. When present, the first whorl organs are cauline leaf-like and they may be separated by long internodes. First whorl organs are often carpelloid with stigmatic tissue at their tips and rudimentary ovules along their margins (Figure 8L). The extent of carpellody of these organs is greater than that observed in the first whorl organs of *ap2-1* flowers. Squamules are present at the base of the flower, where the first whorl organs arise (Figure 8K). Second whorl organs are not present. Although fewer than the wild-type number, morphologically wild-type stamens occupy third whorl positions; the precise positions of third whorl organs are usually ectopic. The central gynoecium is usually normal, failing to fuse properly only in the axillary and apical flowers. The secondary and further inner flowers of basal primary flowers are similar in phenotype to the primary flower except the the extent of carpellody of first whorl organs is increased and fewer third whorl organs are formed. Late in the development of the primary flower, axillary flowers may form towards the base of the pedicel of primary flowers (Figures 8J-L). These are associated with small outgrowths of cells similar to those observed in *ap1-1* flowers (Figure 8K). In contrast to *ap1-1* flowers, many of the axillary flowers of *ap1-1 ap2-1* flowers do not occupy precise lateral or medial positions and do not necessarily arise in axils of first whorl organs.

More apical flowers (approximately the eighth to twelfth primary flowers produced) have only tertiary flowers and by about the twentieth flower only secondary flowers are produced. The more apical primary flowers have phenotypes resembling that of intermediate *ap2* alleles. The outer whorl organs are quite carpelloid when present, being carpelloid leaves, solitary carpels or mosaics of leaf, carpel, and stamen tissue. Second whorl organs are not present and the number of third whorl organs (usually stamens but sometimes just filamentous structures with no anthers) is reduced to approximately three per flower. The secondary and tertiary flowers have a similar phenotype. This trend continues such that the ~25th primary flower produced has a phenotype similar to that observed for a single basal *ap1-1 ap2-9* flower.

agamous-4

All of the previously described *ag* alleles, *ag-1*, *-2*, and *-3*, have similar phenotypes. The outer two whorls develop normally but the third whorl primordia differentiate into petals rather than the wild-type stamens and the cells that would normally give rise to the gynoecium behave instead as if they constituted a new flower primordium. This process is repeated indeterminately producing a double flower phenotype. Thus, one can summarize the *ag-1*, *-2* and *-3* phenotypes as (sepals, petals, petals)$_n$ although the precise pattern of organs interior to the third whorl is variable and sepal-petal mosaic organs are often formed (Bowman et al., 1989, 1991).

In contrast, staminoid organs develop in *ag-4* flowers and the pattern of organs produced by the indeterminate floral meristem differs from the other alleles. The pattern of organs can best be summarized as (sepals, petals, petaloid stamens, [sepals, petaloid

stamens]$_n$) in which the inner sepals and stamens are in ectopic positions and be mosaic organs of sepal, stamen, and carpel tissue. The development of the outer two whorls of *ag-4* flowers (Figures 1P, 9A-F) is identical to wild type; four sepals occupy the first whorl and four petals occupy the second whorl positions (Figure 9B). The third whorl primordia are initiated correctly, but they differentiate into petaloid stamens, which fail to dehisce even though they contain pollen grains (Figures 9C-F). The cells that normally give rise to the central gynoecium behave as if they constituted another flower primordium (Figure 9B). However, the development of this flower primordium is quite abnormal. Usually four organ primordia are produced on its flank. These organ primordia subsequently differentiate into sepalloid organs, but they can also consist of carpel, stamen, or petal tissue (Figures 9D-F). Interior to the fourth whorl, more organ primordia are produced that develop into petalloid stamens like those found in the third whorl (Figure 9F). Again, the remaining floral meristem behaves like another flower primordium producing another whorl of sepalloid organs and more petalloid stamens. This process repeats indeterminately although the positions and numbers of organs in whorls interior to the third whorl are variable (Figure 9C). The organs that develop in the fourth whorl are often mosaic organs comprised primarily of sepal tissue, but may also have stamen or carpel sectors. In sepal-stamen mosaics, the sectors are well-defined and longitudinal with the stamen sectors occupying the marginal positions and the sepal tissue occupying the central portion (Figure 9E). If carpel tissue is present on more than one fourth whorl organ, the organs tend to fuse to each other sometimes resulting in a connate ring of carpelloid sepals (Figure 9D). In summary, the *ag-4* gene product appears to retain some activity do direct the differentiation of stamens and some carpelloid tissues but has lost its activity to promote terminal differentiation in the fourth whorl.

Discussion

Both *SUPERMAN* and *APETALA1* act in pattern formation within the developing flower (Irish and Sussex, 1990; Bowman and Meyerowitz, 1991). Mutations in either result in specific pattern defects primarily in two adjacent whorls, as is the case with the floral homeotic genes *AGAMOUS*, *APETALA2*, *APETALA3*, and *PISTILLATA* (Bowman et al., 1988; Komaki et al., 1988; Bowman et al., 1989; Kunst et al., 1989; Hill and Lord, 1989; Bowman et al., 1991) In contrast to the floral homeotic genes, the alterations in flower structure observed in *ap1-1* and *sup-1* mutants are not strictly homeotic conversions of floral organs. For example, in *superman-1* flowers the cells that would ordinarily give rise to the fourth whorl carpels often develop into several whorls of organ primordia that subsequently differentiate into stamens. That the first whorl organs of *superman-1 ap2-2* flowers are solitary carpels, however, suggests that the *SUPERMAN* gene product is not required for normal carpel development. In *ap1-1* mutants, there appears to be a homeotic (and perhaps heterochronic) conversion of floral meristems to inflorescence meristems, in addition to alterations in the pattern of floral organs. Thus, the *APETALA1* gene may be involved in the establishment of the floral meristem and the initiation of flower homeotic gene expression.

The role of superman

The model presented in Chapter 3 (Bowman et al., 1991) in which different combinations of homeotic genes specify the identity of floral organ was based on a series of genetic experiments. It is clear that cross-regulatory interactions between the homeotic genes are in part responsible for defining the spatial domains of activity of the floral homeotic genes; for example, the mutually antagonistic interactions between *AG* and *AP2* (Chapter 3, Bowman et al., 1991; Chapter 5, Drews et al., 1991). However, this does not thoroughly explain the proposed localization of floral homeotic gene activities to specific whorls. That at least two of the homeotic genes (*AG* and *AP3*) are initially expressed in a

well-defined temporal and spatial manner (Chapter 5, Drews et al., 1991; Tom Jack, unpublished) suggests that they may be responding to a prepattern. The phenotype of *superman-1* flowers suggests that *SUPERMAN* is at least partially responsible for defining the expression patterns of *AP3* and *PI*.

Specifically, we propose that SUPERMAN defines the boundary of the third and fourth whorls by inhibiting the activity of AP3 or both AP3 and PI in the fourth whorl (*AP3* is not expressed in the fourth whorl in wild-type flowers; Tom Jack, unpublished) as shown in Figure 10. *superman* mutants would have both AG and AP3/PI activity in the fourth whorl, resulting in the differentiation of the fourth whorl primordia as stamens according to the organ identity specification model proposed in Chapter 3 (Bowman et al., 1991). This is what is observed in *superman-1* flowers, although some carpel tissue may form interior to the stamens. The variable nature of the boundary between staminoid and carpelloid organs in *superman-1* flowers, in terms of both numbers of organs produced and amount of carpel tissue suggests that the boundary between the third and fourth whorls may be quite plastic indicating that other factors may also play a role in defining the boundary, or alternatively, suggests a variable activity of the *sup-1* mutant allele. The appearance of carpel tissue in *superman-1* flowers as well as the development of solitary carpels in the first whorl of *superman-1 ap2-2* flowers and carpels in the central gynoecium of *superman-1 pi-1* flowers argues against *SUPERMAN* being required for carpel development per se. In addition, the development of wild-type petals, sepals and stamens in *superman-1* flowers suggests that *SUPERMAN* does not have a direct role in the specification of any of the floral organ types.

Predictions for double mutants

If the primary role of SUPERMAN is defining the boundary of AP3/PI activity between the third and fourth whorls, certain predictions can be made about doubly mutant

strains with *superman-1* and the homeotic mutations; these are shown in Figure 10. Additive interactions should be observed in *superman ap2* flowers since ap2 mutations primarily affect organ specification in the outer two whorls. The outer two whorls of *superman ap2* flowers should be the same as *ap2* flowers, while the inner two whorls of the double mutants should resemble *superman* flowers. This is essentially the case seen in *superman-1 ap2-2* flowers. The outer two whorls resemble those of *ap2-2* flowers while the inner whorls are occupied by an increased number of stamens (relative to the number in *ap2-2* flowers) and a reduced amount of carpel tissue. As expected, the number of staminoid organs produced in *superman-1 ap2-2* flowers (2.4), is intermediate between the number produced in *superman-1* flowers (14.6) and the number produced in *ap2-2* flowers (.25; Bowman et al., 1991). These arguments also hold for *superman-1 ap2-1* flowers.

In *superman ag* flowers, AP2 should be active in all whorls due to the absence of AG activity and AP3/PI activity should expand to encompass all whorls except whorl one in the absence of SUPERMAN activity. Therefore, *superman ag* flowers are expected to consist of an outer whorl of sepals with an indeterminate number of whorls (due to the indeterminate nature of *ag* flowers) of petals interior to the sepals. A similar argument can be made to predict flowers with an outer whorl of leaves and an indeterminate number of whorls of staminoid petals for *superman ap2 ag* triple mutants. These predictions are confirmed by the phenotypes of these flowers.

In contrast, an epistatic relationship is observed between *superman* and *pi* mutations, with *pi-1* being epistatic to *superman-1*. *superman-1 pi-1* flowers are morphologically indistinguishable from *pi-1* flowers. This is the predicted result if defining the boundary between the third and fourth whorls is the only role *SUPERMAN* has in the specification of identity of the floral organs. In summary, the phenotypes of all the doubly and triply mutant flowers described here support the hypothesis that

SUPERMAN defines the boundary between the third and fourth whorls by repressing AP3 and/or PI activity in the fourth whorl.

Floral homeotic gene expression in *superman* flowers

Since *superman* mutations primarily affect the fourth whorl, the simplest scenario would be that *SUPERMAN* is expressed in the fourth whorl and autonomously inhibits the activity of *AP3* and/or *PI* in the fourth whorl. If *SUPERMAN* acts in an autonomous manner to inhibit *AP3* and *PI* activity in the fourth whorl, then the expression patterns of the floral homeotic genes should be as shown in Figure 10. Expression patterns of *AP2* and *AG* should be unaltered in a *superman* background as compared to wild type; *AP2* should be restricted to the outer two whorls while *AG* should be expressed in the inner two whorls. Mutations in either *AP3* or *PI* result in a similar phenotype and *ap3 pi* double mutants have a phenotype indistinguishable from the single mutants (Bowman et al., 1989). Therefore, *SUPERMAN* need only restrict the activity of either *AP3* or *PI* to the second and third whorls. In wild-type flowers, *AP3* RNA is restricted to whorls two and three (Tom Jack, unpublished). Therefore, in *superman* flowers the expression pattern of *AP3* should expand to include whorl four in addition to whorls two and three and in *sup ag* flowers *AP3* should be expressed in all whorls interior to the first. Since both *AP3* and *PI* activities are necessary for proper petal and stamen development, *SUPERMAN* need only repress the activity of one of the two genes, *AP3* or *PI*, in the fourth whorl to allow carpel development. Although it is a formal possibility that PI is expressed in the fourth whorl in wild-type flowers, it is most likely the case that the expression patterns of *PI* will be similar to those of *AP3*. Likewise, expression patterns of the floral homeotic genes in double mutants involving *superman* are also predicted in Figure 10.

The center of the flower

The tissue present in the central region of *sup-1* flowers is often a fusion of staminoid and carpelloid tissues. In some cases, it appears that the central organ is merely a congenital fusion of two primordia or portions of primordia that are expressing different fates, stamen and carpel respectively. This results in an organ in which there are only two large longitudinal sectors, one of stamen tissue and one of carpel tissue. In other cases, an organ with a large central carpel sector flanked by thin longitudinal stamen sectors. In these organs, ovules develop not on the margins of the organ but in rows in the middle of the carpel tissue. This may be due to the presence of the flanking stamen tissue. The presence of any carpelloid tissue is not readily explained by the proposed model. Perhaps the AP3/PI activities are eventually reduced in the central region due to the indeterminate nature of the floral meristem in *sup-1* flowers (see below). In *sup-1 ag-1* flowers some sepaloid characteristics are observed in the organs of the inner whorls suggesting that the AP3 and PI activities are also reduced in the inner whorls of these flowers. Alternatively, it is not known that the *sup-1* allele is null, thus *sup-1* may be a partial loss of function and the *sup-1* product may retain some activity.

The fourth whorl of *ag* flowers can be interpreted as either a fourth whorl of sepals (a conversion of the normal fourth whorl carpels to sepals) or as the first whorl of sepals of an internal flower. In *ag-2* flowers that are in an a wild-type *ERECTA* background, pedicel elongation is observed between successive internal flowers. This suggests that the fourth whorl in these flowers develops as the first whorl of an internal flower. In *ag-2 sup-1* flowers in a wild-type *ERECTA* background, there is no significant elongation of the pedicel between any of the inner whorls, all of which are petals (Detlef Weigel and Hajime Sakai, unpublished). This would suggest that in *ag sup* flowers the floral meristem

In *sup-1 ap2-2* and *sup-1 ap2-1* flowers fewer organs are present interior to the second whorl than are observed in *sup-1* flowers. One of the functions of *AG* is to control

the determinacy of the floral meristem possibly by reducing cell division. Thus, ectopic expression may result in a reduction in the number of organ primordia produced as has been proposed for the outer two whorls of *ap2* flowers (Bowman et al., 1991). However, third whorl organ number is also reduced in *ap2* flowers. One hypothesis for this phenomena is that early ectopic *AG* expression reduces the rate of cell division in the entire flower primordium prior to formation of the second and third whorl primordia . This could result in the reduction in number of third and fourth whorl organ primordia in any strain homozygous for *ap2*. In addition, many of the central organs of *sup-1 ap2-2* and *sup-1 ap2-1* flowers display carpelloid characteristics, similar to those observed in *ap2-9* flower when grown at 16°C. One may rationalize these observations as manifestations of ectopic *AG* expression, but these arguments are merely hand-waving and therefore further discussion must await molecular data of *AP2* expression.

Determinacy of the floral meristem

That more than twenty stamens can develop interior to the second whorl in *superman-1* flowers, suggests that the *SUPERMAN* product may have a role in regulating the determinacy of the floral meristem in addition to defining whorl boundaries. It is not simply the case that the fourth whorl primordia, which number two or four depending on the interpretation, develop into stamens. The phyllotaxy of the extra primordia in *superman-1* is whorled, with, in the most extreme cases, four to five distinct whorls developing from those cells that would normally develop into the gynoecium. In *sup-1 ag-1* flowers the floral meristem continues to produce whorls of organ primordia, all of which develop into petals, indeterminately. After several whorls are generated, the floral meristem becomes enlarged and elongated, over 100 μm in length, and produces organ primordia along its entire circumference. This type of fasciated floral meristem has been observed in *ag-1 clavata2* flowers (John Alvarez and David Smyth, unpublished). *clavata2* mutations cause the floral meristem to be enlarged relative to wild type, with the result that

clavata2 flowers have a four-carpelled gynoecium and occasionally extra organs in the other whorls as well. Thus both *superman* and *clavata2* mutations appear to interact synergistically with *ag* mutations resulting in uncontrolled meristematic growth. (Crosses to construct a *sup-1 clv2* double mutants are in progress, Hajime Sakai, personal communication). The clavata (*clv1*, *clv2*, and *clv3*) mutations seem to affect vegetative and inflorescence as well as floral meristems, while *superman* and *agamous* mutations specifically affect the floral meristems. This suggests that *AG* and *SUPERMAN* function in concert with other genes, involved in regulating meristematic cell division throughout the plant, to control determinacy in the floral meristem. Thus, both identified genes that are involved in pattern formation in the innermost whorl of the flower also appear to be involved in directing the floral meristem to be determinate.

The *apetala1-1* mutation alters both inflorescence and flower structure

APETALA1 also appears to have a role in pattern formation in the developing flower as mutations at this locus result in alterations in both inflorescence and flower architecture (Irish and Sussex, 1990). Groups of cells that would normally constitute a single flower primordia in wild-type plants often develop into several flowers and occasionally into an entire inflorescence. In the Landsberg *erecta* background, primordia produced on the flanks of the inflorescence meristem often develop into a branched structure composed of a primary central flower with several younger axillary flowers which arise from the axils of organs of the primary flower or from the pedicel of the primary flower (Irish and Sussex, 1990). In contrast, when the *ap1-1* mutation is in the Wassilewskija background, each individual primordium produced on the flanks of the inflorescence meristem also behaves as an inflorescence meristem rather than a flower meristem. In addition, alterations of number and identity of floral organs, primarily in the outer whorls, are observed in *ap1-1* flowers.

Axillary flower formation in *apetala1-1* flowers

In *apetala1-1* mutants, axillary floral meristems are formed in the axils of the first whorl organs of the primary flower (Irish and Sussex, 1990). This process is reiterated in the axillary flowers resulting in a highly branched structure resembling in some aspects a branching inflorescence (the normally racemose *Arabidopsis* inflorescence resembles in some respects a thyrse inflorescence in *ap1-1* plants). However, in the Landsberg *erecta* background it is not simply the case that each individual floral meristem is completely converted into an inflorescence meristem. The branched structure differs in several aspects from a normal inflorescence meristem. Firstly, the primary or central part of the meristem behaves as a flower meristem developing floral organ primordia. And secondly, the axillary flowers are usually produced in the axils of the primary flower's outer whorl organs and therefore not in a phyllotactic spiral, as are flowers produced by inflorescence meristems.

Although axillary buds are usually associated with the axil of an outer whorl floral organ, which are quite leaf-like in *ap1-1* flowers, the formation of axillary buds is not strictly dependent on their presence since axillary buds may arise in positions that apparently lack an outer whorl organ. In these cases a small outgrowths of cells are present at the base of the axillary flowers. These may be aborted leaf primordia since they have filamentous structures, possibly stipules, flanking the outgrowths. These outgrowths also occur without an associated axillary flower. In these cases a small mound of cells is present in the axil of the outgrowths. These cells may constitute an aborted floral meristem. The axillary flowers do not develop at the expense of any other normal floral organ since another allele of *ap1* (*ap1-LA7.7*; Maarten Koornneef and J. Bowman, unpublished) has flowers with the full complement of floral organs in addition to axillary flowers. Additionally, both second whorl organs and axillary flowers may develop in

ap1-1 flowers. The formation of axillary buds varies acropetally to a great extent such that this aspect of indeterminacy of the floral meristems is lost after the first fifteen or so flowers are produced.

Axillary flowers, a result of phylloidy or conversion of floral meristems towards inflorescence meristems?

A hypothesis proposed for the development of axillary flowers produced in the axils of first whorl organs of *ap2-1* flowers is that they are a secondary result of the conversion of those organs to a leaf-like identity (Bowman et al., 1991). Radical and cauline leaves of *Arabidopsis* plants have associated meristems in their axils. Thus, the meristems produced in the axils of first whorl organs of *ap2-1* flowers are possibly a consequence of the organs' identity as leaves. The axillary meristems are floral meristems since they are formed in the axils of organs which form part of the flower. These axillary flowers only develop in the most basal flowers of *ap2-1* plants (Bowman et al., 1989). The outer whorl organs of *ap2-1* flowers develop carpelloid characteristics in the more apical flowers; this departure from phylloidy could explain the failure to initiate axillary meristems in these flowers. The first whorl organs of *ap1-1* flowers are also quite leaf-like in their development. They have stipules at their bases and have an epidermal cellular morphology of leaves rather than sepals. Thus, the formation of axillary buds in their axils could also be a consequence of their phylloid identity. These organs also exhibit some slight carpellody in more apical flowers. This might explain the decrease in frequency of axillary flower development in the more apical flowers. In *ap1-1 ap2-1* flowers the extent of axillary flower development is exaggerated. Perhaps the first whorl organs of these flowers are more phylloid in character than in either of the single mutants leading to increased production of axillary buds. Axillary flower frequency decreases acropetally, in correlation with the first whorl organs developing carpelloid characteristics in the more apical flowers.

However, this hypothesis is insufficient to explain the presence of axillary buds that develop independently of the identity of first whorl organs in *ap1-1 ap2-9* flowers. Axillary flowers can develop from the pedicels of *ap1-1 ap2-9* flowers, below all four carpelloid first whorl organs. Small outgrowths of cells, which may be aborted leaf primordia developing ectopically on the base of the pedicel of the primary flower, are usually, but not always visible at the base of these axillary flowers. Thus, the development of axillary flowers, in some cases, is independent of any visible (at the level of surface structures) subtending leaf-like organ or primordium. In summary, the data suggest that leaf-associated axillary meristems may increase the occurrence of axillary flowers, but are probably not the primary cause.

A hypothesis for the reason that there are fewer axillary flowers in *ap1-1 ap2-9* flowers than in either *ap1-1* or *ap1-1 ap2-1* flowers is that *AG* is ectopically expressed in *ap1-1 ap2-9* flowers. If *AG* is controlling determinacy by regulating cell division (see above) perhaps ectopic expression of *AG* in the outer two whorls influences the formation of axillary floral primordia. This ectopic expression may affect the formation of axillary floral primordia in the axils of the first whorl organs, which are infrequently observed in *ap1-1 ap2-9* flowers but should have little effect on axillary floral meristems originating lower on the pedicel, like those observed in *ap1-1 ap2-9* flowers, since *AG* is not expressed below the receptacle in *ap2* flowers (Drews et al., 1991).

The homeotic transformation of floral meristems to inflorescence meristems observed at a low frequency in *ap1-1*, *ap1-1 ap2-9*, *ap1-1 pi-1*, and possibly other genotypes involving *ap1-1*, suggests that one function of the wild-type *APETALA1* product is to mediate the transformation from inflorescence meristem to floral meristems.

An enhanced *apetala1-1* phenotype suggests a homeotic conversion of floral meristems to inflorescence meristems

In order to discuss the *ap1-1* phenotype it is necessary to introduce some terms to identify meristems which differ in number, position, and identity of primordia they produce. In *Arabidopsis*, the initial shoot apical meristem that develops during embryogenesis is a vegetative meristem, designated V. This meristem produces several (approximately 5-9 depending on growth conditions) organ primordia in a phyllotactic spiral. Each of these organ primordia develops into a radical leaf with little elongation of internodes. In response both internal and environmental signals, including age, temperature, day length, and nutritional status, the vegetative meristem is converted to an inflorescence meristem in a process called floral evocation or induction. The vegetative shoot apical meristem undergoes reorganization to form the inflorescence meristem. The initial fate of this inflorescence meristem is to produce a small number (one to three) of primordia that subsequently develop into cauline leaves. We call this meristem which has undergone floral induction but produces cauline leaves the I1 inflorescence meristem (I1). Following the production of cauline leaf primordia (with axillary I1 meristems) the inflorescence meristem undergoes another transition after which it produces only flower meristems in an indeterminate phyllotactic spiral. An elongation of internode length is associated with both the I1 and I2 meristems; the lengthening of the internodes does not begin until several flower primordia are produced. We term this inflorescence meristem which produces only flower primordia the I2 inflorescence meristem (I2) and the flower primordia it produces flower meristems (F). For the following discussion the initial I2 meristem that was derived from the vegetative meristem via the I1 meristem will be referred to as the primary I2 meristem.

When the *ap1-1* mutation is in the Landsberg *erecta* background, the first one or two primordia produced by the primary (or first order) I2 meristem occasionally behave as if they are I2 meristems (second order inflorescence meristems), rather than F meristems. This process may be repeated by the first primordium produced in the transformed

meristems resulting in a single third order I2 inflorescence meristem. These transformed meristems (I2 inflorescence meristems) do not produce any cauline leaves, but rather, commence producing primordia on their flanks, in a phyllotactic spiral, which subsequently develop into flowers. When the *ap1-1* mutation is crossed into a Wassilewskija (Ws) genetic background, the *ap1-1* phenotype is altered. *ap1-1* plants in the Ws background, produce the normal number of cauline leaves, but each of the subsequent primordia produced by the inflorescence meristem (the primordia are produced in a phyllotactic spiral) behaves as an I2 meristem (these are second order I2 inflorescence meristems) rather than the normal F meristem. In the most basal primordia produced by the primary I2 inflorescence meristem this process can be reiterated three or four times (secondary I2 meristems produce third order I2 meristems and so on) producing third, fourth, and higher order I2 inflorescence meristems. Thus, primordia that would develop into a single flower in wild-type plants or 7-15 flowers (a primary flower and its associated axillary flowers) in *ap1-1* Landsberg *erecta* plants can give rise to over one hundred primordia in the enhanced *ap1-1* genotype. Eventually, some the primordia produced differentiate into flowers with an *ap1-1* phenotype (see below). Most of the primordia produced, however, fail to develop past the stage at which they are morphologically indistinguishable from normal floral meristems or the transformed secondary I2 meristems. Thus, it is not possible to determine their state of differentiation by morphological criteria. In the more apical primordia produced by the primary I2 meristem, the conversion of F meristems to I2 meristems is iterated less frequently, such that fewer orders of I2 inflorescence meristems are produced before flowers develop. Thus, the enhanced phenotype is a homeotic transformation of flower meristems to I2 inflorescence meristems, although flower meristems do develop following a variable number of iterations of this homeotic transformation. This may also be considered a heterochronic transformation, since the cells of the primordia produced by the primary I2 meristem behave as their ancestors did, like I2 meristems rather than F meristems.

Although the genetics of the enhanced *ap1-1* phenotype is not yet characterized, it is tempting to speculate that the formation of axillary flowers in the axils of *ap1-1* flowers in the Landsberg *erecta* background is at least partially a consequence of the conversion of floral meristems to I2 inflorescence meristems. In this scenario the axillary flowers could be a result of both axillary meristems associated with leaf-like first whorl organs and a primary floral meristem that has only partially made the switch from an I2 inflorescence meristem to a floral meristem.

Another mutation, *leafy* (Haughn and Somerville, 1988; John Alvarez, David Smyth, and Detlef Weigel, unpublished), appears to cause the primordia which would ordinarily be floral meristems to behave as I1 inflorescence meristems. In this case primordia are initiated in a phyllotactic spiral and each differentiates into a cauline leaf-like structure, sometimes with carpelloid characteristics. These cauline leaf-like structures have associated axillary meristems which behave in a similar manner. Eventually, floral structures are produced but not in any particular pattern except that carpelloid structures are common, sepaloid and staminoid structures are less common, are petalloid organs are rare. These floral organs are usually mosaic structures. Thus, the *leafy* mutation might be considered to cause a homeotic transformation of floral meristems to I1 inflorescence meristems.

Analogous mutations are known to exist in *Antirrhinum majus*. Mutations at the *FLORICAULA* locus result in a phenotype similar to *leafy* (Coen et al., 1990). That these genes are also similar at the level of DNA sequence suggests that they are cognate homologs (Coen et al., 1990; Detlef Weigel, unpublished). Mutations at the *SQUAMOSA* locus of *Antirrhinum* appear to have phenotype similar to the enhanced *ap1-1* phenotype (Stubbe, 1966). *SQUAMOSA* is known to encode a putative transcription factor with similarity to SRF, MCM1, *AGAMOUS*, *APETALA3*, and *DEFICIENS*

(Schwarz-Sommer et al., 1990; Tom Jack unpublished). Perhaps the *APETALA1* gene is the *SQUAMOSA* homolog.

Cauliflower

Brassica oleracea, a member of the Brassicaceae family (as is *Arabidopsis thaliana*) has been cultivated for well over two millennia. During its cultivation, variants have been selected and propagated, including several familiar vegetable crops such as cabbage, brussels sprouts, collards, broccoli, cauliflower, and kale (Yarnell, 1956). In *Brassica oleracea* L. var. *botrytis*, otherwise known as cauliflower, a phenotype similar to the enhanced phenotype of *ap1-1* is observed. Morphological studies on the developing curd of cauliflower suggest that in many cultivars of *Brassica oleracea* L. var. *botrytis*, primordia produced on the flank of the inflorescence meristem that would ordinarily develop into floral meristems behave instead as if they constituted what we term I2 inflorescence meristems (Sadik, 1962; Crisp and Walkey, 1974; Margara and David, 1978). This developmental sequence repeats as describe above for *ap1-1* such that several orders of primordia (I2 meristems) are produced. A few of the primordia from each of the orders eventually differentiate into flowers while the majority of the primordia remain undifferentiated. In the process of floral differentiation the pedicels of the flowers elongate to produce a structure very similar to that observed for *ap1-1* (Sadik, 1962; Figure 6K-T). Thus, the morphology of this variant of *Brassica oleracea* is similar in several respects to the enhanced phenotype of *ap1-1* in *Arabidopsis thaliana*. One difference between the phenotypes is that in cauliflower, the flowers that eventually develop are close to wild-type in structure, while *ap1-1* flowers in *Arabidopsis* also have pattern defects in the outer two whorls (see below).

The formation of the curd in *Brassica oleracea* appears to be a semi-dominant trait in crosses between cauliflower and other variants of *Brassica oleracea* (Yarnell, 1956). The genetics of the enhanced *ap1-1* phenotype in *Arabidopsis* is not yet characterized. Among

the several possibilities are: 1) there is a second modifier locus in the Ws ecotype which has no phenotype of own but causes the *ap1-1* phenotype to be enhanced (this modifier locus could be either dominant or recessive and could either be specific to the *ag-2* Ws strain (Yanofsky et al., 1990) or be in all Ws strains) and 2) a mutation, possibly *erecta*, in the Landsberg strain that suppresses the *ap1-1* phenotype. It may be that the enhancer is specific to the Ws background since it has been independently observed twice in crosses involving Ws (Maarten Koornneef, personal communication). Thus, no genetic comparisons can be made at this time.

In one cultivar of cauliflower, 'February-Early-March', curd formation can occur without subsequent flower formation if the plants are not vernalized (Sadik, 1962). In this case, some of the primordia eventually differentiate into bracts. The curds of flowering cultivars may also be devernalized causing the curd primordia to differentiate into leafy shoots. This cultivar may have a second mutation which alters floral evocation.

APETALA1 also influences the pattern of floral organs

The number and identity of organs in the outer whorls of the flower may be altered in *ap1* mutants. The first whorl organs of *ap1-1* flowers have phylloid characters and the second whorl organs are usually absent due to a failure of second whorl organ primordia to develop. However, second whorl organs occasionally do develop into petaloid organs or phylloid organs that resemble those occupying the first whorl. The number of stamens in the third whorl is often fewer than the wild-type six and they may occupy ectopic positions. The gynoecium occupying the fourth whorl is morphologically wild type. These are all characteristics of weak *ap2* alleles in which cauline leaf-like organs occupy the first whorl and the second whorl organs are often missing. In *ap2-1* flowers the second whorl can be occupied by petals or phylloid organs. In addition, the positions of stamens in *ap2-1* and *ap2-1/ap2-2* flowers are often ectopic when fewer than six develop. The first whorl organs

of *ap1* flowers appear to be intermediate in phenotype between sepals and leaves and have been referred to as bract-like (Irish and Sussex, 1990) since they resemble bracts of other species of flowering plants, although *Arabidopsis* plants do not form bracts. The first whorl organs in *ap2-1* flowers differ in phenotype from those in *ap1-1* flowers. The *ap2-1* first whorl organs have numerous stellate trichomes and are wider than those of *ap1-1* flowers. The first whorl organs of both genotypes have stipules at their bases and have cellular morphology characteristic of leaves. Thus, the *AP1* product appears to have a role in the specification of sepal identity in the first floral whorl.

Although the second whorl organs are usually missing, occasional petal tissue does develop in some *ap1-1* flowers. This petal tissue can be a solitary second whorl organ or can be part of a mosaic organ. Petals develop in all four second whorl positions in *ap1-LA7.7* flowers. In addition, petals develop in the inner whorls of *ap1-1 ag-2* flowers. This argues against *AP1* having a role in the specification of identity of the second whorl organs, although it does appear to have a function in the formation of the second whorl organ primordia. These conclusions must be qualified, however, since only two alleles have been examined and there is no evidence that either is a null allele.

The presence of the many outgrowths, small outgrowths of cells and squamules, of cells on the pedicels of *ap1* and *ap1 ap2* flowers remains a mystery. It is of note that these types of outgrowths have been observed in many other species in the Brassicaceae family (Saunders, 1923). It has been speculated that they may be vestiges of bracts that were purportedly lost during the evolution of the Brassicaceae (Saunders, 1923). Considering the many other aspects of the *ap1-1* phenotype, their presence on *ap1-1* flowers could be rationalized in numerous ways such as reappearing vestigial bracts or nebulous and aborted floral primordia.

A Speculative Model

The roles in specification of identity of the first whorl organs and in formation of second whorl primordia of *AP1* suggest that the *AP1* product may act in concert with the *AP2* product early in flower development. Many *ap2* alleles, such as *ap2-9*, have a much more dramatic effect than *ap1-1* on the development of the outer two whorls of floral organs. The phenotype of *ap1-1 ap2-9* flowers, however, does not allow their individual roles to be deduced. Rather, the epistatic nature of their interaction cannot be interpreted to provide evidence for order in a developmental pathway. However, the other phenotypic aspects of *ap1* and *ap2* allow a plausible model for the roles of *AP1* and *AP2* in flower development to be synthesized.

In this model the switch from I2 to F is mediated at least in part (the locus which is causing the enhanced *ap1-1* phenotype must also play a role at least indirectly) by the *APETALA1* gene product. It has been proposed that *APETALA1* and *AGAMOUS* act to establish a determinate floral field (Irish and Sussex, 1990). In the case of *APETALA1*, the establishment of determinacy may be merely a consequence of meristem identity, inflorescence versus floral. *APETALA1*, along with other genes such as *LEAFY* (which may mediate the switch from I1 to I2 inflorescence meristems), is in part responsible for initiating the expression of the floral homeotic genes. *APETALA1* may play a minor role in initiating floral homeotic gene expression since every floral organ type except sepals is observed in *ap1* flowers. This suggests that the lone floral homeotic gene *APETALA1* is only involved in initiating is *APETALA2*, and *APETALA1* can only be a minor contributor to the expression of *APETALA2* since the floral organ phenotypes of *ap1* flowers resemble those of weak *ap2* mutants. It does appear that the *APETALA1* gene plays a major role in the process of organ primordium formation. Whether this is mediated through the action of the floral homeotic genes, for example *APETALA2* and *AGAMOUS*, or independently of them, remains to be determined. Once the expression of the floral homeotic genes is established, interactions between the floral homeotic genes themselves (Bowman et al.,

1991; Drews et al., 1991) and other genes such as *SUPERMAN* (above) define their precise spatial patterns of expression that are responsible for the specification of cell fate in the developing flower.

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Table 1. First whorl organs of *ap1-1* primary flowers.

Flowers 1-5 (n = 17)			Flowers 6-15 (n = 39)		
L	Ab	Ad	L	Ab	Ad
16	10	3	20	23	12

Flowers 1-5 refer to the five most basal primary flowers produced by the inflorescence meristem, flowers 6-15 refer to the next ten primary flowers produced. L, Lateral; Ab, Abaxial; Ad, Adaxial

Table 2. Frequency of axillary flowers in *ap1-1*

	Flowers 1-5						Flowers 6-10		Flowers 11-15	
other	2°		3°		4°		2°		2°	
	L	M	L	M	L	M	L	M	L	M
3	34	17	20	3	1	0	12	7	5	1

Twenty primary flowers were scored in each category, five primary flowers from each of four plants. Flowers 1-5 refer to the most basal five primary flowers produced by the primary inflorescence meristem, flowers 6-10 refers to the next five primary flowers and so on. The flowers classified in other category did not originate in well defined lateral or medial positions, but rather from the pedicel of the primary flower. 2°, secondary; 3°, tertiary; 4°, quaternary; L, Lateral positions; M, Medial positions

Figure 1. Phenotypes of wild-type and mutant *Arabidopsis* flowers.

(A) wild type

(B) *superman -1*

(C) *superman-1 apetala2-2*

(D) *superman-1 apetala2-1*

(E) *superman-1 agamous-1*

(F) *superman-1 agamous-1 apetala2-1*

(G) *apetala1-1*

(H, I) *apetala1-1* in Ws background

(J) *apetala1-1 agamous-2*

(K) *apetala1-1 pistillata-1*

(L) *apetala1-1 superman-1*

(M) *apetala1-1 apetala3-1*

(N) *apetala1-1 apetala2-1*

(O) *apetala1-1 apetala2-9*

(P) *agamous-4*.

Bar = 1 mm.


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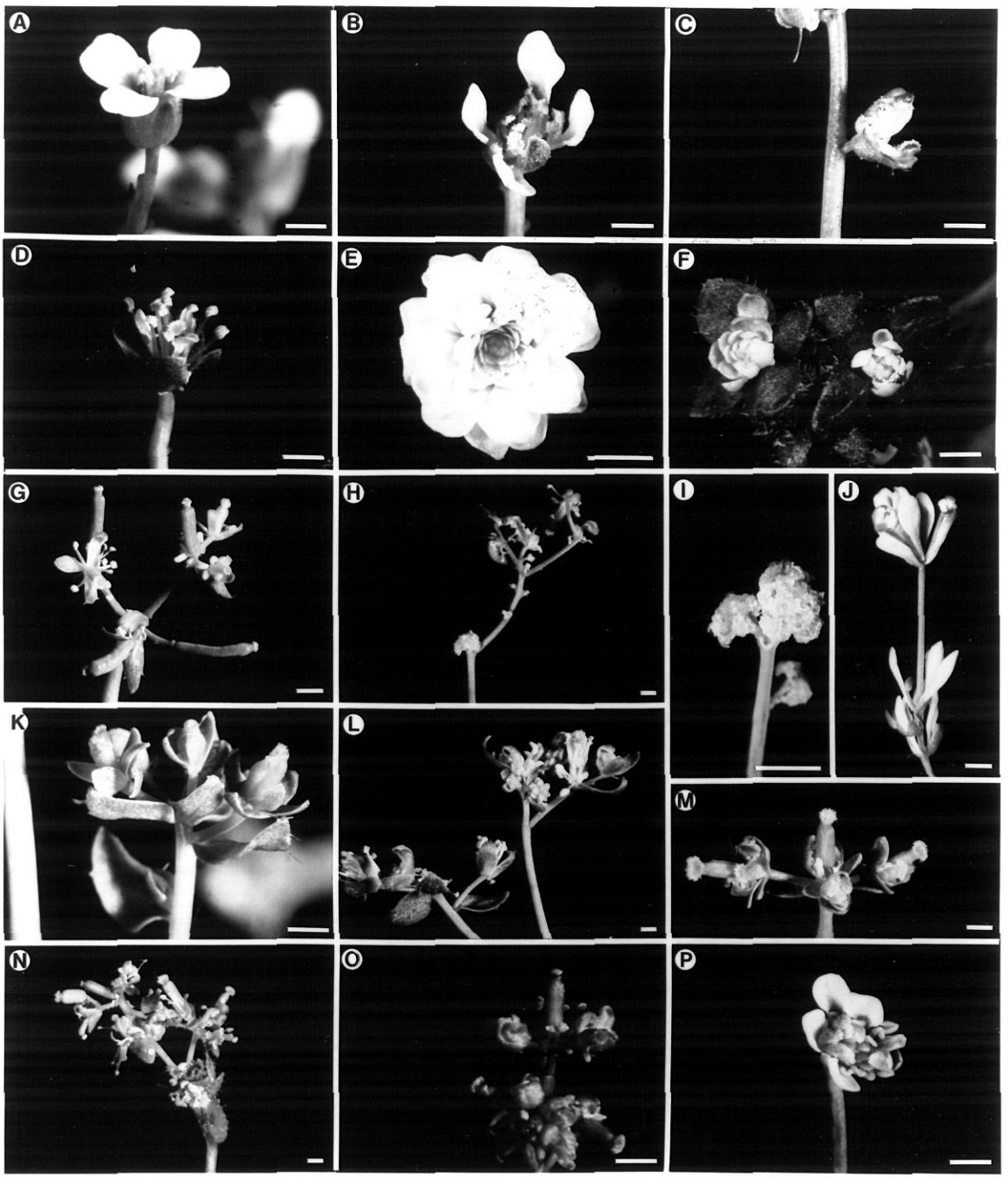
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Figure 2. Scanning electron micrographs depicting the development of wild-type and *superman-1 Arabidopsis* flowers. In many cases the outer whorls of the flowers pictured have been dissected away to reveal the inner whorls.

(A-D) Wild type. **(A)** Inflorescence meristem and flowers in stages 1-4 (Smyth et al., 1990). **(B)** Stage 6 flower. **(C)** Stage 7-8 flower. Second whorl petal (P), third whorl lateral stamen (LS), third whorl medial stamen (MS), and fourth whorl gynoecium (G) primordia are indicated. **(D)** Stage 12 flower.

(E-P) *superman-1*. **(E)** Inflorescence meristem and flowers of stages 1-5. **(F)** Stage 6 flower. **(G)** Stage 7-8 flower. Four organ primordia are developing in a whorled fashion on the flanks of the cells that would normally give rise to the gynoecium. **(H)** Stage 9-10 flower. Five stamen occupy the fourth whorl with three organ primordia in the fifth whorl. **(I)** Six stamens occupy their normal positions in the third whorl (3). Eleven additional stamens are evident, six in the fourth whorl (4) and five in the fifth whorl (5). **(J)** Stage 11 flower. Six stamens, in addition to the normal third whorl of stamens, are present. **(K)** Stage 12 flower. Four whorls of stamens are visible. **(L)** Mature *superman-1* flower. Ten stamens and a central gynoecium lacking most of the ovary tissue are visible. **(M)** The outer two whorls, the sepals and petals have been removed to reveal the thirteen stamens occupying the central region of the flower. **(N)** A filamentous structure, whose epidermal morphology is similar to that of stamen filaments, capped with stigmatic tissue occupies the center of this flower. Nectaries at the base of the third whorl and inner whorl stamens are visible (arrows). **(O)** A mosaic organ of stamen (St) and carpel (C). Each sector is large, well-defined, and longitudinal. **(P)** Mosaic organ in which the sectors are small and less well-defined as those in (O).

Bar = 10 μm in A, B, C, E, F, and G: 30 μm in H; 100 μm in D, I, J, K, M, N, O, and P; 300 μm in L.

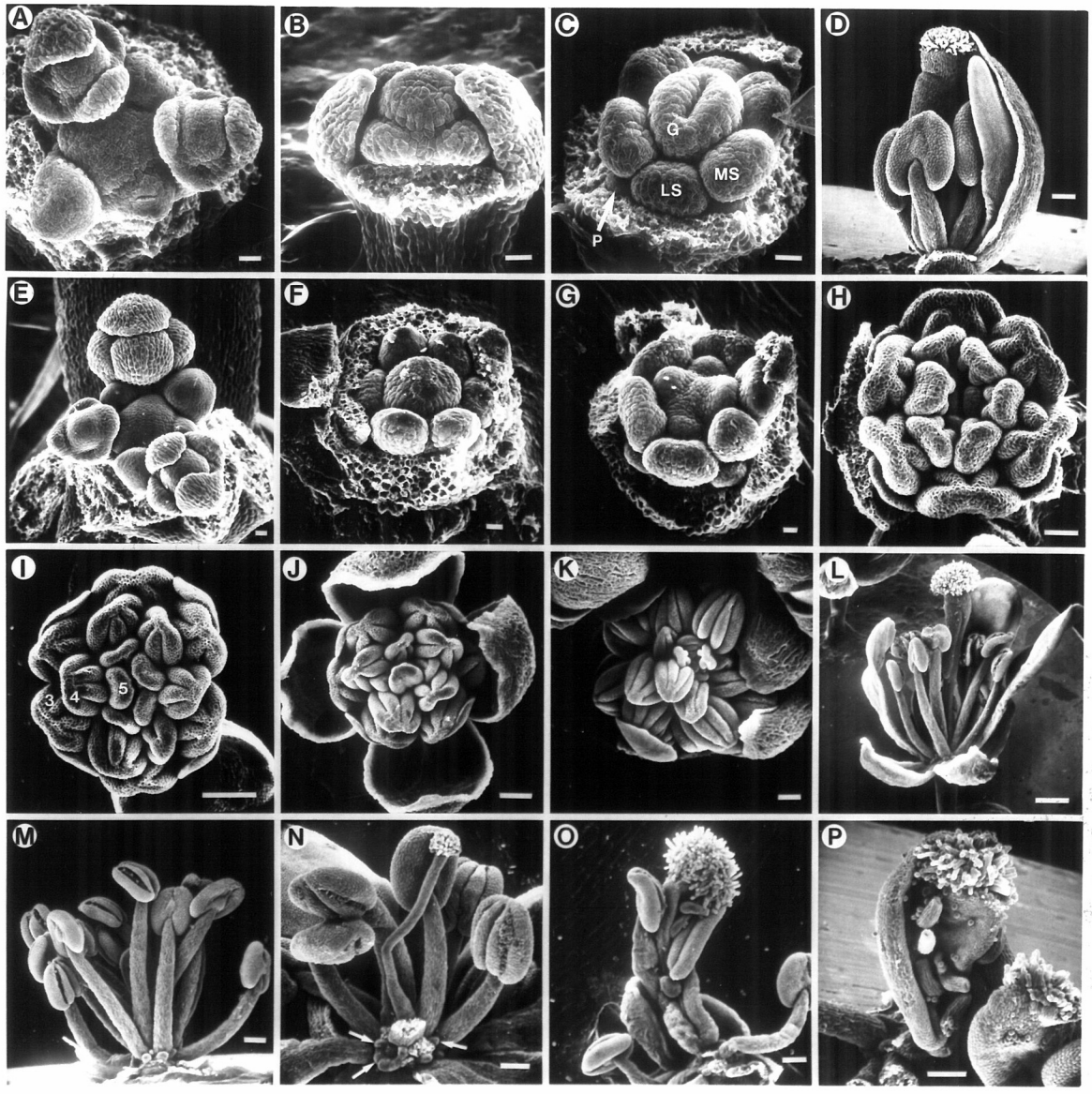
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Figure 3. Scanning electron micrographs depicting the development of *superman apetala2* flowers.

(A-E) *sup-1 ap2-2* flowers. **(A)** Inflorescence meristem and flowers of stages 1-5. **(B)** Stage 7 flower. **(C)** Developing ovules are visible along the margins of the first whorl carpels. Three stamens and a carpelloid organ are visible interior to the first whorl. **(D)** Mature *sup-1 ap2-2* flower. Two medial first whorl carpels and three inner whorl stamens (arrows) are visible. **(E)** Occasionally, *sup-1 ap2-2* flowers consist of merely a two-carpelled gynoecium as shown here.

(F-J) *sup-1 ap2-1* flowers. **(F)** Inflorescence meristem and flowers in stages 1-4. **(G)** Stage six flower. Stipules are present at the base of the first whorl organs. **(H)** The fourth whorl is occupied by two organs whose epidermal morphology is carpelloid. **(I)** The fourth whorl is occupied by three organs, one of which is a stamen. **(J)** Mature *sup-1 ap2-1* flower. Four cauline leaf-like organs are present in the first whorl, four staminoid petals in the second whorl, five stamens in the third whorl, and another four stamens are present in the fourth whorl. The center of the flower is occupied by a filamentous structure that has developing stigmatic tissue at its tip.

Bar = 10 μm in A, B, F, and G; 100 μm in C, D, E, H, and I; 300 μm in J.

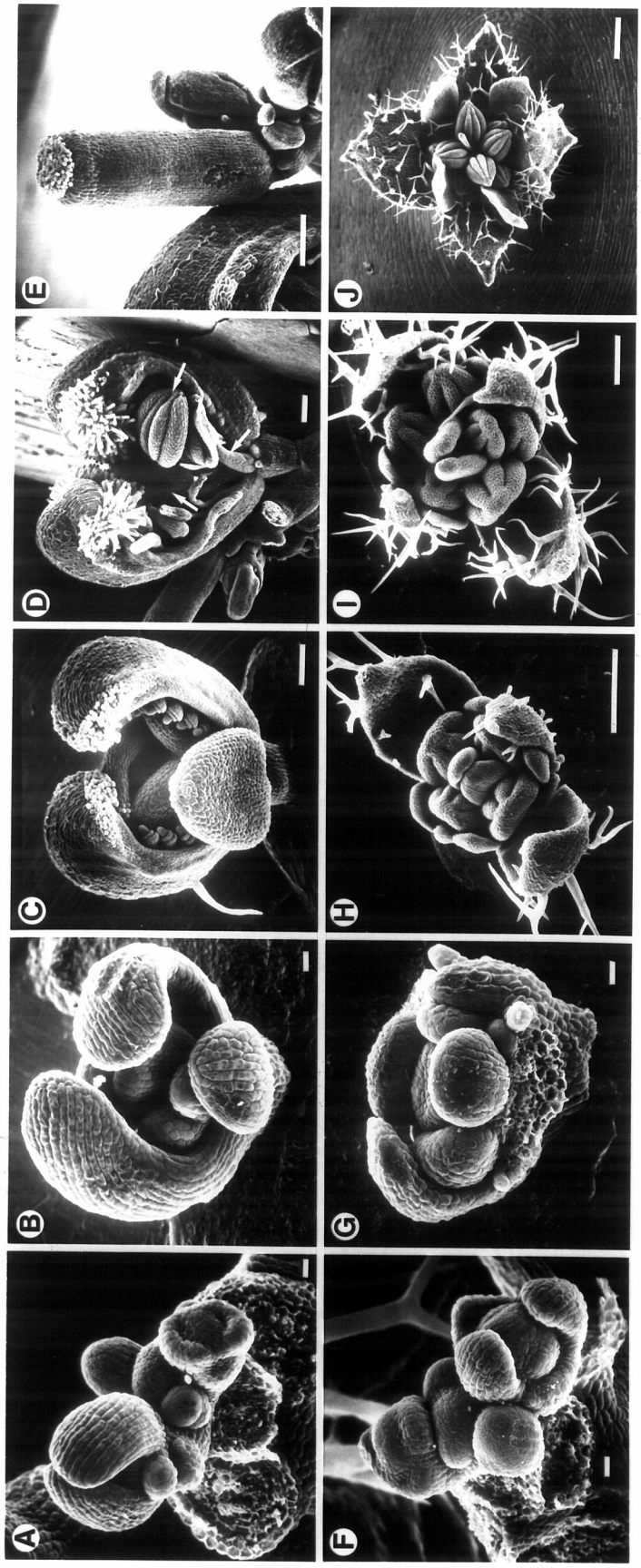
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Figure 4. Scanning electron micrographs depicting the development of *sup-1 ag-1* and *sup-1 ag-1 ap2-1* flowers.

(A-H) *sup-1 ag-1* flowers. **(A)** Inflorescence meristem and flowers in stages 1-4. **(B)** Stage 6 flower. At this stage, *sup-1 ag-1* flowers resemble wild type except that the second and third whorl organ primordia are similar in size. **(C)** The fourth whorl consists of six organ primordia and the floral meristem is pentagonal. **(D)** Organs of the third and fourth whorl are irregular in position and number. The fifth whorl has eight organ primordia. **(E)** The floral meristem continues to produce whorls of several organ primordia. **(F)** *sup-1 ag-1* flower of late developmental stage. **(G)** The floral meristem of flower shown in (F) has become enlarged and elongated, producing organ primordia along its entire circumference. **(H)** Close up of inner whorl organs of *sup-1 ag-1* flower. Many stomata are visible as well as a sector of sepaloid tissue (arrow).

(I-L) *sup-1 ag-1 ap2-1* flowers. **(I)** Inflorescence meristem and flowers in stages 1-5. **(J)** Stage 6-7 flower. Organ primordia of the third whorl are not in the normal positions and the floral meristem is enlarged. **(K)** The first whorl cauline leaf-like organs have developed stellate trichomes while all the organ of the inner whorls are staminoid petals. **(L)** *sup-1 ag-1 ap2-1* flower of late developmental stage.

Bar = 10 μm in A, B, C, I, and J; 50 μm in D, E, and K; 100 μm in G and H; 300 μm in F and L.

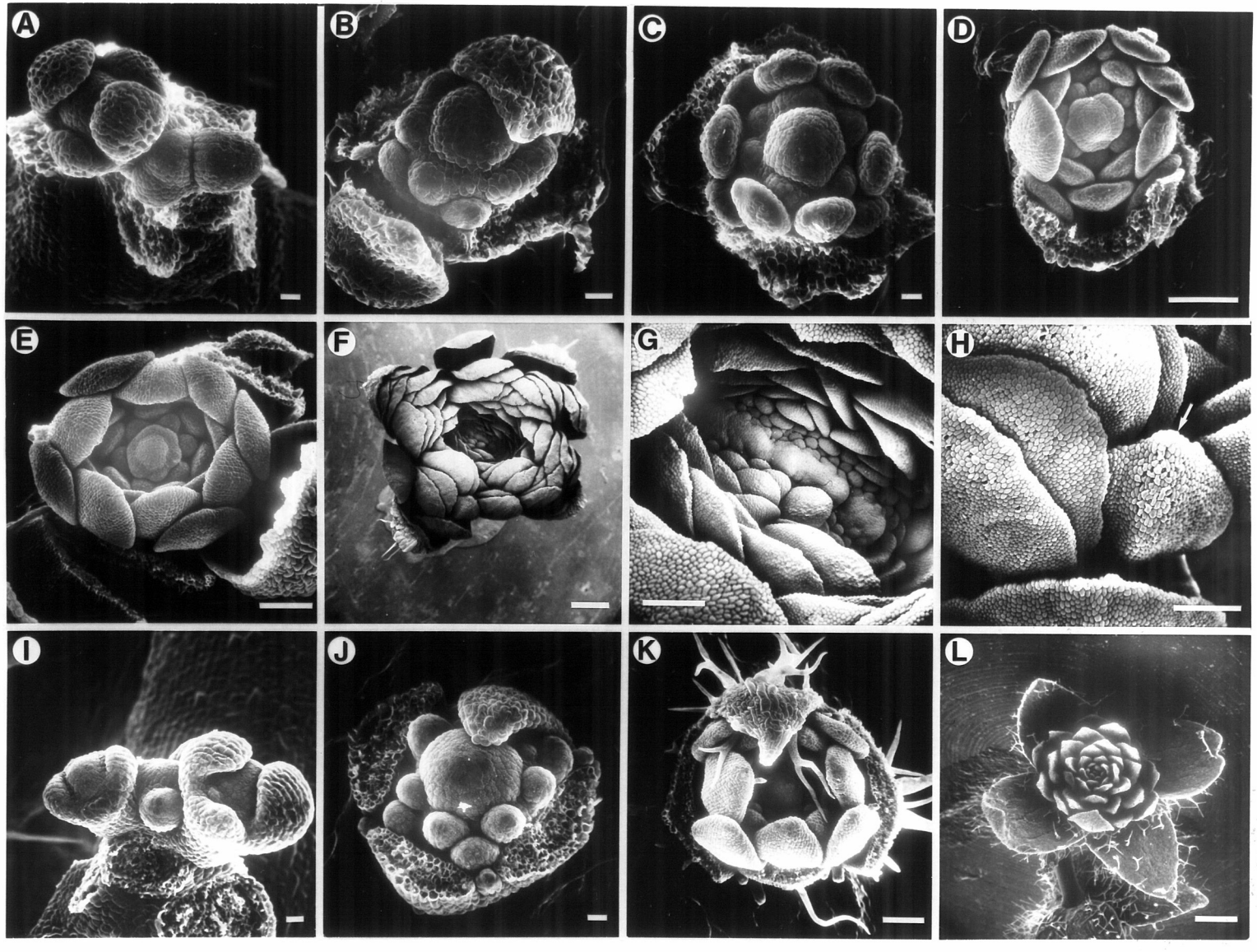
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Figure 5. Scanning electron micrographs depicting the development of *apetala1-1* flowers. See text for a discussion of the terms axillary and orders.

(**A-J**) *apetala1-1* flowers in Landsberg *erecta* genetic background. (**A**) Inflorescence meristem and the first fifteen flowers produced. The first two primordia produced by this inflorescence meristem behaved like inflorescence meristems (1 and 2), producing second order primordia. (**B**) Side view of (A) showing that the second order inflorescence (1) does not produce and is not subtended cauline leaves. (**C**) Stage 7 flower. Axillary floral meristems (arrows) are visible in the axils of two developing first whorl organs. (**D**) Stage 9 flower. An axillary flower (arrow) is present although no primary first whorl organ is present. (**E**) The first second order inflorescence meristem [(1) in (A)] produced by the primary inflorescence meristem. (**F**) The second second order inflorescence meristem [(2) in (A)] produced by the primary inflorescence meristem. The first primordium produced by this second order inflorescence meristem also behaved like an inflorescence meristem (arrow; this is a third order inflorescence meristem). (**G**) Close up of the third order inflorescence meristem in (F). Six fourth order primordia are visible. (**H**) Inflorescence meristem and flowers of stages 1-7 after the inflorescence meristem has produced about fifteen flowers. First whorl organs are often absent and the gynoecium of the oldest flower is not developing properly. (**I**) Structure that may be an aborted first whorl organ primordia (arrow). Structures resembling stipules flank the possible primordia and a group of cells that may be an aborted floral meristem in visible in its axis. Groups of morphologically undifferentiated cells are also visible in the region between the first and fourth whorl organs (double arrow). (**J**) Mature *ap1-1* flower. The primary and two secondary flowers are visible.

(**K-T**) *apetala1-1* flowers in the Wassilewskija genetic background. (**K**) Overview of the entire inflorescence architecture. (**L**) First order inflorescence meristem (1). This meristem is producing second order inflorescence meristems (2) that in turn are producing third order inflorescence meristems (3) and so on. (**M**) Close up of (K). Although most primordia remain undifferentiated, a floral structure is evident (arrow). (**N**) The 1st second order inflorescence meristem produced (1 in K). (**O**) Third order inflorescence meristem (3) of (N). Fourth order primordia (4) do not appear to be simply behaving as either an inflorescence or floral meristem. Some fourth order primordia appear to be differentiating into a floral meristem with axillary flower meristems in the axils of their outer whorl organs; one of these is indicated as a fifth order meristem (5). However, the identity of most of the fourth and fifth order primordia cannot be determined at this stage of development by morphological criteria. (**P**) The 3rd second order inflorescence meristem of (K). All visible third order primordia are behaving like inflorescence meristems. (**Q**) The 6th second order inflorescence meristem of (K). One third order meristem appears to be forming floral structures (arrow). (**R**) The 9th second order inflorescence meristem of (K). One third order primordia is clearly differentiating into a flower (arrow). Another appears to be forming leafy structures (double arrow). The remainder are developing as inflorescence meristems. (**S**) First order inflorescence meristem at a late stage of development, after about fifteen second order primordia have been produced. Most of the second, third, and higher order primordia remain morphologically undifferentiated but some have developed into flowers with an *ap1-1* phenotype. (**T**) Close up of a single second order inflorescence from which a few flowers have differentiated.

Bar = 10 μm in C, G, and H; 30 μm in D, F, and O; 100 μm in A, B, E, I, K, L, M, N, P, Q, R, and T; 300 μm in J and S.

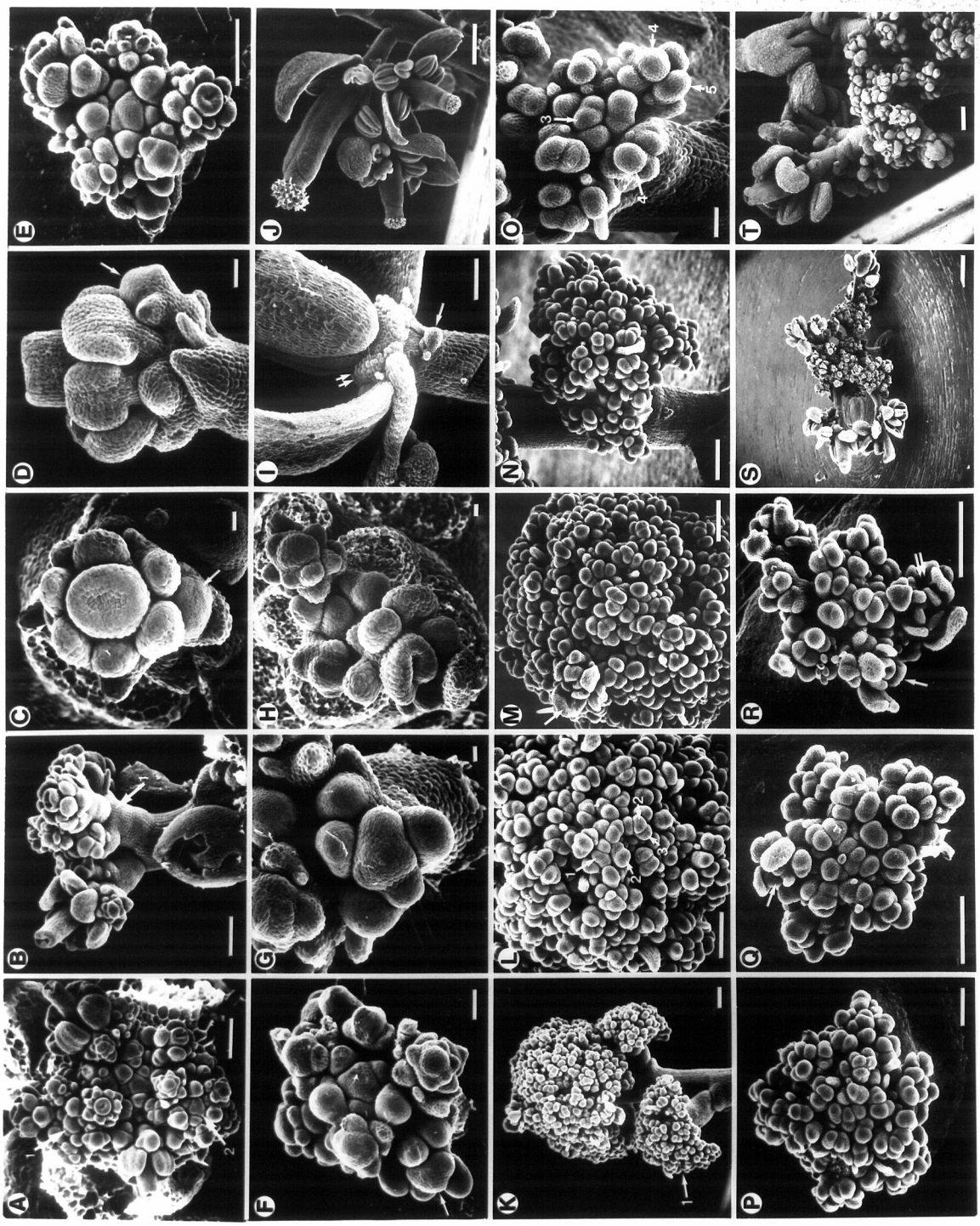
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Figure 6. Scanning electron micrographs depicting the development of *ap1-1 ap3-1*, *ap1-1 pi-1*, and *ap1-1 sup-1* flowers.

(A-D) *ap1-1 ap3-1*. **(A)** Inflorescence meristem and flowers of stages 1-7. **(B)** Stage 7-8 flower. The growth of the central gynoecium is irregular. **(C)** Organs in the third whorl are developing similar to the staminoid carpels observed in the third whorl of *ap3-1* flowers. **(D)** The central gynoecium appears to be forming three carpels. Three secondary flowers are visible.

(E-H) *ap1-1 pi-1*. **(E)** Inflorescence meristem and flowers of stages 1-7. **(F)** Leaf-like primordia are visible in second whorl positions and the gynoecial cylinder is enlarged. **(G)** Five secondary flowers are visible, a case not observed in *ap1-1* flowers. **(H)** Mature ap1-1 pi-1 flower. A tertiary axillary meristem is behaving like an inflorescence meristem (arrow).

(I-L) *ap1-1 sup-1*. **(I)** Inflorescence meristem and flowers of stages 1-7. **(J)** Three stamens have been removed. **(K)** The primary flower consists of only stamens and a single leaf-like organ. **(L)** Mature primary flowers and its two secondary flowers. The primary flower has a carpelloid organ.

Bar = 30 μm in A, B, E, F, I, and J; 100 μm in C, D, G, H, K, and L.

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H
I
J
K
L

Figure 7. Scanning electron micrographs depicting the development of *ap1-1 ag-2* flowers.

(A) Inflorescence meristem and flowers of stages 1-6. **(B)** Approximately stage 7 flower. Four axillary flowers are visible. The organs and organ primordia interior to the secondary flowers do not appear to be in a well-defined pattern. Organs of different size are present in a single whorl. **(C)** Inner whorls organs are not in a well-defined pattern. **(D)** The floral meristem continues to produce primordia that develop into petaloid or leaf-like organs. **(E)** *ap1-1 ag-2* flower of late developmental stage. A secondary flower (double arrows) is arising from the pedicel of the primary flower. It is accompanied by filamentous structures at its base (arrows), possibly stipules of an aborted leaf primordia. The apical meristem is visible at right. **(F)** Close up of inner whorl organs. One (A) has well-defined sectors of leaf-like and petaloid tissue while another (B) has a more graded boundary between petaloid and phylloid tissue.

Bar = 10 μm in A, B, C, and D; 100 μm in E and F.

A
B
C
D
E
F
B
A

Figure 8. Scanning electron micrographs depicting the development of *ap1-1 ap2-9* and *ap1-1 ap2-1* flowers.

(A-F) *ap1-1 ap2-9*. **(A)** Inflorescence meristem and flowers of stages 1-7. Congenital fusion of the first whorls organs is visible. **(B)** Four carpelloid organs are present in what appears to be first whorl position. In addition an outgrowth of cells is visible on the pedicel below the receptacle (arrow). **(C** and **D)** Later stages of development of flowers similar to (B). **(E)** A secondary flower consisting of two partially fused carpels is visible (arrow). A small outgrowth of cells is at the base of the secondary flower. **(F)** Two secondary (2) and one tertiary (3) flowers are visible. Both of the secondary flowers arise from the pedicel below the organs of the primary flower. One secondary flower consists of a two-carpelled gynoecium whereas the other is similar in phenotype to the primary flower.

(G-L) *ap1-1 ap2-1*. **(G)** Inflorescence meristem and flowers of stages 1-8. **(H)** Opposite side of inflorescence meristem shown in (G). **(I)** Secondary flower (arrow) without accompanying first whorl organ although stipule-like structures flank the base of the flower. **(J)** Both developed secondary flowers (arrows) are subtended by a first whorl leaf-like organ. The secondary flowers exhibit a phenotype characteristic of strong *ap2* mutants. **(K)** Close up of (J). An outgrowth of cells (O) that may be an aborted leaf-like organ and its associated stipules. In its axil may be an aborted meristem. Several squamules that are quite stipule-like are present at the base of the flower. A nectary (N) is present at the base of one stamen. **(L)** Both secondary flowers have carpelloid first whorl organs. One secondary flower has a tertiary flower. An outgrowth of cells, similar to that shown in (K), is visible on the pedicel (arrow).

Bar = 30 μm in A, B, and C; 100 μm in D, E, F, G, H, I, J, and K; 300 μm in L.

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D
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2
3
2
N
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Figure 9. Scanning electron micrographs depicting the development of *ag-4* flowers.

(A) Inflorescence meristem and flowers of stages 1-6. **(B)** Stage 7 flower. Four organ primordia have been produced on the flank of the cells that would normally give rise to the gynoecium. These fourth whorl primordia usually develop into sepals although they may also have staminoid or carpelloid characteristics. **(C)** The pattern of organ primordia formation interior to the third whorl may be irregular as shown here. **(D)** Flower in which the fourth whorl organs (arrow) are carpelloid and have fused along their margins forming a connate ring of carpelloid sepals. Fusion occurs congenitally. The floral meristem continues indeterminately interior to this ring of organs. **(E)** Close up of flower shown in (F). A fourth whorl organ has sectors of stamen (arrow) and sepal (double arrow) tissue. **(F)** Although the third whorl stamens are only slightly petaloid, they do not dehisce.

Bar = 10 μm in A and B; 30 μm in C; 100 μm in D and E; 300 μm in F.

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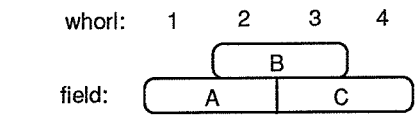
Figure 10. A schematic representation of the model depicting how SUP could regulate the activity of AP3 and PI in the fourth whorl of the flower. A section through one-half of a floral primordium is represented as a set of boxes, with the regions representing each whorl shown at the top of each column. Each box represents a single field (defined in Bowman et al., 1991); these are also shown at the top of the first column. In *sup* flowers field B, the domain of AP3 and PI activity, expands to include the fourth whorl. The genotype under consideration is listed at the left with the predicted distribution of gene products present in each genotype indicated by uppercase letters within the boxes. The predicted phenotype of the organs in each whorl are shown under the diagrams. Se = sepal, P = petal, St = stamen, C = carpel. P/St = petaloid stamen, an organ, present in *sup ap2 ag* flowers, with characteristics of both petals and stamens. L = leaf or carpelloid leaf, either can be found in the positions indicated depending upon the allele of *ap2* present. The * is a reminder that in each genotype containing *ag*, there are several whorls of organs interior to the fourth whorl. Details of the model are in the Discussion.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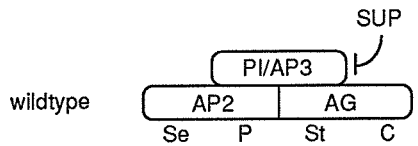
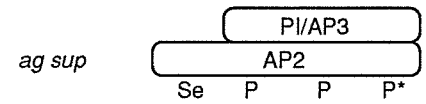

SUP
PI/AP3
wildtype
AP2
AG
Se P St C


PI/AP3
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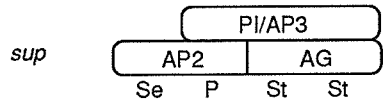
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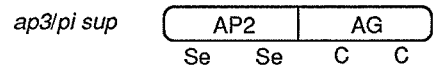
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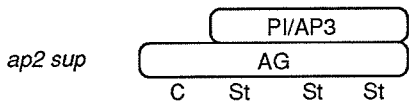
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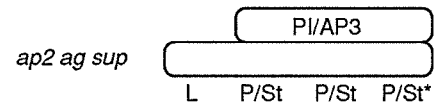
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CONCLUSIONS

How Do Cells Become Different?

A fundamental question in developmental biology is the origin of complex structures from a single apparently unordered cell or group of cells. At the cellular level this translates to the question of how does the mitotic division of a undifferentiated cell result in the production of two daughter cells each with a different developmental potential or fate. This process is easily visualized in the development of echinoderm and amphibian embryos in which a large amorphous fertilized egg is divided into a morphologically complex structure consisting of many small cells. Early experiments on these embryos led to the realization that the developmental potential of individual cells was limited or determined depending on both the position of the cell within the embryo and the developmental age or cleavage stage of the cell (see Davidson, 1986 for review). For example, each of the four cells of the 4-cell stage sea urchin embryo is capable of developing into a complete larva but by the 8-cell stage, the developmental potentials of all cells are no longer equivalent. The idea that there are localized determinants, or morphogens, specifying different fates that are segregated to the daughter cells followed this realization.

Lessons From Animal Development

Until the early part of this century experimental embryology and genetics were considered to be unrelated fields. The discovery that genes, and subsequently DNA, contained the information required for development resulted in a merger of the two fields. This, in turn, led to the notion of cascades of sequential gene activation as a mechanism to specify cell fate. It was soon realized, however, that it is not simply the case that localized cytoplasmic determinants specified cell fate (i.e., there are not gene products that specify each of the possible cell fates of an embryo localized in the egg). Rather, a more complex

picture of development emerged in which many factors, such as cell-cell interactions and cell migrations in addition to localized determinants influence developmental pathways. Investigations into the development of several different phylogenetic branches of animals has shown that the mechanisms by which different organisms achieve the goal of cell fate specification are as diverse as the organisms examined (see Davidson, 1990; Melton, 1991 for reviews). Early cleavage patterns of the egg may be variable (mammals) or invariant (*C. elegans*). Cell migrations play a major role in zebrafish, for example, while little cell migration is observed in some other animals (*Drosophila*). Some eggs use cytoplasmic localizations to determine one (*Xenopus*) or two (*Drosophila*) axes while in others no axis is specified in the egg (mammals). Some embryos exhibit rigid cell lineages (*C. elegans*) while in others every individual has a different cell lineage, relying on cell-cell interactions to specify fate (zebrafish). To further complicate the situation, cells in some lineages that appear to be specified autonomously, may in fact be responding to positional information within the developing embryo. When these cells are moved from their normally stereotyped position within the developing embryo, they may acquire a different developmental fate (e.g., Wood, 1991).

Despite this wealth of diversity in developmental mechanisms, a common theme of cascades of sequential gene activation being the underlying cause for developmental processes emerges. The regulatory principles controlling the differential activation of genes in the organism are ultimately responsible for the developmental program of that organism. These principles may vary from organism to organism, but in general the genes responsible can be placed into a regulatory hierarchy based on their temporal expression and their function in development (see Davidson, 1990, for review) Briefly, there are initial patterning genes which provide global positional information in the organism. These include classic cytoplasmic determinants that are responsible for specifying axes within the organism. These gene products activate, either directly or by inductive processes, other

patterning genes or region specific genes. These include the homeotic genes which seem to be responsible for specifying the identity of large regions of an organism. Next in the hierarchy are local patterning genes, which are responsible for detailed patterning of cell types within a small region of the organism. The fine patterning can be a response to a local morphogen gradients (e.g., Sternberg and Horwitz, 1989) or apparently stochastic processes (e.g., Campos-Ortega, 1988; Seydoux and Greenwald, 1989). The next lower level is occupied by regulatory genes which control the expression of cell-type specific gene batteries (i.e., groups of muscle specific genes). At the lowest level are those genes directly responsible for the biochemical and morphological differentiation of cell types, the histospecific structural genes. The order in which levels of the hierarchy are first activated varies from organism to organism, and in some cases within an organism. For example, histospecific structural genes are activated prior to regional specification genes in some lineages of *C. elegans* and sea urchin embryos. In contrast, the order of activation in *Drosophila* embryos is, in general, from global patterning to cell type specific genes.

Drosophila as a Model System

Recently, a molecular genetic approach has been successful in identifying many of the genes utilized during the initial patterning events of *Drosophila* embryogenesis. These studies are briefly summarized here because they have provided a paradigm for dissecting developmental processes in systems amenable to molecular genetics, such as *Arabidopsis*. In addition, several parallels may be drawn between the genes controlling pattern formation during *Drosophila* embryogenesis and those of floral morphogenesis in *Arabidopsis*.

In *Drosophila*, the genetics of homeotic genes, whose mutant phenotypes are the transformations of one segment type to another, provided the first indication that the development of the *Drosophila* embryo relies on positional information (Lewis, 1978). In the homeotic mutants, cells in the developing embryo appear to misinterpret their position.

These cells or their descendents subsequently differentiate into inappropriate cell and organ types. As an approach to uncovering the basis of these phenotypes, saturating mutageneses were performed in which a large number of embryo pattern mutants were isolated and analyzed (see Nüsslein-Volhard et al., 1987; Akam, 1987, for reviews). These genetic studies and the subsequent molecular cloning of many of the genes involved have provided a reasonably coherent picture of the genes utilized to specify segment fate in the *Drosophila* embryo.

The segmental character of *Drosophila* is produced by a cascade of gene activation, with many of the genes encoding transcription factors (see Nüsslein-Volhard et al., 1987; Akam, 1987; Ingham, 1988, for reviews). The identity of each segment is then specified by the combinatorial activity of a set of homeotic selector genes which are activated in response to the positional information embodied in the expression patterns of a previously acting cascade of gene activation. This cascade of gene activation is rather hierarchical with several distinct levels or classes of genes in terms of the spatial domains in which they act. At the top of the hierarchy are localized maternal determinants, such as *bicoid* and *nanos*, which specify the antero-posterior axis of the embryo. *bicoid*, a transcription factor, is distributed in a gradient along the antero-posterior axis and acts as a classic morphogen in specifying cell position in a concentration dependent manner (see Nüsslein-Volhard et al., 1987; Ingham, 1988; Melton, 1991, for reviews). The highest concentration of *bicoid* protein is at the anterior end of the embryo while *nanos* is primarily localized to the presumptive abdomen. The spatial domains of activity of these morphogens are a result of their polarized deposition or subsequent localization in the egg due to maternal factors, which is governed by a number of genes. Thus, the initial gradient which specifies the antero-posterior axis of the embryo is a consequence of a polarity already present. The two maternal activities are responsible for activating the next level of the hierarchy, the gap genes.

The embryo is subdivided by the zygotic gap genes, each of which is a transcription factor, that are activated in specific regions by a combinatorial interactions with the *bicoid* and *nanos* groups. Their final domains of activity are refined by interactions amongst the gap genes themselves. The gap genes are in turn responsible, at least in part, for activating another set of genes called the pair-rule genes (also transcription factors) whose expression patterns further subdivide the embryo in segments (or parasegments). The boundaries of the parasegments are defined as a result of interactions between the pair-rule genes. The polarity of each parasegment is established due to the expression of the segment polarity genes whose activation is dependent upon the activities of the pair-rule genes. Up to this point expression patterns of genes at one level of the hierarchy are influenced, sometimes directly, by the expression patterns of genes in each of the higher levels; for example the segment polarity genes can be acted upon not only by segment polarity and pair-rule genes but also by gap genes. However, the early (higher) levels of the hierarchy are not influenced by the genes in the later (lower) levels. The expression of the maternal, gap, and pair-rule genes is transient. Thus, the embryo is subdivided into parasegments, each uniquely identified by the combination and levels of segmentation genes expressed, by a transient cascade of spatially localized transcription factors. The positional information specified by these transient expression patterns is translated into a stable set of positional addresses identified by the expression patterns of the segment polarity and homeotic genes. The homeotic genes are expressed in a combinatorial manner in response to activation (or repression) by the gap, pair-rule, segment polarity, and homeotic genes. Each parasegment acquires its own stable positional address as a consequence of the combinatorial set of homeotic genes expressed.

The homeotic selector genes specify parasegmental character (see Lewis, 1978; Gehring and Hiromi, 1986; Peifer et al., 1987; Ingham, 1988, for reviews). The genes encode transcription factors with a common DNA binding motif called the homeobox and

each is expressed primarily in the regions altered when mutant. The initial expression patterns of the homeotic genes is dependent upon the gap, pair-rule, and segment polarity genes. Once activated, however, the precise boundaries of expression are in large part determined by interactions among the homeotic genes, while still other loci are involved in maintaining these patterns of expression.

In *Drosophila*, the homeotic genes form two large gene complexes (*Antennapedia* and *bithorax*), although in most other animal species in which homologous complexes are found, all the genes are present in a single complex. Within the complexes, the individual genes are arranged on the chromosome in the same manner as they are expressed in the embryo (antero-posteriorly). Each is expressed in several usually contiguous parasegments, although the levels of expression varies between parasegments. Mutations in the homeotic genes result in the transformation of a segments' identity to that of another. Depending on the extent of homeotic gene deficiency, from a single segment to nearly all segments can be transformed. Due to interactions among the homeotic genes, recessive mutations result in transformations to more anterior segmental identity while dominant mutations result in transformations towards posterior identity. Thus, the homeotic genes are relaying the positional information generated by the segmentation genes to downstream genes that are responsible for morphological differentiation within each parasegment.

Individual homeotic genes are expressed in strikingly different patterns in different parasegments. This may reflect the fact that a homeotic gene may have very different roles in adjacent segments and may be a result of differential activation by the segmentation genes. The positional information supplied by the combination of homeotic genes is interpreted differently in different contexts (i.e., in different parasegments). Differential expression of combinations of homeotic genes between cells in the same parasegment may play a role, but how the positional information is translated at the molecular level is still a matter of speculation. Thus, although we know that the homeotic genes represent region

specific positional information and histospecific genes represent cell-type specific gene expression, the relationship between the two groups is a gap in our knowledge of development at this time.

The Plant Kingdom

Plant development offers many contrasts when compared to animal development (e.g., Burgess, 1985). Most higher plant cells are totipotent, as has been demonstrated with protoplasts of nucleated cell types from many species. Fully differentiated cells, such as secondary phloem cells, can dedifferentiate to produce totipotent cells. This indicates deposition in the egg cell of localized determinants specifying positional information are not required (although possibly utilized) for the proper development of any plant structure. In contrast to animals in which organogenesis occurs predominantly during embryonic development, organogenesis occurs throughout the lifetime of plants with an indeterminate number of organs produced from meristematic cells that are continually being regenerated. Thus, positional information required during development must either be continually generated or be maintained in the meristems throughout the lifetime of the plant. Another difference between plant and animal cells is the ability to migrate. Each plant cell is surrounded by a rigid cell wall, synthesized simultaneously with cell division. The result is that there is little opportunity for cell migration in plants.

Communication between plant cells can occur through structures called plasmodesmata. Plasmodesmata offer cytoplasmic, as well as extracellular and endoplasmic reticular lumens, bridges between adjacent cells that usually, but not necessarily share a common recent developmental history. Although there is evidence that these bridges may play a communicative role in the development of fern gametophytes, the extent that plasmodesmata are used for cell-cell communication during development of higher plants is unknown. Long range communication is evident in plant responses to the

environment, such as light, gravity, and infection by pathogens, as well as many aspects of general developmental processes. Although the mediators of many of these responses remain elusive, phytohormones are known to act in several developmental phenomena. Each of the known phytohormones have pleiotropic effects when their normal *in vivo* level or sensitivity is altered (by exogenous application, mutation, or *in vivo* over expression). For example, a reduction in *in vivo* auxin levels results in decreased apical dominance, inhibition of root growth, and reduced xylem differentiation (e.g., Romano et al., 1991). Several lines of evidence (see Chapter Two) argue against the known phytohormones having a role in the developmental processes considered in this thesis.

Cell lineage patterns in plants are highly variable. A single meristematic cell may give rise to several different tissues including both vegetative and reproductive organs. The extent of clonally derived sectors is variable both within and between plants. Thus, there are no lineages set aside specifically for reproductive or vegetative structures. Despite this variability in tissue type derived from cell lineages, some generalizations can be made. Three distinct cell lineage layers are present in the plant meristem, each of which give rise to characteristic cell layers within floral and vegetative organs (see Chapter Five). Thus, cell layers rather than entire organs are more likely to be clonally derived.

In general plants are much simpler organisms than most animals, consisting of only a fraction of the cell and organ types found in animals. Despite this, the molecular bases of plant development have remained, until recently, relatively obscure. The application of molecular genetics to the plant kingdom has led to a beginning of unraveling the mysteries of plant morphogenesis. Despite the fundamental differences between the animal and plant kingdoms and their distant phylogenetic separation, some of the molecular mechanisms of pattern formation in plants appear similar to basic concepts identified in animal development.

Flowers

Angiosperms, or flowering plants, are the dominant order of plants living today, as they have been for the past 100 or so million years. The most complex structure of angiosperms, the flower, provides a useful system in which to study pattern formation and the specification of cell fate in plants. The enormous diversity of inflorescence and flower structure has long been used in taxonomy to infer phylogenetic relationships. Flowers of each species consist of a precise pattern of several organ types, with each organ type occupying one or more whorls of which flowers are typically composed. Interspecific variants of flower structure have been noted for over two millennia (see Meyerowitz et al., 1989 for review). As early as the sixteenth century, special attention was paid to specific types of abnormalities. These aberrant flowers exhibited normal floral organ types but in ectopic positions within the flower. J. W. von Goethe termed this phenomena 'metamorphy' (1790) which was replaced by Bateson with the term 'homeosis' a century later (1894). The term homeosis has been used since, and its application extended to similar transformations of identity in metameric series in many species of animals as well. In the mid-nineteenth century these floral variants were categorized by nature of abnormality although no distinction was made between inherited abnormalities and individual aberrant flowers due to environmental factors or chance (see Meyerowitz et al., 1989 for review).

By the late nineteenth century it was realized that some of the flower abnormalities are heritable, and these mutants were among the first used in crosses to test for Mendelian segregation following the rediscovery of Mendel's work in the early twentieth century (see Meyerowitz et al., 1989 for review). Although the development of wild-type flowers of many species was examined in detail and abnormal flowers were known in these species, in only a few instances were these studies combined. Recently, a molecular genetic

approach undertaken in two different species, *Arabidopsis* and *Antirrhinum*, has given insight into some of the molecular mechanisms underlying flower development. *Arabidopsis thaliana* (see Appendix Three for review of *Arabidopsis*) was used as a model system to investigate regional pattern formation during flower development.

Arabidopsis flowers

Flowers of *Arabidopsis thaliana* are produced in a phylotactic spiral on the flanks of the inflorescence meristem (Chapter One). Each flower consists of four concentric whorls of organs. The organs of each whorl have stereotypical positions relative to the inflorescence meristem, to other organs within the same whorl, and to organs in the other whorls. Four sepals occupy the first whorl, four petals are found in the second whorl, six stamens reside in the third whorl, and a two-carpelled gynoecium occupies the innermost or fourth whorl.

A large number of mutations were collected, either isolated in our laboratory or obtained from other generous laboratories (Ken Feldmann, Maarten Koornneef, Gerd Jürgens, and David Smyth), each of which appears to cause cells in the developing flower to misinterpret their positions and subsequently differentiate inappropriately. The mutations have no vegetative phenotype. Some of these mutations (*agamous*, *apetala2*, *apetala3*, and *pistillata*) represent the type of abnormalities recognized by Goethe and others and can be termed homeotic. Other mutations (*apetala1*, *clavata1*, and *superman*) cause pattern defects in the developing flower but the nature of their phenotypes are not genuinely homeotic. The genes represented by the homeotic mutations appear to encode products which provide positional information within the developing flower, much as the homeotic genes of *Drosophila* specify positional information within the developing embryo. Each of the mutations affects the identity of organs in two adjacent whorls of the flower; the phenotypes are described in detail in Chapters Two, Three, and Seven. Genetics

experiments with several alleles at each of the homeotic loci has led to a testable model of some of the molecular mechanisms underlying flower development (presented in detail in Chapter Three).

Models of flower development

In this century two major classes of flower development models have been proposed. One class of model involves communication between adjacent whorls of developing floral organs, leading to sequential specification of organ identity (Heslop-Harrison, 1963; McHughen, 1980; Green, 1988). The signals between the whorls were proposed to be either biochemical or biophysical. Thus, developing sepals would send a molecular signal to the next interior whorl of organ primordia and instruct them to become petals, and so on. It is clear from the genetic data presented here, in addition to the extensive teratological evidence compiled from other species, that it cannot be that the proper differentiation of any particular whorl is dependent on the correct differentiation of neighboring whorls, either inner or outer. For example, petals form normally in the second whorl of *ap2-1* and *ag-1* flowers despite the improper differentiation of leaf-like organs in the first whorl of *ap2-1* flowers and the incorrect differentiation of the third whorl into petals in *ag-1* flowers. A model that is better supported by the data is one in which the flower primordium is divided into embryonic fields by the expression patterns of the homeotic genes. This type of model allows for cellular communication in the flower primordia during the process of setting up the field pattern which in turn specifies the organ pattern without implicating communication between specific organ types. Models of this type have been proposed to explain morphological abnormalities of some flower mutants although the nature of the substances identifying the extent of the fields was left unspecified (Brieger, 1935; Holder, 1979). In the model presented here the homeotic genes, each a gene regulatory factor, are proposed to act alone and in combination to specify the identity of each whorl in the flower. Details of the model can be found in Chapters Three and

Seven. Similar models have also recently proposed for the specification of floral organ identity in *Antirrhinum* (Carpenter and Coen, 1990; Schwarz-Sommer et al., 1990). These models are consistent with and subsets of the one presented in Chapter Three.

In Chapter Three several predictions were made about the temporal and spatial characteristics of expression patterns of the homeotic genes. Thus far, predictions have been confirmed with two genes, *AG* (Chapters Five and Six) and *AP3* (Tom Jack, unpublished) and this verification, especially the expansion of the spatial domain of *AG* expression in *ap2* mutants, is support for the validity of the model. Four major conclusions may be drawn from the observed patterns of RNA expression of these genes. Firstly, the expression patterns correspond to the whorls of the flower which are disrupted when the genes are mutated. This is consistent with these genes being responsible for the specification of floral organ identity and is analogous to the expression patterns observed for the homeotic genes of *Drosophila.* Secondly, the homeotic genes encode regional positional information but do not singularly identify specific organ types, again similar to the *Drosophila* homeotic genes. In other words, there is no gene that uniquely specifies stamens, but rather a limited number of genes act in a combinatorial fashion to specify the different floral organs. For example, there are no stamens produced in *ag* flowers, however, the development of organs with characteristics of stamens can occur in the absence of AG activity if AP2 activity is also absent. Thirdly, expression or repression of the homeotic products must be maintained throughout flower development, at least in the case of *AG* and probably *AP3*, in order for proper cellular differentiation to occur. Ectopic late expression of the homeotic genes leads to homeotic transformations of certain cell types in an otherwise properly differentiated organ (see Chapter Six). And fourthly, although the final spatial expression patterns are in part defined by interactions between the homeotic genes themselves (*AP2* and *AG*, see Chapters Three and Five), the homeotic genes are

responding to an already spatially complex prepattern. Again, these final two points are also analogous to some situations observed in *Drosophila.*

Organ primordia and their subsequent specification?

Which comes first, the floral organ primordia or the patterns of gene expression that specify their identity? It appears that the formation of organ primordia and their specification of identity are somewhat independent processes. Mutants at each of the four homeotic loci alter both organ primordia position and number as well as their identity. Both *AG* and *AP3* are expressed in the floral meristem before the organ primordia to which they are localized morphologically arise (Chapter Five; Tom Jack, unpublished). In addition, the temperature sensitive period of *ap2-1* begins before the affected organ primordia arise (Chapter Two). Similarly, it can be inferred that *PI* is likely to be expressed before the second and third whorl primordia arise since the third whorl primordia fail to form properly in *pi-1* flowers (Chapter Two). Thus, the homeotic genes responsible for the specification of identity of floral organs are activated before the floral organ primordia arise.

Regions of the floral meristem that are specified to differentiate into carpelloid tissue seem to 'know' if adjacent tissue also has been specified to differentiate into carpels. The fourth whorl carpels are the only organs in the wild-type *Arabidopsis* flower that fuse. The carpel tissue in the fourth whorl normally behaves as a single primordium, probably as a fusion of two carpel primordia. In mutant flowers, however, nearly all adjacent organ primordia that have a carpel fate fuse congenitally, in either intra- or inter-whorl fusions (Chapters Two and Three). The extreme example of this is the enlarged gynoecium of *pi-1* flowers in which all cells that would ordinarily give rise to the third and fourth whorls develops into a single gynoecial cylinder.

These observations suggest that the expression patterns of the homeotic genes are probably established before the organ primordia, whose identity they specify, arise. Thus, the flower primordium is divided into fields of differential gene expression specifying cell fate at about the time or before the cell divisions forming the individual organ primordia are complete. This helps explain many of the mosaic organs observed in mutants flowers. The organ primordia which give rise to mosaic organs are simply derived from cells from more than one cell fate specification field (see Chapter Three for more discussion of this point).

Even though the fate of floral organ primordia may be determined at about the same time they arise, the determination is not irreversible. For example, in *ap3-1*, the fates of both the second and third whorl organ primordia can be altered by temperature shifts after the primordia arise (Chapter Two). Thus, sustained activity of homeotic gene activity may be necessary for the maintenance of the determined state. An additional point is that, although the fate of organ primordia is not irreversibly determined when they form, their possible fates may be restricted. For example, as shown by temperature shift experiments, the second whorl organ primordia choose between sepal/petal and stamen/carpel fates by stage four, but do not choose between sepal and petal fates until a later time, stages 7-8. This is most likely a consequence of early competition between the *AP2* and *AG* products in the outer whorls of the flower. Once *AP2* expression is established it precludes *AG* expression and the stamen/carpel fates, and this event may occur before the onset of irreversible AP3/PI activity in the second whorl.

Two other aspects of flower development, the determinacy of the floral meristem and the formation of floral organ primordia, are obviously controlled in part by the floral homeotic genes. These two processes remain puzzling with the data presently available, but some speculative ideas are presented in Chapters Three and Seven.

Activators and repressors

Each of the homeotic genes described have been proposed to act as both activators and repressors of gene expression. For example, AP2 is proposed to repress AG activity in the outer two whorls as well as activate genes responsible for sepal and, in combination with AP3/PI, petal development. Likewise AG is proposed to activate genes responsible for carpel formation and, in combination with AP3/PI genes, responsible for stamen differentiation. At the same time AG is proposed to repress AP2 activity in the inner two whorls as well as genes regulating cell division. There are several precedents in which a single factor is known to act as both an activator and repressor of gene expression. In the case of MCM1, which activates and represses cell-type-specific genes in yeast, other factors, products of the MAT locus, interact with MCM1 to define the effect that MCM1 has on gene expression (see Herskowitz, 1990, for review). In addition, a factor bound the same binding site can affect gene expression in different ways depending on the presence or absence of other factors (e.g., Diamond et al., 1990: Graham and Gilman, 1991). Therefore, many scenarios can be envisioned by mixing and matching homeotic gene products with common factors to give the desired result. For example, AP2 could activate sepal differentiation genes when it is the only specific factor present, however, the same genes could be repressed if AP3/PI are present along with AP2. In this context, with AP2, AP3, and PI all present, petal specific genes could be activated. There is no need to postulate repression of the petal specific genes in the outer whorl if AP2 alone can not activate them. The mutual antagonism of AP2 and AG is more complex as other, possibly general, factors must be involved. All the factors necessary for the activation of AG must be present in the outer whorls although the presence of AP2 activity precludes its activation. How and why AP2 appears to only be activated in the outer whorls initially must be a consequence of earlier patterns of gene expression. In summary, the biochemical

context in which the floral homeotic genes are expressed influences their impact, positive or negative, on subsequent gene expression.

AP3 is an SRF homologue and *PI* may likely be. The argument for this is that there are two genes, *DEFICIENS* and *GLOBOSA*, in *Antirrhinum* majus (Stubbe, 1966; Sommer et al., 1990; Schwarz-Sommer et al., 1990) that when mutated result in a phenotype similar to the *AP3/PI* phenotypes. It has been shown that *AP3* is a cognate homologue of *DEFICIENS* (Tom Jack, unpublished) therefore *PI* may be the *GLOBOSA* homologue. *GLOBOSA* also encodes a putative transcription factor with homology to SRF (Schwarz-Sommer et al., 1990). SRF binds to DNA and effects transcription as a dimer (Norman et al., 1988). Thus, two speculative possibilities exist. First, AP3 and PI could both form homodimers, with both species of homodimer necessarily being present to effectively influence transcription. Second, AP3 and PI could form a heterodimer that binds to DNA to effect transcription. In both cases, mutations in either one of the genes would result in the same mutant phenotype, as is observed. The *SUPERMAN* gene is proposed to negatively regulate the activity of AP3 and PI in the fourth whorl (Chapter Seven). The *SUPERMAN* product could repress the activity of either *AP3* or *PI* or both in the fourth whorl. That *AP3* is not expressed in the fourth whorl of wild-type flowers and that the expression of *AP3* expands to include the fourth and inner whorls in *superman-1* flowers indicates that the expression (at the RNA level) of at least *AP3* is repressed in the fourth whorl by SUPERMAN (Tom Jack, unpublished).

Autonomy and induction

Although it has been shown that *AG* (and *AP3*; Tom Jack, unpublished) is expressed in all three germ layers, L1, L2, and L3, of the floral meristem (see Chapter Five) it is not known if AG activity is necessary in all germ layers. There is some evidence from genetic mosaics that the L1 germ layer, which primarily gives rise to the epidermis,

can direct differentiation of the inner layers, L2 and L3, in the flowers of some species (see Meyerowitz et al., 1991 for brief review). This implies that the epidermal cells become specified to a certain fate and somehow signal the adjacent inner cells to differentiate accordingly. That cells in the floral primordium are communicating to some extent is suggested by the fusion of presumptive carpel tissue of adjacent whorls in *ap2* flowers (see above).

RNA or even protein expression patterns, although often corresponding to activity patterns, do not necessarily predict where a particular product needs to be active to exert its effects. Therefore, to determine conclusively in which cell layers the floral homeotic genes are active, genetic mosaics must be constructed and analyzed.

What controls the floral homeotic genes?

The final patterns of floral homeotic gene expression can in part be explained by interactions between the homeotic genes themselves (the mutually exclusive patterns of expression of *AP2* and *AG*, Chapters Three and Five) and with other genes (*SUPERMAN* inhibiting *AP3/PI* in the fourth whorl, Chapter Seven). However, that *AG* (and *AP3*), are initially expressed in a spatially complex pattern suggests that they are responding to a pre-existing pattern of gene expression. Two genes that may be involved in this prepattern are *APETALA1* (Irish and Sussex, 1990) and *LEAFY* (Haughn and Somerville, 1988; John Alvarez, David Smyth, and Detlef Weigel, unpublished). *APETALA1* was discussed in Chapter Seven as a candidate for controlling the conversion from an indeterminate inflorescence meristem to a determinate floral meristem as well as possibly having a minor role in regulating *APETALA2*.

The *LEAFY* gene shares substantial homology at the nucleotide level as well as the phenotypic level with the *Antirrhinum* gene, *FLORICAULA* (Coen et al., 1990). The phenotypes of *leafy* and *floricaula* mutants are discussed in the Introduction.

FLORICAULA displays a complex RNA expression pattern as revealed by *in situ* hybridization. It is expressed transiently and sequentially in the primordia which develop into bracts, sepals, petals, and carpels in wild-type *Antirrhinum* flowers (Coen et al., 1990). The phenotypes of mosaic organs in weak *leafy* mutants suggests that in these mutants the homeotic genes are only partially activated spatially, and perhaps, quantitatively (Detlef Weigel, unpublished). This in turn suggests that *LEAFY* may be a direct regulator of the homeotic genes. Many scenarios can be envisioned in which these genes, *APETALA1* and *LEAFY*, could set up a prepattern to which the homeotic genes could subsequently respond. The sequential nature of the production of the floral whorls may also play a role in how these patterns are accomplished at a molecular level, although the initial expression patterns of *AG* and *AP3*, which exert their effects in different spatial domains, do not appear to significantly differ temporally. That *FLORICAULA*, and most likely *LEAFY*, is also initially expressed in a complex spatial and temporal pattern suggests that there are even earlier patterns of gene expression in the inflorescence meristem. The foundation for these patterns are beyond the realm of this review as well as our present knowledge.

The relationships between the floral homeotic genes and these earlier acting genes in *Arabidopsis*, may be somewhat analogous to that observed in *Drosophila* between the segmentation and homeotic genes in that species. For example, the expression of the gap, pair-rule, and segment polarity genes, on whose activity the later-acting homeotic genes depend for their initial expression patterns, is transitory in nature. Interactions among the homeotic genes, once there expression is initiated by the segmentation genes, help define their final expression patterns. These final expression patterns are relatively stable, but must be maintained to ensure proper cellular differentiation. The effects of homeotic gene activity are usually cell autonomous. The expression of *LEAFY*, a proposed floral homeotic gene regulator, is also likely to be transitory (see above). Again, once the floral

homeotic genes' expression is initiated, interactions among them help define their domains of activity and, at least in the case of *AG* and *AP3*, they continue to be expressed until late in development. This late expression seems to be necessary for proper cellular differentiation. In addition, the effects of floral homeotic gene activity appear to be relatively cell autonomous. The original patterning event in the *Drosophila* embryo is a morphogenetic gradient which is dependent on the polarity of oocyte production. It is unclear what the initial patterning event is in the floral meristem, although it is likely to be derived from patterns in the inflorescence meristem.

A few mutants have been isolated in which the production of floral meristems from the inflorescence meristem is altered (see Introduction). However, there must be many genes involved in this complex process. Two possible reasons that more genes have not been identified are firstly, a saturating mutagenesis screen for such mutants, or any floral mutants for that matter, has not been systematically undertaken and, secondly, genes involved in this processes may also be used in the production or maintenance of other meristems, and a lethal phenotype would result if the activities of the genes were eliminated. Efforts to alleviate these deficiencies need to be addressed if we wish to investigate the inflorescence meristem with a genetic approach.

How is the positional information encoded by the floral homeotic genes translated into patterns of cellular differentiation

Once the identity of floral organ primordia are specified, the subsequent process of cellular differentiation within the organs remains a mystery. The *FLORICAULA* gene of *Antirrhinum* appears to be transiently expressed in a non-uniform pattern within floral organ primordia (Coen et al., 1990). Perhaps this initial asymmetry is part of the basis for establishing relative position within individual floral organs. The floral homeotic gene *AGAMOUS* is progressively restricted to certain cell types as cellular differentiation takes

place in stamens and carpels (Chapter Six) suggesting that *AG* may also have a role in the process. In *Drosophila*, the translation of positional information encoded by the homeotic genes into patterns of cellular differentiation is also obscure. However, the identification of some genes downstream, or selector genes, of the *Drosophila* homeotic genes as transcription factors suggests that there may be more levels of regulatory hierarchies than are presently characterized in the translation of positional information into cellular patterning. In light of this, possible candidates for selector genes of *AG* would be *AGL1* and *AGL2*, both transcription factors whose spatial expression patterns are a subset of *AG*'s and whose initial temporal expression pattern is subsequent to that of *AG* (Ma et al., 1991).

In summary, some of the genes involved in regional pattern formation during flower development have been identified. In addition, some candidates for genes involved in earlier developmental processes, in global patterning of the inflorescence meristem, and later developmental processes, in the specification of cell fate, have also been identified. However, the identification of such genes is merely a beginning in the quest to understand the underlying molecular mechanisms by which the fate of cells in the developing flower are specified.

Evolutionary considerations

Apparently homologous mutations described in other species of dicots (including *Antirrhinum*, *Matthiola*, *Cheiranthus*, *Petunia*, and *Primula*; see Meyerowitz et al., 1989 for review) resemble the homeotic flower mutations of *Arabidopsis*. Thus, the mechanisms used in flower development of *Arabidopsis* are likely to be utilized in other flowering plants as well. Flowering plants appeared recently in the fossil record (120-140 million years ago; lower cretaceous) and rapidly became the dominant class of plants (80-90 million years ago; upper cretaceous; Friis and Crepet, 1987). Due to the sparse nature

of the early fossil record, it is difficult to ascertain the relative order of appearance of the floral organ types, but it is apparent that their evolution was rapid since the four basic types were present even in early flowers (120-140 million years ago). It has been suggested that the floral organs are modified leaves and that carpels evolved from sporophylls of primitive vascular plants (Stebbins, 1976). When all three types of flower homeotic genes are mutant in *Arabidopsis*, a flower in which all organs are leaves or carpelloid leaves, which could be interpreted as sporophylls, is produced. This suggests that the three classes of genes represented by the known *Arabidopsis* homeotic genes may be responsible for the majority of fundamental features associated with the flower.

Many of the cloned genes identified in floral development encode putative transcription factors. A large subset of these factors, at least *AGAMOUS* and *APETALA3* of *Arabidopsis* and *DEFICIENS*, *GLOBOSA*, and *SQUAMOSA* of *Antirrhinum* and possibly the other corresponding cognate homologues between the two species, share homology in their putative DNA-binding motif, called the MADS box (Sommer et al., 1990; Schwarz-Sommer et al., 1990; Yanofsky et al., 1990; Tom Jack, unpublished). Each of these members of the same gene family are involved in distinct developmental processes in pattern formation in the flower. In addition, several other genes (*AGL1*, *2*, *4*, *5*, and *6*) specifically expressed in flowers are also members of the same family of proteins (Ma et al., 1991). The other genes involved in pattern formation, *FLORICAULA* in *Antirrhinum* and the *Arabidopsis* homolog, *LEAFY*, that have been cloned are not members of the MADS box family of genes, although they do possess some features of transcription factors (Coen et al., 1990; Detlef Weigel, unpublished). Still, it is possible that much of the genetic machinery required for the development of flowers may have evolved from a small number of progenitor genes. Early duplication and divergence could produce a small number of gene families whose members direct the development of the basic floral structure. In contrast to the situation in *Drosophila*, in which a tandem array of duplicated genes is responsible for the specification of the linear segments of that organism,

the MADS box genes of *Arabidopsis*, which are at least in part responsible for specifying the identity of the radial whorls of the flower, are distributed throughout the genome.

One of the most conspicuous and facsinating aspects of biology is the extent of variation between even phylogenetically similar organsims. The enormous diversity of flower structure among the angiosperms indicates that, although a common basic developmental program mat be used, there is tremendous variation on the common theme. Uncovering the mechanisms of this variation lead us back to our original question of how do cells, and thus complex structures, become different.

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APPENDICES

The four appendices that follow represent work with which I was involved but did not fit into the main body of the thesis for various reasons. Appendix One is not directly related to the biological problem investigated in the thesis. Appendix Two provides a historical perspective on the study of flowers, but was not included in the main body of the thesis because I was not a major contributor to the work. Appendix Three provides a review on the knowledge of *Arabidopsis thaliana* as of late 1987. Although out of date, it does provide some useful background information on *Arabidopsis*. Appendix Four contains data collected over the years on segregation data in various crosses. It was not published when the genetics involved were published and is thus included here for completeness.

APPENDIX ONE

RESTRICTION FRAGMENT LENGTH POLYMORPHISM LINKAGE MAP FOR *ARABIDOPSIS THALIANA*

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Genetics

Restriction fragment length polymorphism linkage map for *Arabidopsis thaliana*

(genetic map/Brassicaceae)

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ABSTRACT **We have constructed a restriction fragment length polymorphism linkage map for the nuclear genome of the flowering plant *Arabidopsis thaliana*. The map, containing 90 randomly distributed molecular markers, is physically very dense; >50% of the genome is within 1.9 centimorgans, or ≈270 kilobase pairs, of the mapped DNA fragments. The map was based on the meiotic segregation of markers in two different crosses. The restriction fragment length polymorphism linkage groups were integrated with the five classically mapped linkage groups by virtue of mapped mutations included in these crosses. Markers consist of both cloned *Arabidopsis* genes and random low-copy-number genomic DNA clones that are able to detect polymorphisms with the restriction enzymes *Eco*RI, *Bgl* II, and/or *Xba* I. These cloned markers can serve as starting points for chromosome walking, allowing for the isolation of *Arabidopsis* genes of known map location. The restriction fragment length polymorphism map also can associate clones of unknown gene function with mutant phenotypes, and vice versa.**

Cloning of genes known only by mutant phenotype and genetic map position can be accomplished by successively isolating overlapping clones from a DNA library until the desired locus is reached (1, 2). This approach, known as "chromosome walking," is facilitated by having starting clones in close proximity to the endpoints of interest. To provide such starting points in the flowering plant *Arabidopsis thaliana*, we have constructed a genetic linkage map of the *Arabidopsis* nuclear genome consisting of 90 molecular markers that are distinguished on the basis of restriction fragment length polymorphisms (RFLPs). RFLPs are codominant, typically neutral, genetic markers. They are provided in abundance by differences between allelic DNA sequences that result from nucleotide substitution, rearrangement, insertion, or deletion. Accordingly, they can be used to construct detailed genetic linkage maps, follow inheritance of genetic diseases and other heritable traits, and examine variation between and within populations (3–5). This RFLP linkage map consequently enhances the utility of *Arabidopsis* for experiments in plant molecular genetics.

Previously recognized attributes of *Arabidopsis* include an extremely small genome, rapid generation time, fecundity, ease of growth, and an extensive background of classical genetic analysis (6). Chromosome walking in *Arabidopsis* should be facilitated by the small genome and virtual absence of dispersed repetitive DNA sequences [an average spacing of 125 kilobases (kb) between repeats as compared to 1.4 kb in tobacco and 0.3 kb in pea (6)]. The end of a chromosomal walk in *Arabidopsis* can be determined by complementation of the mutant phenotype through transformation (7). Complementation has been demonstrated in an *Arabidopsis* alcohol dehydrogenase null mutant transformed with the *Arabidopsis* alcohol dehydrogenase gene (C.C. and E.M.M., unpublished work). Numerous mutations affecting a range of developmental processes, various enzyme activities, and hormone synthesis and response have been isolated in *Arabidopsis*, and approximately 80 of these mutations have been ordered into a genetic linkage map (8, 9). The RFLP map is consistent with this map, and the two have been partly integrated.

Over half of the genome is within 1.9 centimorgans (cM) of the RFLP markers on the map. On average, 1 cM in the *Arabidopsis* genome represents 140 kb, since the genome consists of 501 cM and ≈70,000 kb (10).

MATERIALS AND METHODS

Cloned *Arabidopsis* Genes. Cloned *Arabidopsis* genes used as RFLP markers were kindly provided by the following individuals: chalcone synthase (λCHS2) by R. Feinbaum and F. Ausubel (Massachusetts General Hospital); nitrate reductase (λAt-24) (11) by N. Crawford (University of California, San Diego); actin (pAtc4) by R. Ferl (University of Florida); phytochrome (λPh.Ara.1) by R. Sharrock, C. Gatz, and P. Quail (U.S. Department of Agriculture, Albany, CA); and acetolactate synthase (pGH1) (12) by G. Haughn and C. Somerville (Michigan State University). The following cloned genes were from this laboratory: λfAt3012 (alcohol dehydrogenase) (13), sAt2105 (12S seed storage protein), and nAt1511 (small seed RNA-coding) (P. Pang, R. Pruitt, and E.M.M., unpublished results). The remaining mapped fragments were chosen from a genomic DNA library.

***Arabidopsis* Strains.** Ecotypes Niederzenz (Nd-0) (14), Landsberg (La-0) (15), and Columbia (Col-0) (14), and mutations, *an* (16), *gl-1* (16), *tz* (17), *er* (18), *ap-2* (19), and *clv-1* (7), have been described. To perform crosses, flowers of the female recipient were hand-emasculated and anthers from the donor were applied to the stigmatic surface of the recipient.

Library Screening. The *Arabidopsis* (Col-0) genomic λ library was screened by standard techniques (20).

Isolation of DNA. Rapid λ DNA preparations were as described in ref. 21, except that the clear lysate was incubated with 1 μl of DNase I (1 mg/ml) and 1 μl of RNase A (1 mg/ml) for 20 min at 37°C and then incubated with 5 μl of diethylpyrocarbonate for 5 min at room temperature, prior to treatment with $NaDodSO_4$.

Whole-plant DNA was extracted from 3- to 6-week-old plants by either of two methods described in refs. 22 and 23.

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Abbreviations: RFLP, restriction fragment length polymorphism; cM, centimorgan(s).

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The procedure in ref. 23 was modified as follows. Stirring of the lysis mixture and the first phenol/chloroform extraction were omitted. Instead, after adding the $NaDodSO_4$, the mixture was Vortex mixed and incubated at 65°C for 10 min. Two extractions were performed using a 2:1 phenol/chloro form molar ratio. The DNA was precipitated in 1 vol of 1 M KOAc/isopropanol at room temperature. The DNA was centrifuged ($2.55 \times 10^5 \times g$ for 20 hr) in a CsCl gradient and the CsCl was removed by ethanol precipitation of the DNA.

Genome Blot Hybridizations. Whole-plant DNA (0.5–2.0 μg) was digested with 10–20 units of restriction enzyme (*Eco*RI, *Xba* I, or *Bgl* II) and 1 mM spermidine (Sigma) for 2–4 hr and then subjected to gel electrophoresis in 0.8% agarose gels at 0.5–2 V/cm until the orange G (Sigma) loading dye migrated ≈13 cm. (The digested DNA was loaded into 5 × 1.5 × 5 mm wells.) Gels were blotted and the DNA was cross-linked to Hybond-N filters (Amersham) according to the manufacturer's instructions. ^{32}P-labeled DNA probes ($2–3 \times 10^8$ cpm/μg) were produced by nick-translation (24) of λ clone DNA (21). Filters were prehybridized for 1–10 hr and hybridized (10^6–10^7 cpm/ml) for 18–48 hr at 65°C in a solution containing 5× SSPE (900 mM NaCl/50 mM $NaH_2PO_4 \cdot H_2O$/40 mM NaOH/5 mM Na_2EDTA), 5× Denhardt's solution (1× Denhardt's solution = 0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin), 0.5% $NaDodSO_4$, and denatured salmon sperm DNA (20 μg/ml). Filters were washed at 65°C in 2× SSPE for 30 min, in 2× SSPE with 0.1% $NaDodSO_4$ for 30 min, and in 0.1× SSPE for 15 min. Preflashed Kodak XAR-5 film was exposed to filters for 1–7 days at −70°C with an intensifying screen. Filters were re-used up to 30 times after removing the previous probe by submerging filters in boiling 0.1% $NaDodSO_4$.

Most of the detected RFLPs were distinctly resolved codominant alleles. However, 12% of all RFLPs were the dominant/null allele type in which there are new (or absent) fragment(s) in one of the lines with no apparent alterations in any of the other fragment lengths. These polymorphisms probably did not result from sequence duplications since polymorphisms were not revealed by all three enzymes. Most likely, they are caused by the presence of a common restriction site within and a polymorphic restriction site outside of the probe sequence; if the polymorphic site is far enough from the probe sequence to produce a restriction fragment that is larger than the mean size of the isolated DNA, then it will not be detected. These RFLPs were not mapped or included in the calculations of RFLP frequencies given in the results.

Linkage Analysis. Multipoint linkage analysis was performed by using the MAPMAKER computer program (25, 26). Initially two-point linkage analysis was performed to determine the maximum likelihood recombination fraction and the associated lod score for each pair of loci. The lod score reflects the degree of support for linkage (27) and is defined as the $\log_{10}$ of the ratio of the probability that the data would arise if the loci were actually linked at a given recombination fraction divided by the probability that the data would arise if the loci were unlinked. Pairs of loci were considered linked if the lod score exceeded 3.0. (The traditional threshold for declaring linkage in human genetics is a lod score of 3.0. Since the *Arabidopsis* genome is roughly seven times smaller in genetic length than the human genome, the use of a lod score threshold of 3.0 in this study is quite conservative.) Once the linkage groups were assembled, three-point and subsequently *n*-point linkage analysis was performed on each linkage group.

RESULTS

Construction of the *Arabidopsis* RFLP map involved the following three steps: (*i*) collecting and identifying potential RFLP markers, (*ii*) performing the genetic crosses, and (*iii*) determining the linear order of RFLP loci and the map distances between them by examining segregation of the RFLPs.

Obtaining RFLP Markers. Cloned inserts for the first step should be large and have a low copy number in the genome to increase the probability of detecting polymorphisms while at the same time avoiding RFLPs at multiple loci. Both these criteria are met by large genomic clones of *Arabidopsis* due to the low amount of dispersed repetitive sequences. Our primary source of RFLP clone candidates was a collection of random low-copy-number clones obtained by screening (20) an *Arabidopsis* total genome λ library (22) (estimated average insert size of 12.5 kb). The probe was total *Arabidopsis* DNA, and an internal λ vector DNA sequence (to detect nonrecombinant phage), that had been ^{32}P-labeled by nick-translation (24). In agreement with the proportion predicted by genome studies (10, 22), 60% of the screened recombinant clones gave either a weak or undetectable signal, presumably because they represent sequences of low copy number in the nuclear genome. These clones were isolated and rescreened by either plaque screening or clone DNA blotting (20), resulting in the isolation of 280 low-copy-number clones. We also used 18 single-copy *Arabidopsis* clones (isolated from the same DNA library (22)), and 8 cloned *Arabidopsis* genes.

To screen plant DNA for the presence of RFLPs, the clones were used as ^{32}P-labeled probes in hybridizations to gel blots containing genomic DNA of three *Arabidopsis* strains, digested separately with one of three restriction enzymes (Fig. 1). The following strains were used: (*i*) Niederzenz (Nd-0); (*ii*) a triply marked line (which we designate as C) in a background that is largely Columbia (Col-0) carrying the recessive visible mutations angustifolia (*an*), glabra-1 (*gl-1*), and thiazole requiring (*tz*); and (*iii*) Landsberg (La-0) carrying the recessive visible mutation erecta (*er*). [Nearly all the available and mapped mutations in *Arabidopsis* have been induced in Landsberg erecta (8).] By using 202 clones, we screened all three lines for RFLPs that

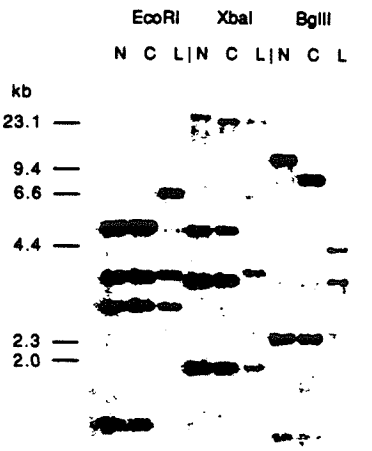

FIG. 1. Autoradiogram of a typical genomic blot to screen for RFLPs between different parental lines. The figure shows several RFLPs detected by clone 305 hybridized to Nd-0 (N), C (C), and La-0 *er* (L) DNA digested with *Eco*RI, *Xba* I, or *Bgl* II.

are revealed by at least one of the three enzymes *Eco*RI, *Xba* I, and *Bgl* II. Nd-0 and C were screened with 23 additional clones, and C and La-0 *er* were screened with 3 additional clones. The overall frequency of clones that detected RFLPs with at least one of the three enzymes for at least two pairs of lines was 54%; the frequency was 37% between Nd-0 and C, 46% between Nd-0 and La-0 *er*, and 39% between C and La-0 *er*. The frequency of the clones detecting fragments of different lengths in all three strains was 15%. The three restriction enzymes were found to be equally useful for RFLP detection.

A survey of all RFLPs (including dominant/null types) indicated that Nd-0 and La-0 *er* differ in ≈1.4% of the nucleotides in their low-copy-number genomic DNA, C and Nd-0 in ≈1.3% in their low-copy-number genomic DNA, and C and La-0 *er* in ≈1.1% of their low-copy-number genomic DNA. In this calculation we assumed that (*i*) there is one nucleotide substitution per RFLP, (*ii*) there are four restriction sites detected by a probe per enzyme, and (*iii*) all sites are nonoverlapping. The above results suggest that a cross between any two of the three strains is suitable for RFLP mapping.

Genetic Crosses. Our RFLP map is based on two crosses, each including a set of visible mutations for the purpose of positioning RFLP linkage groups relative to the five linkage groups established by mapping morphological and biochemical mutations. One of the two crosses was between Nd-0 and the triple mutant line C with mutations on chromosomes 1, 3, and 5. The other cross was between Nd-0 and La-0 with the recessive visible mutations clavata-1 (*clv-1*), *er*, and apetala-2 (*ap-2*) on chromosomes 1, 2, and 4, respectively. For each cross, the resulting F_1 plants were allowed to self-pollinate to produce F_2 individuals. F_2 plants were allowed to self-pollinate to each produce a pool of F_3 plants. Although segregation of RFLPs is present in F_2 individuals, the F_3 plants enabled us to score the genotypes of F_2 plants for the recessive visible markers. DNA was prepared from pools of 15 or more F_3 plants per F_2 individual, providing a greater amount of tissue, and thus of DNA, than would have been provided by a single F_2 plant.

Segregation of RFLPs. To examine the segregation of RFLPs, DNA probes were hybridized to genome blots containing digested F_3 DNAs of both crosses. An example for two probes is shown in Fig. 2. For each of the enzymes *Eco*RI, *Xba* I, and *Bgl* II, a set of genome blots was prepared

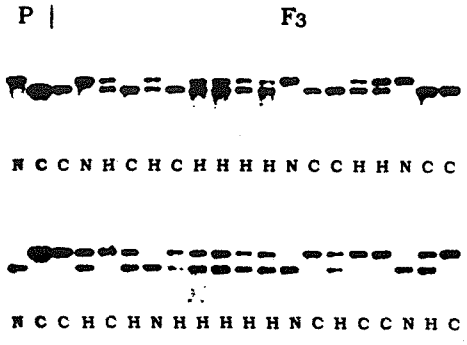

FIG. 2. Segregation of RFLPs in pooled F_3 progeny is shown in autoradiograms of the same genome blot hybridized sequentially with two unlinked RFLP probes. The blot contains genomic DNA samples digested with *Eco*RI. (*A*) Blot was probed with clone 322. (*B*) Blot was probed with clone 214. The first and second lanes contain DNA of the parental lines Nd-0 (N) and C (C), respectively. The 18 lanes to the right contain DNA of pooled F_3 progeny derived from the cross between Nd-0 and C, scored as either N, C, or H (heterozygous).

by using the F_3 DNAs of each cross. RFLP probes were hybridized sequentially to filters of the appropriate digest and cross. Each F_3 pool was scored with respect to the parental DNA restriction patterns as being homozygous for one of the parental types, heterozygous, or unscorable for technical reasons. By using the Nd-0 × C cross alone, for which 106 F_3 pools were scored, 41 RFLPs were mapped. By using the Nd-0 × La-0 cross alone, for which 119 F_3 pools were scored, 25 RFLPs were mapped. To align linkage groups of clones mapped in the separate crosses, 24 additional markers were mapped in both crosses. The resolution afforded by 225 F_3 pools is 0.22 cM—i.e., one recombinant divided by 450 chromatids, which represents on average 30 kb in the *Arabidopsis* genome.

All but two of the clones appeared to detect single loci. Clone nAt1511 (which contains a gene encoding an abundant seed-specific mRNA) hybridized to two pairs of segregating RFLP alleles; one RFLP was within the cloned copy and the other was within an uncloned homologous sequence whose existence was indicated by a comparison between genome blots and the restriction map of the clone. We mapped both RFLPs and found them to be closely linked. Probe 281 hybridized to two polymorphic fragments that mapped to different chromosomes. This probe may hybridize to nonadjacent members of a gene family or may contain two unrelated sequences that were ligated together during the library construction. When such markers are used to initiate a chromosome walk, a clone specific to the RFLP locus of interest must be obtained.

Linkage Analysis. The genetic map was constructed by using the MAPMAKER computer program (25, 26) to analyze the combined data from the two crosses. Two-point linkage analysis was first performed to determine the maximum likelihood recombination fraction and the associated lod score (27) for each of the 4560 pairs of the 96 loci. Pairs of loci were considered linked when the lod score exceeded 3.0. The 96 loci fell into five linkage groups that were assigned to the five chromosomes by virtue of the visible markers with previously assigned genetic positions.

Three-point linkage analysis was next used to narrow down possible genetic orders for the loci (a–b–c, a–c–b, b–a–c). For each order, we computed the likelihood that the data would have arisen given the maximum likelihood three-point map for that order. A particular three-point order was ruled out if some alternative order for the loci was at least 100,000 times more likely to have given rise to the data. For each linkage group, we determined all genetic orders that contained no suborders that had been ruled out by the three-point analysis. Typically, some 20–50 genetic orders for the linkage group met this criterion.

Finally, full *n*-point linkage analysis was performed for each remaining genetic order to compute the most likely recombination fractions and the chance that the map would have given rise to the data. Genetic orders were discarded that were at least 1000 times less likely to give rise to the data.

A single genetic order emerged for each chromosome, which was unique up to the possible uncertainty in the order of certain pairs of loci separated by <4 cM. In the final map, shown in Fig. 3, map distances have been corrected for the discrepancy between actual crossovers and the observed recombination frequencies by using the Kosambi (28) function. Any order that is supported by odds of <1000:1 is indicated. As a confirmation, the entire process was repeated with just the data for the Nd-0 × C cross alone and the Nd-0 × La-0 cross alone; the resulting genetic orders and maps were consistent with the map shown in Fig. 3.

The orientation of RFLP chromosomes 1 and 5 with respect to the standard map has been determined by using two visible markers on each chromosome: *an* and *clv-1* on chromosome 1, and a recessive visible mutation pistillata

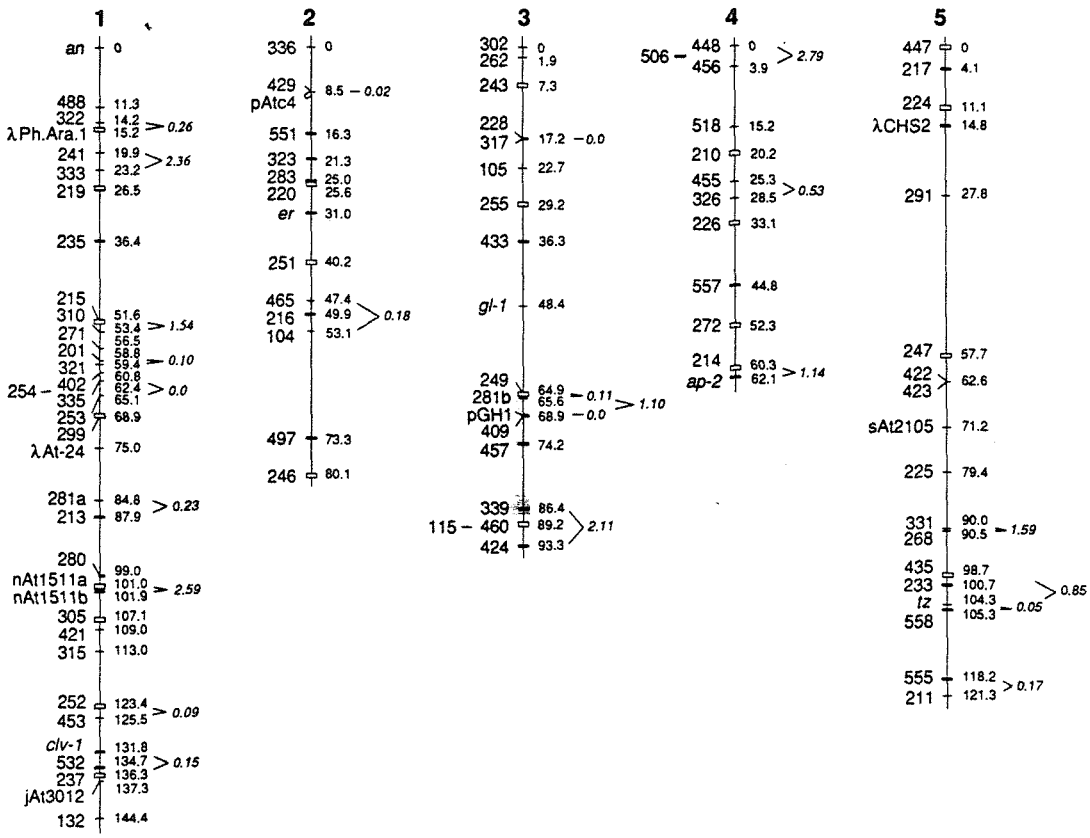

FIG. 3. An RFLP linkage map of the five chromosomes of the *Arabidopsis thaliana* genome. Map distances are shown in centimorgans. (We arbitrarily assigned a position of zero to the top-most marker on each chromosome.) Markers are designated by a clone number for the random low-copy-number clones and by a clone name for the known genes. The letter a or b after the marker name indicates that the clone detects more than one map locus. Markers scored in both crosses are indicated by an open box. Markers scored only in the cross between Nd-0 and La-0 are indicated by a solid box. The remaining markers were scored only in the cross between Nd-0 and C. Markers, placed in an order that is <1000 times more likely to have given rise to the data than any alternative order, are bracketed to the right of the map distances; the numbers by the brackets are the $\log_{10}$ of likelihood ratio of the order shown to the next most likely alternative order. In three separate cases (115, 254, and 506), the order of markers could not be resolved—i.e., the likelihood for any of the possible intervals was virtually the same. The approximate positions of these markers are indicated to the left of the other markers.

from a third cross (J.L.B. and E.M.M., unpublished results) and *tz* on chromosome 5. Chromosomes 3 and 4 are shown in the orientation most likely to agree with the standard map based on the map position of an acetolactate synthase (ALS) mutation, chlorsulfuron resistance, on chromosome 3 (ref. 29; G. W. Haughn and C. Somerville, personal communication) for which we have mapped the ALS gene clone and on the distal position of *ap-2* on chromosome 4. The orientation of chromosome 2 relative to the standard map is not known.

DISCUSSION

We have constructed an RFLP linkage map of 90 molecular markers that can be integrated with the standard linkage map of approximately 80 mutation markers. The RFLP map contains linkage groups of approximately 144, 80, 93, 62, and 121 cM for a total of 501 cM, which corresponds well with the standard genetic map showing linkage groups of 126, 51, 91, 69, and 100 cM for a total of 437 cM (9). The positions of the visible markers that are common to both maps are also in essential agreement. On the standard mutation map, *an* and *clv-1* are on chromosome 1 at positions 0 and 114.6 cM, respectively, *er* is on chromosome 2 at 15.9 cM, *gl-1* is on chromosome 3 at 40.9 cM, *ap-2* is on chromosome 4 at 58.6 cM, and *tz* is on chromosome 5 at 82.4 cM. The RFLP map is also consistent with the cytological karyotype (30, 31). The positions of centromeres in the RFLP map might be determined by the use of existing telotrisomic lines (8). One reason this is of interest is that the C-banded heterochromatin associated with the centromeres is likely to present a barrier to chromosome walks across centromeres.

RFLP mapping of cloned genes (which have been obtained by other means) may associate the genes with mapped loci that are responsible for mutant phenotypes. For example, the RFLP map position of the chalcone synthase gene clone suggests a correlation between this gene and the pigment mutation transparent testa-4 (9); and the map position of the nitrate reductase gene clone suggests a correlation between this gene and the mutation chlorate resistance-3 (9) in which nitrate reductase activity is reduced. Thus the combined maps have the potential to provide information on clones having unknown gene function or to associate mutant phenotypes with gene products.

The RFLP map should facilitate cloning of genes known only by mutant phenotype by providing starting points for

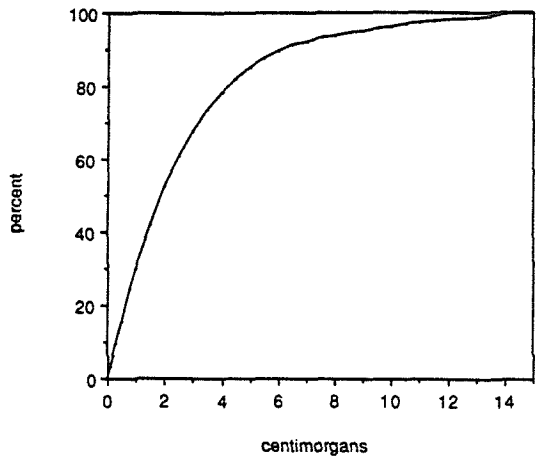

FIG. 4. A graph depicting the completeness of the RFLP linkage map. For given genetic distances (N) in centimorgans, the percent of the genome within N cM of the RFLP markers was calculated. The results of these calculations are depicted for all values of N from 0 to 15 cM. To avoid assuming lengths for the ends of the chromosomes, the genome is considered here to be the sum of the RFLP chromosome lengths, where the two ends of each chromosome are defined by the outermost RFLP markers. All (100%) of the genome is within 15 cM of one or more of the RFLP markers; >50% is within 1.9 cM. On average, 1 cM is equal to ≈140 kilobase pairs.

chromosome walking. The labor involved in chromosome walking depends upon the physical distance between the starting clone and the desired gene. Although an actual correlation of physical distance and genetic distance has yet to be established for specific regions of the genome, a general measure is 140 kb per cM (the genome size in kilobases divided by the RFLP map genome size in centimorgans). In terms of averages, the mean distance between RFLP markers is 780 kb, and the mean walking distance to reach an arbitrary gene, therefore, is 195 kb. A more descriptive measure of the map's usefulness than the mean spacing between markers is to examine the percent of the genome (the genome being defined by the RFLP map) within a given distance from any RFLP marker (Fig. 4). This calculation shows that more than half of the genome lies within only 1.9 cM (≈270 kb) of an RFLP marker, and if one is willing to walk about three times that distance, then one has access to 90% of the genome. The required walking distances can be reduced by increasing the density of RFLP markers on the map. The distances between consecutive loci closely fit the negative exponential distribution that would be expected from randomly picking points along linear chromosomes, which indicates that current gaps in the map are likely to be filled.

We are grateful to the following people for their valuable contributions to this work: Robert Pruitt for constructing the triply marked line and performing the cross with Nd-0; Mark Daly for excellent assistance with the data analysis; Stanley Tamaki, Bonnie Pruitt, and Patty Pang for assistance with plant DNA preparations; Michael Ahn for assistance with phage DNA preparations; Sherry Kempin for assistance with gel blots and phage DNA preparations; and members of the laboratory of E.M.M. for comments on the manuscript. This work was supported by a National Science Foundation grant (DCB-8703439) to E.M.M. J.L.B. was supported, and C.C. was partially supported, by a National Institutes of Health training grant (5T32-GM07616). E.S.L. was partially supported by a National Science Foundation grant (DCB-8611317) and by a System Development Foundation grant (G612).

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APPENDIX TWO

ABNORMAL FLOWERS AND PATTERN FORMATION IN FLORAL DEVELOPMENT

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Abnormal flowers and pattern formation in floral development

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"From our acquaintance with this abnormal metamorphosis, we are enabled to unveil the secrets that normal metamorphosis conceals from us, and to see distinctly what, from the regular course of development, we can only infer."

– J. W. von Goethe (1790)

Key words: pattern formation, homeosis, flower development, plant genetics, evolution.

Introduction

The development of flowers is a mystery. Each flower starts as a small clump of undifferentiated cells, but develops into a complex structure in which different organs occupy precisely defined positions. In addition, each organ has its own characteristic cell types, organization and function. Since there is no cell migration in higher plant development and since flowers can develop normally without positional information being specified maternally, each cell in the developing floral primordium must somehow learn its position relative to other cells, and differentiate accordingly. Mutant plants in which cells do this improperly have recently become a focus of attention. In this review, we will briefly describe past work in morphological analysis of abnormal flowers and in the inheritance of floral abnormalities. While many instances of inherited alterations in flower development have been recognized and studied to answer genetic or evolutionary questions, there has, surprisingly, been little use of such material in experiments directed to understanding flower development. Recently such work has started, though, with several plant species, and in several laboratories. We will examine the past work and will describe recent work focussed on understanding the molecular mechanisms by which cells in developing flowers recognize their positions in space and in developmental time, and thus differentiate into appropriate organs in appropriate places.

Monstrous flowers as curiosities

Monstrous flowers have been recognized as curiosities by botanists for more than two thousand years: Theophrastus mentions double roses (flowers containing many more than the normal number of petals) in his *Enquiry into Plants*, written before 286 BC; double roses were also described by Pliny in the first century. Double peonies were known and selected by around 750 AD in China, and two Thang Dynasty authors described these flowers in the ninth century (Needham, 1986). Double flowers were described by the herbalists of the Renaissance, as well: Dodoens published such a description in 1568, and Gerard (1597) has illustrations of many double flowers beside their wild-type counterparts. Double flowers are thus the earliest recognized examples of floral abnormalities and of floral mutants. They have been of interest from the origins of botany to the present: Masters (1869) and Worsdell (1915–6) describe many of them, as do Reynolds & Tampion (1983) in a recent book that classifies the different developmental origins of the extra petals in different species with double flowers. Double flowers are at least as common in present-day gardens as they were in those of the herbalists: hybrid tea roses, carnations, double camellias, columbines and stocks, and so on, are all familiar examples (Reynolds & Tampion, 1983).

There are many additional, characteristic types of floral abnormalities, with homologous examples in many different species. One type of abnormal flower of considerable interest to developmental biologists is that said by early plant teratologists to exhibit 'metamorphosis' or 'metamorphy', the appearance of a normal organ in the place where a homologous organ of a different type is typically found (Figs 1 and 2). The use of metamorphosis to refer to the replacement of one organ with a homologue was used by Goethe (1790), who in an essay on flower development called the appearance of one type of floral organ in a site normally occupied by an organ of a different type 'abnormal

metamorphosis'. Goethe emphasized the homology of the organs in successive whorls of flowers, homology between organs having been suggested to him by cases of abnormal metamorphosis. Perhaps the earliest compendium of such abnormalities is that of Moquin-Tandon (1841), which summarizes a large number of reports of unusual flower forms published from the seventeenth to the early nineteenth centuries. The classic English work on floral abnormalities is by Masters (1869). Both Moquin-Tandon and Masters categorized the abnormalities according to the nature of the departure that they showed from the normal flower of the same species; neither considered seriously the distinction between inherited abnormalities and freaks. Moquin-Tandon (1841) traces the concept of metamorphosis to Jung in the seventeenth century; a critical review of the various definitions and interpretations of this concept is found in Sachs (1906).

In his 1894 classification of animal abnormalities, Bateson refers extensively to Masters' earlier work, and replaces Masters' 'metamorphy' with another word, 'homoeosis'. Bateson defined homoeosis as 'the assumption by one member of a Meristic series, of the form or character proper to other members of the series...' and goes on to state "In the case of plants such Variation is very common and is one of the most familiar forms of abnormality." In flowers the meristic, or repetitious, series refers to the successive whorls of different organs that constitute the flower; "formation of sepaloid petals", as shown in the mutant flowers depicted in Fig. 1C,D and F and Fig. 2E and F, is one of Bateson's examples of homeotic floral variation. Sattler (1988) has recently reviewed some of the literature on homeosis in plants, pointing out that some authors after Bateson have redefined the term to include phenomena not recognized as homeosis or metamorphy by the earlier authors, such as appearance of similar organs in different places in plants of different species.

There have been other encyclopedic catalogues of floral abnormalities since Moquin-Tandon and Masters, including those by Penzig (1890–4), arranged taxonomically, and Worsdell (1915–6), arranged by type of abnormality. Remarkably, not even the latter, compiled 15 years after the rediscovery of Mendel's experiments, differentiates between regularly inherited abnormalities and individual aberrant forms resulting from environmental treatments, insect infestations, and chance. A more recent review (Meyer, 1966) summarizes many of the findings of the earlier authors, and the categories of homeotic conversions of floral organs that have been observed in a wide variety of plant species.

Evolutionary interpretation

One early consideration of abnormal flowers as something other than curiosities was by Linnaeus, who in 1744 described a mutant flower phenotype in toad flax (*Linaria vulgaris*), and briefly recognized that the occurrence of mutations casts doubt on the then-current view (earlier propounded by Linnaeus himself) of the immutable fixity of species (Moquin-Tandon, 1841; DeVries, 1903; Gustafsson, 1979). The type of abnormality recognized and named by Linnaeus is peloria, in which a plant of a species with flowers that are ordinarily bilaterally symmetric (such as snapdragon, *Antirrhinum*) has radially symmetric flowers. Peloric flowers are known in many families of flowering plants (DeVries, 1906). The *Antirrhinum cycloidea* mutation shown in Fig. 1B is an example.

In the nineteenth and present centuries, the nature of floral monstrosities has been used to make inferences about the evolutionary homologies of different plant parts, and, by arbitrarily declaring certain mutations to be atavistic, about the structure of ancestral flowers. Darwin employed this type of reasoning cautiously (Darwin, 1876); his successors were not always cautious. For example, many instances of speculative use of teratological data are found in Worsdell (1915–6), a plant teratology work that is a successor to Masters' compendium, but which adds to the rigorous descriptions of Masters an imaginative evolutionary interpretation of many of the floral abnormalities. "In very many cases the so-called 'freaks' and 'monstrosities' represent reversions or harkings-back, in one form or another, to an ancestral condition..." The uncritical acceptance of

Fig. 1. Homeotic flower mutants of various plant species. (A–D) Phenotypes of flowers of the snapdragon, *Antirrhinum majus* (see Stubbe, 1966). (A) Wild-type morphology. The plant is homozygous for the anthocyanin mutation *nivea* which arose by insertion of the transposable element *Tam1* into the chalcone synthase gene. Pigment is absent in the corolla except where a reversion has occurred following excision of the transposable element during growth. Transposons Tam1 and Tam3 will be useful in tagging homeotic mutations in *Antirrhinum* (Coen & Carpenter, 1986). (B) The *cycloidea*^{radialis} phenotype in which the five petals of the corolla tube all resemble the central petal of the lower lip. The corolla is now radially symmetrical whereas in the wild type it is zygomorphic. (C) The *deficiens*^{chlorantha} phenotype. In this mutant line the petals are reduced in size and partially green. (D) A more extreme allele, *deficiens*^{globifera}. In this case the sepals grow in place of petals. Also in place of the stamens, a tube is formed around the gynoecium with a stigmatic surface at the top and a ring of ovaries at the base. (*Antirrhinum* plants photographed at the Max-Planck-Institut für Züchstungforschung, Köln, with thanks to Ekkehard Lönnig.) (E) Flowers of the green rose, *Rosa* cv. 'Viridiflora', in which all flower organs resemble leaves. (Photographed at the Huntington Botanical Gardens, San Marino; California) (F) Phenotype of the green petals mutation of *Petunia hybrida* (see de Vlaming *et al.* 1984). (Photographed at the Department of Genetics, Vrije Universiteit, Amsterdam.) (G) The *heptandra* mutant phenotype of the common foxglove, *Digitalis purpurea* (see Saunders, 1911; Shull, 1912). The wild-type flower has a tubular corolla with five lobes, two in the upper lip and three in the lower lip. Four stamens are attached inside the corolla tube. In the *heptandra* mutant the three petals of the lower lip are often replaced by three stamens. The resulting flowers have seven stamens. (Photo: Keith Roberts)

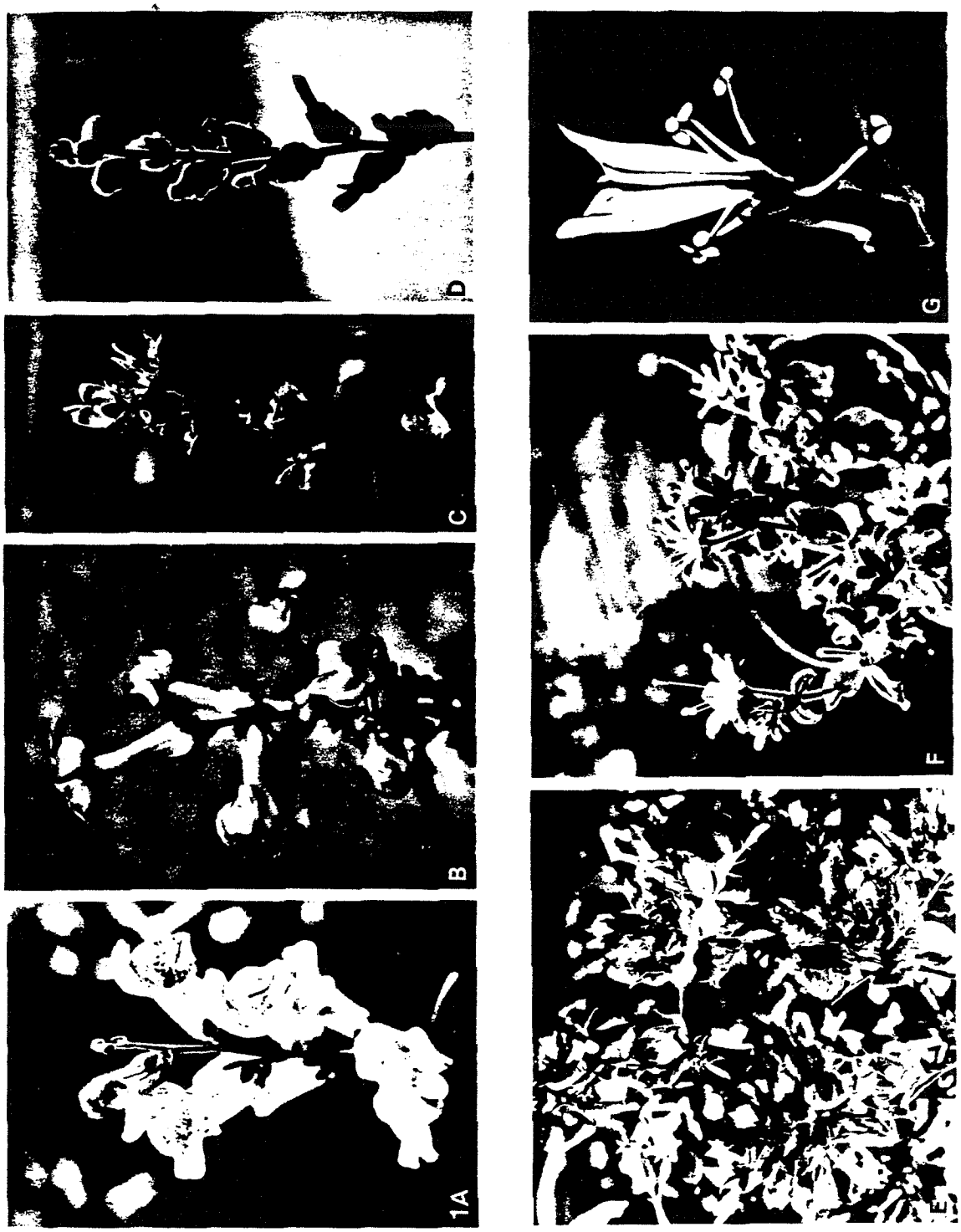
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Fig. 2. Wild-type and homeotic mutants of *Arabidopsis thaliana* (see Bowman *et al.* 1989). In each case the phenotype is illustrated in the left panel while the right panel shows a floral diagram typifying the first flowers on plants raised at 25°C. (A) Wild-type inflorescence of the Landsberg *erecta* laboratory strain. Each flower has four sepals, four petals, six stamens (two lateral and four medial) and a bicarpellate ovary. (B) Inflorescence of an *agamous* plant (mutant S310) in which the stamens are replaced by petals and a new flower arises in place of the gynoecium in a serially repeated pattern. (C) Vertical view of an *apetala2-1* mutant plant. The sepals are leaflike and the petals have some of the properties of stamens. (D) The inflorescence of an extreme *apetala2* mutant, ap2-2. Here the two medial sepals are carpelloid and all other organs except the gynoecium are usually absent. (E) An *apetala3* mutant flower in which the petals are replaced by smaller, sepallike organs and free carpels arise in place of stamens. (F) The *pistillata* phenotype (mutant S244). In this mutation, reduced sepals arise where petals normally occur and stamens are absent.

this point of view was in low repute well before Worsdell published it, as evidenced by Goebel's (1900) statement "We can only consider it as an error to look upon these kinds of malformations as *reversions*..." Nonetheless, there are many subsequent examples.

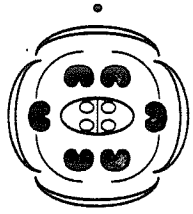
For instance, numerous uses of this sort of speculation are found in the long debate over the constitution of the pistils in mustard flowers. These pistils contain two chambers, separated by a septum. Since at least 1828 (Lindley, 1828) there has been a debate on the nature of the pistils: some believe them to be made of two carpels (the supposed evolutionary unit structure in the formation of gynoecia, recognizable in some but not all flower types), others believe them to be made of four (or even six). To pick a single example of the unacceptable use of evolutionary interpretation of abnormal flowers in this debate, we cite Saunders' (1923) work on abnormal fruits in the mustard *Matthiola*, which relates the structure of abnormal fruits with four rather than two valves. By assuming that this represents an ancestral form ("...there can be no doubt that in all these cases we are witnessing the reappearance of an ancestral character..."), she claims it as support for a multicarpellate ancestor, and thus as support for the plausibility of the four-carpel model. Arber (1931*a*,*b*) specifically criticizes this work, giving a detailed refutation of the type of arguments used by Saunders and calling specious all claims that morphological abnormalities can serve as geneological data. Despite this, the debate continued for many years, with at least one author (Puri, 1941; 1945) arguing both sides. The assumption that abnormal flowers show ancestral forms is a continuing tradition (Guédès, 1966), and a continuing source of controversy, as detailed by Carlquist (1969), who briefly reviews some of the arguments in which teratological speculation has played a role, stating as a general conclusion that "Data from teratology is not useful in the study of the evolution of flowers."

Genetics

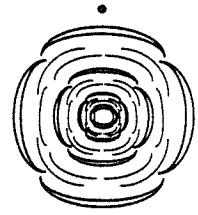
In contrast to the speculative use of floral abnormalities as a clue to ancestral characteristics, the use of genetic analysis to study both patterns of inheritance and the processes by which morphological evolution occurs is a viable and continuing tradition. Even before the publication of Mendel's work in 1866, it was known that pelorism in *Linaria* flowers is recessive to zygomorphy (Naudin, 1865); before the rediscovery of Mendel in 1900, Godron (1874) and Darwin (1876) studied the inheritance of floral monstrosities. Mutations that cause abnormal flowers were among those whose mode of inheritance was studied after the rediscovery of Mendel's paper as well. These striking and convenient characters were employed in crosses as tests of Mendelian patterns of segregation (Bateson *et al.* 1905; Baur, 1910; Keeble *et al.* 1910; Saunders, 1910; Bateson, 1913; White, 1914). Comprehensive bibliographies of these studies were published by Matsuura (1933) and Warner *et al.* (1934). A similar type of work, though for a different purpose, involved crosses between members of different species, as a way of using Mendelian ratios to infer the number and nature of the individual genetic differences between species with differing flower morphologies. One large research program of this type was started by Shull by 1906 (Shull, 1907), involving crosses of different species of Shepherd's Purse (*Capsella*). This work was summarized by Shull in 1929. He found that some strikingly different forms (such as a type with a 4-chambered ovary and the usual two-chambered type) differed in only a single gene that affected the character under study. A well-known example of work in this tradition is that cited by Beadle (1939) showing a close genetic relation between teosinte and maize, despite the different morphology of their female inflorescences and seeds, thus providing evidence that teosinte could be the direct ancestor of maize. Gottlieb (1984) has recently reviewed the many similar studies that were made in the first half of this century, and encourages a renaissance of this sort of work, since we still do not know how typical it is that marked morphological differences between species result from polymorphism at one or a few genes. All of these genetic studies do, however, show that changes in flower structure and organ differentiation can result from single-gene alterations, and that a wealth of material from old experiments is available for present analysis.

Floral mutants in the study of development

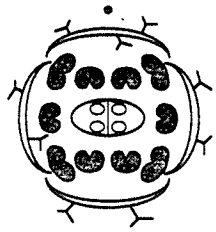
Despite the long history of the knowledge of heritable abnormalities of flowers, and the long history of detailed studies of the development of normal flowers (Payer, 1857; Sattler, 1973), the study of abnormalities and the study of development have been separate traditions: there are only a handful of experiments in which mutant flowers have been used as a means of understanding the mechanisms by which flowers develop. Indeed, some recent hypotheses for the mechan-



wild type

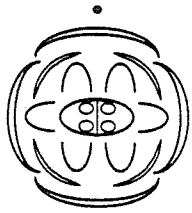


agamous

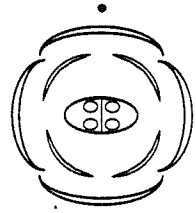


apetala2-1

apetala2-2



apetala3



pistillata

isms by which floral organs appear in appropriate patterns can be seen to be incorrect from teratological evidence. Heslop-Harrison (1963) proposed a relay model for flower development, in which the organs of each whorl differentiate as a result of activation of an organ-specific gene complex. At the same time, this gene complex produces an organ-specific signal, which activates a second gene complex in the next inner whorl; this new gene complex then directs differentiation of the organs of the inner whorl, and produces another specific signal to activate differentiation in the next inner whorl. Green (1988) has proposed a related model, in which the shape changes in the floral primordium created by the development of individual floral organs in an outer whorl or whorls creates localized regions of stress closer to the center of the primordium. The position of these regions specifies the position of the inner organs, and the exact nature of the stress pattern, which depends on the type of organ primordium present in the outer whorls, specifies the type of inner organ that develops. In each of these models, the nature of the organs found in inner floral whorls is determined by the nature of the organs that are differentiating in the adjacent outer whorl or whorls. Thus, if mutant flowers were found in which stamens regularly develop in the positions normally occupied by petals, for example, both models would be shown to be incorrect. Such flowers have been recognized since at least 1821, and mutant plants that regularly produce such flowers have been described many times (Masters, 1869; Worsdell, 1915–6; Dahlgren, 1919; Shull, 1929; De Vlaming *et al.* 1984; Pruitt *et al.* 1987; Komaki *et al.* 1988; Bowman *et al.* 1989). In fact, it is clear from the literature on abnormal flowers that almost any type or number of organs can occupy any whorl, regardless of the character or number of organs in the adjacent whorls (Masters, 1869; Worsdell, 1915–6; Meyer, 1966; Lyndon, 1979*a*,*b*; Kinet *et al.* 1985; Bowman *et al.* 1989).

There were earlier authors who understood the significance of floral abnormalities, but lacked the concepts and methods necessary to use such abnormalities for studying the mechanisms of development. Masters (1869) states quite clearly "The term metamorphosis [by which he meant what we now call homeosis], then, really implies an alteration in the organizing force, taking effect at a very early period in the life of the flower, at or before the period when the primitive aggregation of cells, of which it is at that time composed, becomes separated or 'differentiated' into the several parts of the flower. In other words, the 'development' of the flower pursues a different course from what is usual." This was echoed by Goebel (1900), who attempted to separate evolutionary and developmental interpretations of abnormal flowers, stating "Our idea of metamorphosis is then primarily an ontogenetic one..."

In fact, Goethe (1790) proposed a theory for the differentiation of different organs in different floral whorls, based in part upon the frequent observations of petals differentiating in positions appropriate for stamens or pistils. His theory was that the sap, as it rises higher in the plant, becomes more refined, and thus induces successive, different floral organs: "...the foliar organs are refined, the operation of the unadulterated saps becomes purer and stronger, and the transformation of the parts is rendered possible". He considered the abnormal flowers to be "retrograde", instances of the failure of this mechanism.

More recent plant developmental biologists have also recognized that inherited floral abnormalities may provide important keys to the nature of the gene products that regulate development in flowers (Wardlaw, 1965). Despite this recognition, the use of flower mutations in the study of development has not been a major area in plant developmental biology.

There do exist examples of experimental use of mutant flowers to understand floral development. Brieger (1935) studied the development of several mutant forms of *Primula sinensis* and *P. kewensis*. One mutation studied was *pistilloid* in *P. sinensis*, an X-ray-induced recessive mutation showing a carpelloid development of the primordia that would usually give rise to stamens. Brieger reported that wild-type flowers show a single ring-shaped primordium for both petals and stamens; this later divides into an outer zone that forms petal primordia, and an inner zone that forms the primordial stamens. In the mutant, the two ring zones are separate from the beginning, thus showing that the mutation acts long before the differentiation of any floral organs. Brieger proposes a model for organ specification in which organ-type-specifying 'hormones' reside in concentric rings in the flower primordium, and any primordium arising in the zone of a particular hormone differentiates into the organ specified by that hormone. The initial displacement of the stamen primordium toward the center of the flower thus explains its carpelloid development in the mutant. While such a model is unproven, it is noteworthy both because it is similar to a more recently published model for flower development (Holder, 1979), and because it avoids the problems of the models described earlier: it allows for cellular communication in establishment of what in animal embryology would be called embryonic fields, but does not require that differentiating organs communicate.

A more recent study of the development of double petunias (Natarella & Sink, 1971) leads to similar conclusions. In the development of these flowers, numerous extra primordia occur centripetal to the calyx. They differentiate into petals if they arise near the sepals, into stamens if they appear near the center of the flower, and into organs intermediate between petals and stamens if they arise between these positions. Again, a concentric zone model is suggested. That concentric fields of determined cells are not the sole determinant of fate choices in developing flowers is shown by the mutant *blind* of *Petunia hybrida*, which results in a corolla whose much-reduced limb bears supernumerary anthers. This mutation does not affect the time of initiation and location of petal primordia, but rather later stages of differentiation, with the upper

limb developing as anthers and the lower tube differentiating normally, as petals (Vallade *et al.* 1987).

A different sort of flower mutation has led Stebbins and his co-workers to emphasize a different set of processes in flower development. They have studied a number of morphological variants in barley; the best-studied floral one is *hooded* (Stebbins & Yagil, 1966). Flowers from plants bearing this single-gene semidominant mutation show a variable phenotype that in its most extreme form includes two extra rudimentary florets appearing at the apex of the lemma of the basal (normal) flower, with one growing from the other. The first of the extra flowers is upside-down relative both to the basal flower and to second (apical) extra floret. The earliest differences seen in the development of wild-type (awned) and hooded flowers are well after the differentiation of organ primordia has started; patterns of cell division and cell elongation vary. This fact led Stebbins (1986) to propose that plant mutations with morphological pattern phenotypes are generally due to disturbances in cell division patterns, and are mutations in genes whose primary products are either cytoskeletal proteins, or proteins associated with intercellular membranes.

The hooded mutation of barley might be interpreted as a disturbance in the relative timing of developmental events, with a prolonged period of cell division occurring prior to the start of organ differentiation. Another example of the effect of changes in developmental timing on flower development is found in maize (Poethig, 1988). The phenotype of each of three non-allelic, semidominant mutations, *Teopod1, Teopod2,* and *Teopod3* includes certain floral organs, particularly in the tassel, becoming larger and more leaflike than in wild type. An analysis of these phenotypes indicates that each mutation prolongs vegetative development, such that the succeeding reproductive developmental program is disturbed. It seems that the floral effects are secondary consequences of an earlier primary effect of each mutation.

The potential involvement of known plant hormones in flower development is revealed by the work of Sawhney & Greyson (1973*a*,*b*) on the *stamenless-2* gene of tomatoes. Plants homozygous for this recessive gene have flowers in which the stamens are carpelloid, at times including marginal external ovules. The developing primordia of these organs appear identical to developing stamen primordia in wild-type flowers in their very early development; only after reaching a length of about 100 μm can the differences in the organs be detected. Further studies showed that application of gibberellic acid (at 10^{-3} M) to mutant floral primordia caused the development of the flowers to be almost wild type. This suggests that the mutation may act by interfering with hormone action. However, more recent work (Sawhney, 1983; Sawhney & Polowick, 1986) shows that the *stamenless-2* mutation is temperature-sensitive, and that gibberellin treatment mimics the effects of low temperature in the development of the mutant flower. Thus, it is not clear if the effect of gibberellin on these plants is specific, or if a variety of different stresses might all have the same effect.

Whether hormones are involved in other morphogenetic mutants of flowers is not known. That mutants in *Arabidopsis* similar to *stamenless-2* in tomato are not influenced by exogenous application of gibberellins or other hormones (Bowman *et al.* 1989) indicates that hormones are not necessarily deficient in all abnormal flowers.

The possibility of involvement of another set of known substances in pattern formation in flower development is demonstrated by recent work on polyamine mutants of tobacco (Malmberg, 1980; Malmberg & McIndoo, 1983, 1984; Malmberg *et al.* 1985; Malmberg & Rose, 1987). These authors selected for tissue culture cells of tobacco that had mutations in the polyamine biosynthetic pathway, and regenerated plants from the mutant cells. Many of the plants were dwarfed, and all had some floral abnormalities, ranging from plants that did not flower, to male or female sterility and homeotic conversions of organs. One phenotype that arose in several independent regenerants was staminoid ovules; other observed abnormalities included petaloid anthers, stigmoid anthers, leaflike sepals, and partial flowers developing within the ovary. Only two of the regenerated lines showed enough fertility in either sex to be analyzed genetically; in both cases the floral abnormality and polyamine lesion segregated as nuclear dominants in the small F_1 generations obtained, and cosegregated in the small numbers of progeny obtained from backcrosses of the F_1 plants to wild type.

While there was a remarkable correlation between floral abnormalities and polyamine biosynthetic lesions in these experiments with tobacco, studies on polyamine levels in four different floral mutants of *Petunia* (Gerats *et al.* 1988) showed no disturbance in polyamine levels in three of the mutants (including green petals, Fig. 1F), and a disturbance in the fourth that could as easily be an effect of the abnormal morphology as a cause. Some normal-flower lines of *Petunia* were also shown to have different polyamine levels. Therefore, not all floral phenotypes result from polyamine lesions, and not all polyamine variation is associated with alterations in floral morphology.

Thus, despite a wealth of available mutations in which floral pattern formation is regularly and profoundly disrupted, there has been little use of abnormal flowers in understanding floral morphogenesis. What work has been done in the past has not answered questions of developmental mechanism. Nonetheless, it has been valuable in providing tests of some theories for mechanisms of flower development, in providing facts on which new general theories may be based, and in indicating the possible involvement of known substances in flower development. Furthermore, the existence of single-gene mutations with profound effects on organ patterns in developing flowers demonstrates that genes exist whose products play a key role in specifying the number and position of organ promordia, and in directing the cells of those primordia to differentiate to appropriate cell types.

New approaches

The past few years have seen the development of methods for eukaryotic gene analysis at the molecular level, and the means for the molecular cloning of genes about which no more is known than their genetic map position. Recent use of these methods in the cloning of genes whose mutant phenotypes are disruptions of developmental pattern has led to rapid progress in unraveling the mechanisms by which genes direct pattern formation in the developing embryos of *Drosophila* (French *et al.* 1988). These experiments make it clear that the next stage in understanding flower development is to find the products of the genes known to be of critical importance in flower development, and to determine their sites of action, and activities. Current work is thus turning to those plants with extensive existing series of mutations that have profound effects on flower morphology, and from which it is possible to clone genes when no more is known about the genes than their phenotype.

Two methods, each developed initially in microorganisms, are available for molecular cloning of plant genes when no biochemical information on their products is available. They are transposon tagging and chromosome walking. The former requires mutations in the gene of interest to have been caused by insertion of an active mobile element for which DNA hybridization probes are available. The element, and the adjoining gene, are then recovered from a library of genomic fragments of the mutant line. In the latter method, the DNA of a genetically mapped morphological mutation is recovered by isolation of successive overlapping DNA segments, starting at a nearby cloned DNA fragment. This method is only practicable (at present) in species with small genomes and little dispersed repetitive DNA, whose genetic maps have a large number of closely spaced DNA markers to use as starting points.

The snapdragon, *Antirrhinum majus*, is amenable to the first approach (Martin *et al.* 1985). Three different active transposons are known in this species, Tam1, Tam2 and Tam3 (Coen & Carpenter, 1986). Each was identified through its unstable insertion into genes controlling flower pigment production. When the element leaves the mutant gene, a phenotype approaching wild type may at times be restored. If this happens often during the growth of the corolla, a variegated flower results (Fig. 1A). The DNA sequence of Tam1 and Tam2 shows them to be related, and the elements appear to interact *in vivo* (Hehl *et al.* 1987; Hudson *et al.* 1987). On the other hand, Tam3, which is 1000 times more active at 15°C than at 25°C (Carpenter *et al.* 1987), has a unique DNA sequence and transposition properties (Sommer *et al.* 1985).

Many genes that affect flower structure are known in *Antirrhinum* (Stubbe, 1966). Among these are the multiallelic series at the *cycloidea* locus, the most extreme allele *cycloidea*^{radialis} causing the corolla to become peloric (radially symmetrical) (Fig. 1B). Another multiallelic series of mutations, at the *deficiens* locus, results in changes to the corolla and androecium (Fig. 1C,D). In the extreme mutant *deficiens*^{globifera}, the petals become sepaloid and carpelloid organs replace the stamens, with stigmatic tissue and ovules present. If one were to clone these genes and study their products, it might be possible to gain insight into the mechanisms that produce the normal lateral asymmetry of the corolla, and also to learn about the early developmental decisions that control the determination of petal and stamen primordia.

A successful strategy to clone these genes might involve activating Tam elements by growing plants in nonrepressed genetic backgrounds (Hudson *et al.* 1987) or at low temperatures (Tam 3) and screening succeeding generations for new mutations at these loci. These could then be examined for new sites of Tam insertion by DNA probes, and the adjacent plant DNA cloned. It may not be necessary to produce new, Tam-induced alleles at these loci at all if the unstable mutants already known (Stubbe, 1966) have arisen through past Tam insertions (Coen & Carpenter, 1986). Continued instability might well be the consequence of regular somatic loss of the element, as in the case of genes producing variegated pigment patterns.

Many maize genes have already been cloned by transposon tagging with *Ac* and other endogenous transposable elements (Fedoroff *et al.* 1984; O'Reilly *et al.* 1985; Paz-Ares *et al.* 1986; Wienand *et al.* 1986; Cone *et al.* 1986; Schmidt *et al.* 1987); and there do exist mutations affecting development of the maize inflorescences (Coe & Poethig, 1982; Poethig, 1988). For the most part, these seem to have primary effects on general plant growth, however, and only secondary effects on flower development. Examples are the *Teopod* mutations, with general effects on juvenility discussed earlier, and the andromonoecious dwarf mutations, which cause reduced plant height and leaf length while at the same time allowing development of the normally suppressed stamens in ears. There are also maize mutations that affect the degree of branching in tassels, and others that allow development of the ordinarily suppressed pistils in tassel florets, but these mutants do not show the types of profound alterations of cell fate and developmental pattern that would indicate that their products act directly in specifying the early events of flower development.

In contrast to *Antirrhinum* and maize, the *Arabidopsis* genome is not known to contain active transposons that can be used in gene tagging. The prospects for gene tagging in *Arabidopsis* are thus limited to the introduction of heterologous transposons, such as *Ac* from maize (Van Sluys *et al.* 1987), and to insertional mutagenesis by T-DNA from the Ti or Ri plasmids of *Agrobacterium* (Feldmann & Marks, 1987; Feldmann *et al.* 1989). *Arabidopsis* does have the small genome (70 000 kilobase pairs, Leutwiler *et al.* 1984) and low level of interspersed repeat sequences (Pruitt & Meyerowitz, 1986) which aid chromosome walking. Further, a restriction fragment length polymorphism (RFLP) genetic map exists with enough DNA fragments mapped so that more than 50 % of the genome is

within less than 1·9 centimorgans (on average about 250 kilobase pairs) of the mapped DNA fragments (Chang *et al.* 1988). In addition, complementation of mutations by introduction of wild-type genes *via Agrobacterium*-mediated transformation is possible (Meyerowitz, 1987). Thus, available materials provide starting points for chromosome walks, and a means of knowing when the desired gene has been cloned; as well as the possibility for insertional mutagenesis.

A number of *Arabidopsis* mutations whose phenotype is homeotic transformation of the organs in different whorls, loss or gain of organs in different whorls, or abnormal differentiation of organs have been described (McKelvie, 1962; Conrad, 1971; Koornneef *et al.* 1983; Meyerowitz & Pruitt, 1984; Pruitt *et al.* 1987; Meyerowitz, 1987; Haughn & Somerville, 1988; Komaki *et al.* 1988; Bowman *et al.* 1989). The phenotypes of several mutants homozygous for these recessive, single-gene mutations are depicted in Fig. 2. The range of phenotypes is similar to that described for many other species, including double flowers (*agamous*), staminoid petals and leaflike sepals (*apetala2-1*), carpelloid sepals with absence of organs in the petal or stamen whorls (*apetala2-2*), sepaloid petals and carpelloid stamens (*apetala3-1*), sepaloid petals with no differentiation of organs in the stamen whorl (*pistillata*) and extra organs in various whorls (several different *clavata* loci).

The study of these mutations has gone beyond a description of the mutant phenotypes in mature flowers: the development of four genotypes of mutant flowers have been studied with the scanning electron microscope, and the phenotype of most of the possible double mutants have been described (Bowman *et al.* 1989), allowing conclusions on the interactions and roles of the wild-type products of these genes. One clear conclusion is that different single mutations and double mutant combinations can allow the regular differentiation of almost any organ in almost any whorl, and that the differentiation of any organ type is independent of the organ type developing in adjacent whorls. In addition, each of the homeotic flower mutations so far described in *Arabidopsis* affects organ identity or development in two or more adjacent whorls. These observations are consistent with those models for flower development in which genes establish separate identities for concentric rings of cells prior to any overt cellular differentiation in the flower primordium. Furthermore, the time of action of the products of the *apetala2-1* and *apetala3-1* genes has been determined using temperature-sensitive alleles and temperature-shift experiments (Bowman *et al.* 1989), and the times are different, implying that sequential action of several genes is necessary to fully specify the fate of the cells in each ring. The *apetala2* product is necessary only in the earliest stages of flower development, before the appearance of any organ primordia, for petals to develop normally. The temperature-sensitive period of *apetala3-1* is later, at a time when the affected organ primordia are present, but before any differentiation of the cells in the primordia is visible. This shows that the cells of the primordia are not irreversibly determined until after the number and position of the primordia has been established.

Scanning electron microscope views of these mutants also show that single organs can develop as mosaics of cell types usually found in different floral organs, such that abnormal organs are formed largely from normal cell types in ectopic sites. This emphasizes the point that these sorts of mutations result from cells losing positional information, and not from being unable to differentiate normally. Understanding the role of these genes in the developing flower primordium awaits their molecular cloning, and an analysis of their products.

It is likely that new methods and new mutations will allow similar approaches to the molecular cloning of genes that direct flower development in many species in addition to those already mentioned. Whatever species and whatever means are used to clone these genes, the extension of findings to a wide range of further species with different flower forms will be potentially simple and rapid. Genes with important roles are likely to be found universally, and their isolation by DNA cross-hybridization straightforward. The result may eventually be an understanding both of the molecular mechanisms of flower development, and of the differences in developmental programs that give rise to the varied forms of flowers in different species.

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APPENDIX THREE

ARABIDOPSIS THALIANA: A REVIEW

John L. Bowman, Martin F. Yanofsky, and Elliot M. Meyerowitz

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ARABIDOPSIS THALIANA: A REVIEW

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INTRODUCTION

Arabidopsis thaliana is a member of the mustard family and, although it has no economic value, it belongs to the same family as important crop plants such as cabbage, broccoli and horseradish (For recent reviews see Haughn and Somerville, 1987; Meyerowitz, 1987; Pang and Meyerowitz, 1987; Meyerowitz and Chang, 1988). The mature plant is small, reaching about 30 cm in height, with a rosette of small leaves at its base and an inflorescence at the apex of its shoots. The flowers are similar to those of other members of the mustard family and although they normally self-fertilize, they are easily cross-pollinated in the laboratory. During the two weeks after fertilization, the ovary develops into a silique which contains 30-60 seeds, each of which weighs about 20 µg. A single plant may have more than 200 flowers in its life and thus may produce more than 10,000 seeds. A variety of factors affect the life cycle and size of the plant. The commonly used ecotypes, Columbia and Landsberg, have a generation time of roughly six weeks at 25°C under continuous light. Short generation times are induced by conditions such as crowding, low nutrition, long day length and high temperature. However, these conditions also result in smaller plants with a smaller number of seeds.

Although *Arabidopsis* has been used for experiments in classical genetics for over forty years (Laibach, 1943; Reinholz, 1947), it has only recently gained attention as being useful in molecular genetic studies as well. This is largely due to the small size and simple organization of its genome, as discussed below.

GENOME ORGANIZATION

Genome size varies among Angiosperms over a range of nearly a thousand-fold. There is no apparent correlation between genome size and organism complexity in any order of plants or animals (Table 1) (Bennett and Smith, 1976). Several early experiments indicated that the genome of *Arabidopsis* is one of the smallest among flowering plants (Sparrow and Miksche, 1961; Sparrow et al., 1972; Bennett and Smith, 1976). These results are supported by the reassociation kinetic experiments performed by Leutwiler et al. (1984) who estimated that the haploid *Arabidopsis* genome contains approximately 70,000 kilobase pairs of DNA. This is only five times the size of the genome of the yeast, *Saccharomyces cerevisiae*, and implies that genomic libraries representing the *Arabidopsis* genome can be considerably smaller than those of other flowering plants.

The results of Leutwiler et al. (1984) also demonstrate that of *Arabidopsis* DNA isolated from whole plants, approximately 14% consists of highly repetitive sequences, 25% of middle-repetitive sequences and greater than 50% are single copy sequences. It was further shown that the majority of the middle repetitive sequences were derived from the chloroplast and not the nuclear genome. These conclusions were extended by Pruitt and Meyerowitz (1986) who characterized 50 random genomic lambda clones with an average insert of 12.8 kb, 45 of which were derived from the nuclear genome. The largest class of nuclear clones was represented by 32 clones which were composed entirely of single copy sequences. The second most abundant class contained 8 clones composed of DNA encoding ribosomal RNAs; the ribosomal DNA repeat unit is approximately 10 kb in length and is present in roughly 570 copies per haploid genome. The third class contained three clones, all of which contained repeated sequences that were interspersed with unique sequences. A fourth class was represented by only 2 clones and these contained sequences which are repeated only a few times per genome. An example of cloned genes that fit into this class are the genes encoding the chlorophyll a/b binding proteins which are present within a single cluster in three copies (Leutwiler et al., 1986). Overall, *Arabidopsis* has a genome with dispersed repeats separated on average by over 100 kb of single-copy DNA, with one or more large blocks of tandem rDNA repeats.

In general, the organization of gene families in *Arabidopsis* is simpler than similar gene families from other plants. This simplifies the study of particular gene families and is perhaps, in part, responsible for the small genome size. However, exceptions to this rule do occur. Thus, whereas the alcohol dehydrogenase (Chang and Meyerowitz, 1986), chlorophyll a/b binding proteins (Leutwiler et al., 1986), 12S seed storage proteins (P. Pang et al., in preparation) and other gene families all contain fewer members in *Arabidopsis* than any other characterized higher plant, tubulin gene families (Ludwig et al., 1987) are larger in *Arabidopsis* than those of a number of other angiosperm species.

Table 1. Haploid genome size and average size of single-copy DNA sequences interspersed with repetitive sequences in various flowering plants (see Meyerowitz and Pruitt, 1985).

Plant	Haploid genome size (kb)	Average size in predominant class of single copy sequences (kb)
Arabidopsis	70,000	120
Mung bean	470,000	>6.7
Cotton	780,000	1.8
Tobacco	1,600,000	1.4
Soybean	1,800,000	<3
Pea	4,500,000	0.3
Wheat	5,900,000	1

CLASSICAL GENETICS AND BIOCHEMISTRY

Arabidopsis is well suited for classical genetic studies. The short generation time of six weeks, high fecundity, diminutive size (dozens can be grown to maturity in a small pot), minimal growth requirements, ease of self- and cross-fertilization and ease of mutant isolation, all contribute to making *Arabidopsis* useful for classical genetic studies. Several thousand mutant seeds can be germinated in a single petri dish, allowing the routine screening of very large numbers of plants for induced mutations. Thus even though many other plants (including crop plants such as maize and tomato) have a more extensive base in classical genetics, the ease with which genetic manipulations can be done in *Arabidopsis* is allowing its genetics to approach that of slower growing plants.

Arabidopsis seed collections from various ecotypes have been made for many years (see Röbbelen, 1965a) and in early studies it was determined that the haploid chromosome number is five (Laibach, 1907; see also Steinitz-Sears, 1963). In addition, several early mutagenesis experiments produced morphological mutants (Laibach, 1943; Reinholz, 1947a; Reinholz, 1947b), most of which have not been propagated. Since then, many visible, biochemical, physiological and conditional-lethal mutations have been isolated in a number of laboratories using a variety of mutagens (Röbbelen, 1957; McKelvie 1962; McKelvie, 1963; Burger, 1971; Somerville, 1982; Koornneef et al., 1983a; Meyerowitz and Pruitt, 1984; Meinke, 1986). Many mutant lines and ecotypes are available through the *Arabidopsis* Information Service seed bank located in Frankfurt, Germany (Kranz, 1978, 1987a, 1987b). In early linkage studies, Rédei and Hirono (1964) assigned a large number of mutants to six linkage groups, which Lee-Chen and Steinitz-Sears (1967) assigned to the five chromosomes by means of

Steinitz-Sears (1967) assigned to the five chromosomes by means of five primary trisomics isolated in the Columbia ecotype. Koornneef (1983a; 1987) constructed an internally consistent genetic map consisting of five linkage groups corresponding to the five chromosome pairs of the diploid plant (Figure 1). The order and distance of the nearly one hundred visible, conditional-lethal and hormonal mutations were established by analysis of the segregation of different mutations in meiosis. Using telotrisomic lines, the location of the centromeres on chromosomes 1, 3 and 5 have been determined with respect to the positions of visible markers (Koornneef, 1983a, 1983b) and the centromere on chromosome 2 has been localized on the basis of double reduction in primary trisomics (Sears and Lee-Chen, 1970). The entire genetic map totals 437 centimorgans with chromosomes ranging in length from 51 centimorgans for chromosome 2 to 123 centimorgans for chromosome 1. Cytological studies (Sears and Lee-Chen, 1970) have shown that the physically largest chromosome, 4, is the major nucleolar chromosome with a large heterochromatic block possibly comprising an entire arm. The cytogenetic studies also support a median or submedian location for the centromeres on the medium sized chromosomes, 1, 3 and 5. Chromosome 2 is the shortest chromosome cytologically, as well as genetically. Several multiple marked lines are available for mapping studies including a strain (W100) with two markers on each of the five chromosomes (Koornneef, 1987).

Mutagenesis

Mutagenesis has been performed on seeds using ionizing radiation (X-rays, fast neutrons and gamma rays) and over 80 chemical mutagens (Rédei, 1970). Each seed contains a small number of cells that give rise to the reproductive organs of the mature plant (Müller, 1965: Harle, 1972; Meinke and Sussex, 1979). If a mutation is induced in one of these cells in the mutagenized (M_1) seed, it is replicated in the cell's progeny and thus a sector of the reproductive tissues of the mature plant will be heterozygous for the induced mutation. When flowers in these sectors self-fertilize, one quarter of the seed set (M_2 seeds) in that sector will be homozygous for the newly induced mutation. If the phenotype is recessive sterile or lethal one can retrieve the mutation from the heterozygous seed obtained from the same siliques in which the homozygous mutant seeds were found. Scoring siliques of M_1 plants for recessive embryo lethals has been used to determine the mutagenic potential of seed treatments (Müller 1963; Rédei, 1970). Ethyl methanesulphonate (EMS) has proven to be highly effective in inducing visible mutations without a high incidence of death or sterility (Röbbelen, 1962; McKelvie, 1963; Müller 1965).

Mutagenesis and screening of large numbers of seeds can be performed readily due to their small size. The diminutive size of the mature plant enables thousands of M_1 plants to be grown in the laboratory and harvested for M_2 seed either *en masse* or individually,

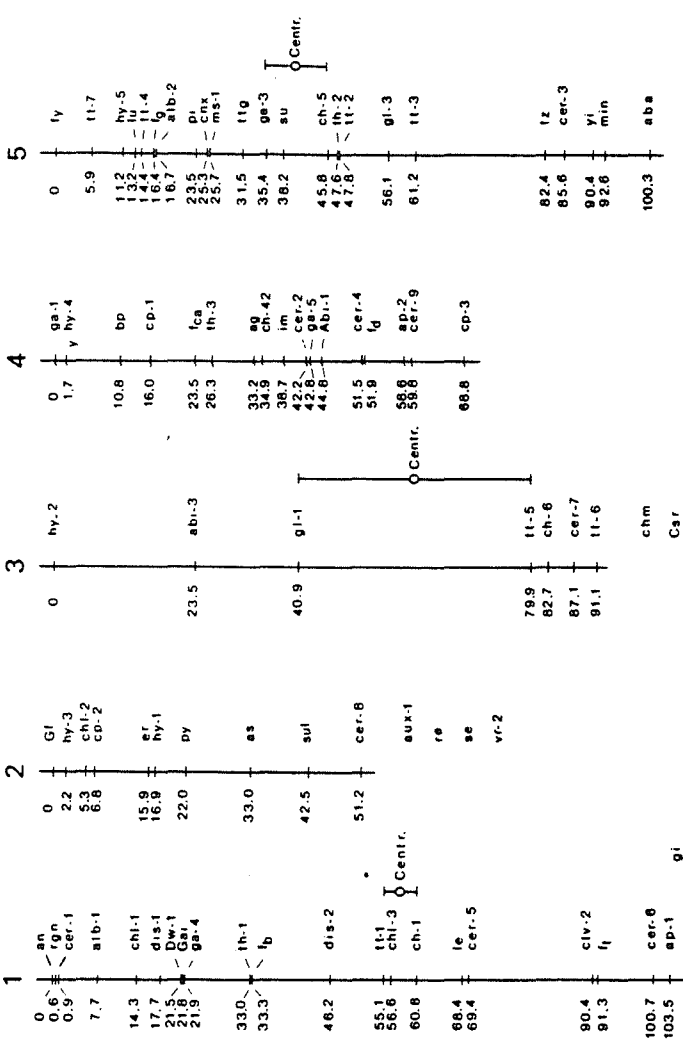

Figure 1. Genetic map of Arabidopsis thaliana *showing the five linkage groups of visible, biochemical and hormone mutants. Location of centromeres on chromosomes 1, 3 and 5 are shown. All distances are in centimorgans (Reprinted from Koornneef, 1987).*

thus producing large quantities of seeds to be screened for the desired mutant phenotype. If selection can be performed at the germination stage, several thousand M_2 seeds can be screened on a single petri dish and, if the desired mutant phenotype can only be effectively selected after germination, the small size of the plant greatly facilitates screening of large numbers of M_2 plants. Once the desired mutation is isolated, it is easily maintained in a homozygous form, providing it is fertile and non-lethal, through normal self-fertilization.

Morphological mutants and colour variants

The majority of characterized mutations have been induced in the Landsberg ecotype carrying the *erecta* mutation (*er*) (Rédei, 1962a) by soaking seeds in EMS solutions, or exposing dry or imbibed seeds to X-rays. Morphological mutations affecting almost every aspect of the plant comprise the largest class of characterized mutants (McKelvie 1962, 1963; Koornneef et al., 1983a; Meyerowitz and Pruitt, 1984). The stature of the entire plant is altered by the *erecta* (Rédei, 1962b, 1964) and *compacta* (Koornneef et al., 1980a, 1983a) mutations. The *erecta* mutation is characterized by a compact rosette, short petioles and blunt fruits. This reduces the plant's overall size and makes plants homozygous for *erecta* useful laboratory lines. Numerous laboratories use this marker homozygous in the Landsberg ecotype as their standard wildtype strain. Leaf morphology is altered in the mutations *asymmetric leaves* (Rédei, 1964) and *angustifolia* (Rédei, 1962b; Lee-Chen and Steinitz-Sears, 1967), which cause narrow leaves; epidermal wax morphology is altered in the numerous recessive *eceriferum* mutations (Dellaert et al., 1979). Many mutations affecting the number and organization of trichomes are available (Feenstra, 1964; Lee-Chen and Steinitz-Sears, 1967; Koornneef et al., 1983a), one of which, *transparent testa glabra* (Koornneef 1981), also lacks anthocyanins and is deficient in seed mucilage. This unique combination of pleiotropic effects suggests a common early step in the biochemistry or development of these structures.

Colour variants and variegated mutants comprise another class of visible mutants; these may be due to a simple deficiency of a light absorbing pigment or they may have a much more complex cause. Absence of anthocyanins in the *transparent testa* mutations (Koornneef, 1981 et al., 1983a) produces plants with yellow seeds and, depending on the allele, may cause older plants not to display the characteristic reddish-purple colour of older wildtype plants. A lack of, or reduction in, levels of chlorophyll b results in a yellow-green plant (Hirono and Rédei, 1963) in the *chlorina-1* mutants.

The recessive mutant *immutans* (Rédei, 1967; Röbbelen, 1968) displays variegated white and green leaves with accumulated inorganic phosphorus in the white sectors. Heterozygotes never show variegation. The extent of the white sectors depends on the intensity of the incident light during the development of a leaf, with high light intensities being lethal. Variegation is also seen in plants homozygous for the nuclear recessive *chloroplast mutator* mutation (*chm*) which induces abnormal plastid morphology (Rédei 1973a,

1973b). The extent of the mutated sectors increases as the plant grows older so that mature plants display varying degrees of the mutant character, depending on the age of the sectors. The newly induced chloroplast mutation which induced the abnormal chloroplast morphology is maternally inherited autonomously and is retained through inheritance. Homoplastidic lines with all chloroplasts showing the same altered morphology may be obtained by crossing the *chm* locus away from the induced abnormal chloroplasts. It has been suggested that the *chloroplast mutator* gene induces abnormal chloroplasts by causing errors in chloroplast DNA replication (Rédei 1973a). Thus, elucidating the roles of genes in the chloroplast genome might be facilitated by using this chloroplast-specific mutator and subsequent isolation of homoplastidic lines.

Biochemical mutants

A third class of mutants are those that are defective in a biochemical pathway or specific enzyme. Typically these are isolated by screening for plants that are resistant to normally toxic compounds or resistant to high concentrations of substrate analogues. Plants selected for resistance to the herbicide chlosulfuron have been shown to have mutations in the *acetolactate synthase* gene (Haughn and Somerville, 1986). Selection for chlorate resistance may have produced plants with mutations in the *nitrate reductase* gene, which has recently been cloned (Crawford et al., 1986), plus additional mutations concerned with the uptake of chlorate (Oostindier-Braaksma and Feenstra, 1973; Braaksma and Feenstra, 1982). Selection for seeds surviving allyl alcohol treatment produces plants with mutations in the single *alcohol dehydrogenase* gene of *Arabidopsis*. These mutants lack alcohol dehydrogenase activity, which converts allyl alcohol to a toxic aldehyde (Dolferus and Jacobs 1984). Conditional-lethals which require exogenous thiamine (*th-1*, *th6-2*) (Langridge, 1955, 1958; Li and Rédei, 1969), either thiamine or the precursor 4-methyl-5-hydroxyethyl thiazole (*tz*) (Feenstra, 1964; Rédei, 1965), or thiamine or the precursor 2,5-dimethyl-4-amino pyrimidine (*py*) (Feenstra, 1964; Rédei, 1965) have been isolated. A number of amino acid analogue resistant mutants have been found as well, including ones resistant to analogues of proline (Verbruggen and Jacobs, 1987; Estill and Lehle, 1987; Dandekar et al., 1987), arginine (McCourt, 1987), histidine (Lalonde and Fink, 1987) and aspartate (Learned and Fink, 1987). These mutants are being used to study the amino acid biosynthetic pathways and the possible role of proline during osmotic stress tolerance. In addition, an adenine analogue resistant mutant (Moffatt and Somerville, 1987) has been identified. Selection for both growth in a non-photorespiratory environment, characterized by an atmosphere enriched in carbon dioxide and lethality in normal respiratory environments has facilitated the isolation of nuclear recessive mutations in several genes encoding enzymes related to photorespiration (Somerville, 1982).

Developmental mutants

Of particular interest to plant developmental biologists are mutations which alter the differentiation and development of *Arabidopsis*. Ontogenic programmes, combined with environmental factors, control the growth and development of all organisms and many aspects of responses to variations in environment (light, gravity, day length, temperature, sources of minerals and water) are unique to plants. In the past these responses have been extensively studied phenomenologically but the underlying molecular mechanisms remain obscure.

Fundamental differences exist between plant and animal development. In contrast to animals, where some cells become committed to particular developmental fates at an early stage of development, meristematic tissues in plants remain embryonic and retain the ability to produce any adult tissue, including germ cells, throughout the life cycle of the plant. Individual differentiated plant cells can de-differentiate and later regenerate into entire, fertile organisms, a process not seen in animals. Although plant cells are developmentally more flexible, they lack the ability to migrate, which is an important feature of animal development.

The use of, and response to, hormones is different in plants and animals, in that most or all cells in a plant are involved with the activity of hormones at some time during growth. Hormones have long been recognized as having an important role in the development of plant tissues; for example, the relative levels of two classes of hormones, auxins and cytokinins, can determine which type of tissue arises from undifferentiated callus tissue.

To understand better these unique growth and differentiation processes, a substantial collection of mutants affecting either single regulatory steps in the pathways involving phytohormone metabolism, or homeotic genes, are being collected in several laboratories. Many genes controlling hormone action or metabolism have been described in different plant systems (maize, tomato, barley) (Phinney, 1960; Pelton, 1964; Tal and Nevo, 1973; Zobel, 1973; Hiller et al., 1980). The ability to isolate rapidly and characterize these types of genes from the small genome of *Arabidopsis* makes it an attractive system for such studies.

Phytohormone mutants

Arabidopsis mutants in five complementation groups which result in the absence of gibberellins are known (Koornneef et al., 1980a). Plants homozygous for these mutations are dwarfs with reduced petals and stamens and, depending on the allele, may yield seeds that fail to germinate. Exogenous gibberellins restore growth to nearly wildtype levels with elongation occurring in the stem, petals and stamens, indicating a role for gibberellin in cell elongation. Leaky alleles, which germinate but apparently do not produce sufficient GA for normal elongation growth, have been isolated for 3 of the 5 complementation groups (*ga-1*, *ga-2*, *ga-3*). It has been proposed that

these loci are involved in the early steps of GA synthesis.

Auxins have been implicated in a wide variety of plant growth and development patterns including phototropism, vascular tissue differentiation, root and fruit growth, and abscission. Both recessive (*aux-1, aux-2*) (Maher and Martindale, 1980; Mirza et al. 1984; Mirza, 1987) and dominant *Dwarf* (*Dwf*) (Mirza et al, 1984; Olsen et al., 1984; Estelle and Somerville, 1987) mutations conferring resistance to high levels of the synthetic auxin 2,4-dichlorophenoxy acetic acid (3 fold to 2000 fold higher than wildtype depending on the mutation) have been selected in *Arabidopsis*. *Dwf* results in a phenotype of an agravitropic dwarf when heterozygous and is lethal when homozygous. The roots of *aux-1* seedlings are agravitropic while the roots of *aux-2* mutants display normal gravitropism; both mutant lines show increased root growth. The prevailing theory that amyloplasts with starch grains act as statoliths and form a primary part of gravity perception in roots has not been supported by studies performed on *aux-1* mutants (Mirza et al., 1984) and a recently isolated starchless mutant in *Arabidopsis* (Casper et al., 1987). The starchless mutant, which is unable to synthesize leaf starch due to a deficiency in the plastid isozyme of phosphoglucomutase, has been shown to be gravitropically competent. The plastids in the mutant contain no starch and do not respond to gravity, indicating that plastid sedimentation is not necessary for gravity perception (Casper et al., 1987).

In addition to the well-documented negative phototropic and positive gravitropic responses of the root, horizontal root curvatures have been shown to be influenced by both light and gravity in studies with wildtype *Arabidopsis* and *aux-1* mutants, which retain their phototropism despite being agravitropic. A clockwise root curvature is always observed perpendicular to the synergistically acting gravity and light vectors (Mirza, 1987).

Many physiological responses appear to be mediated by phytochrome, the only known pigment in plants that senses light signals from the environment and transduces them into changes in gene expression (e.g. expression of light harvesting chorophyll binding protein genes). Spectral studies with mutants that do not show the hypocotyl elongation inhibition response to light (*hy-1* to *5*) (Koornneef et al. 1980b; Spruit et al., 1980) support the idea that phytochrome at least partially mediates this response. Mutations that inhibit the response of *Arabidopsis* to long day conditions, which normally induce flowering, have been reported (Rédei, 1962b; Van der Veen, 1965; Koornneef et al, 1983a). Langridge (1957) showed that application of gibberellic acid (GA) to *Arabidopsis* plants grown in short days promoted flower formation, but the mechanism by which GA mediates this response and its relationship with other factors affecting flower induction, such as phytochrome, vernalization and photoperiodism, remains unclear.

Mutants affecting other known phytohormones have been isolated including abscisic acid (ABA) deficient genotypes that show symptoms of withering and absence of seed dormancy (Koornneef et al., 1982; Karssen et al., 1983; Koornneef et al., 1984). Embryonic ABA has been

suggested to have a role in induction of seed dormancy. A dominant mutation which makes plants completely insensitive to the usual effects of ethylene application, such as seed germination, cell elongation, accelerated leaf senescence and regulation of ethylene biosynthesis, still produces plants which are relatively normal phenotypically (Bleecker et al., 1987).

Homeotic mutants

Another approach to understanding development is the study of homeotic and developmentally arrested mutants. An impressive collection of embryo lethal mutants has been classified into stages of developmental arrest and are being characterized. These have been extensively reviewed recently (Meinke, 1986).

Mutations affecting floral morphogenesis have been described in many plant species and are prevalent among the visible mutations arising from mutagenesis procedures performed on *Arabidopsis* (McKelvie, 1962; Arnold, 1965; Röbbelen, 1965b; Reinholz, 1966; Usmanov, 1970; Buggurt and Röbbelen, 1970; Burger, 1971; Conrad, 1971; Relichova, 1976; Kranz, 1978; Korrnneef et al., 1983a; Goto et al., 1987). Among these are a few mutations which may be considered homeotic, as described by Bateson (1894), in that they cause transformation of one plant structure into another, usually adjacent, structure. Homeotic mutants have been described in segmented organisms, such as *Drosophila* (Lewis, 1978) and the silkmoth *Bombyx mori* (for a review of homeosis, see Gehring and Hiromi, 1986), and a conserved sequence in the coding region of some homeotic genes in *Drosophila* termed the homeobox has been found in such diverse organisms as *Xenopus*, mouse and man (McGinnis et al., 1984). The homeobox region is thought to encode a DNA binding domain whose possible function is regulating the transcription of developmentally regulated genes (Laughon et al, 1984; McGinnis et al., 1984a). Although the *Arabidopsis* inflorescence has some characteristics of segmented construction, the conservation of genes between plants and the segmented animals seems unlikely. Thus the investigation of these genes from the plant kingdom is particularly intriguing.

Arabidopsis flowers (Figure 2A), which are typical of Cruciferae, consist of four white petals, alternate and interior to four sepals; six stamens, four long and two short, each consisting of a filament capped with a pollen-bearing anther; and a gynoecium of two fused carpels topped with a papillate stigma (Vaughn, 1955). The bilaterally symmetric flowers are initiated in a phyllotactic spiral. Sepal primordia are initiated first in an abaxial towards adaxial manner followed by simultaneous initiation of four petal primordia alternate to the sepal primordia. Next, the four tall stamens are initiated in pairs in front of the abaxial and adaxial sepal primordia, followed by the two short stamens, which initiate in front of the lateral sepal primordia, all interior to the petal primordia. The remaining floral meristem develops into the ovary (Hill et al., 1987). Petal enlargement lags behind stamen development until anthesis approaches and stamen development lags behind gynoecium development until anthesis, allowing the possibility of cross

pollination. At anthesis, petals rapidly expand causing the flower to open and the stamen to elongate sufficiently to facilitate self-pollination.

Among those mutations considered homeotic is *apetala-2* (Figure 2B), which causes conversion of sepals to leaves that are occasionally capped with stigmata and a partial conversion of petals to stamens (Koornneef et al., 1980c; Pruitt et al., 1987). In *apetala-3*, petals are converted to sepals and stamens are partially converted to carpel-like structures with external ovaries and stigmata (Figure 2C) (Koornneef, personal communication; Meyerowitz, 1987). *Pistillata* (Figure 2D) causes the transformation of petals to sepals and the elimination of the stamens (Koornneef et al, 1983a; Pruitt et al., 1987). Other mutations which are not strictly homeotic but which affect positions and multiplicity of organs in floral morphology include *apetala-1* (McKelvie, 1962, 1963; Koornneef et al., 1982a), in which petals are reduced and are sometimes apparently replaced with entire flowers, these in turn also display the *ap-1* phenotype. *Agamous* (Figure 2E) and *multipetala* (Conrad, 1971; Koornneef 1980a; Pruitt et al, 1987) cause the reproductive organs of the flower to be replaced with numerous petals and sepals. Other mutations, such as those in the *clavata* genes (Koornneef et al., 1983a), only affect the number of organs within whorls. All of the mutations mentioned above are single nuclear recessive mutations with the stated phenotypes produced during growth conditions of continuous light at 25°C and 70% relative humidity.

The phenotypes of most of these mutations vary, depending on the age of the plant and growth conditions such as temperature. A trend of conversion towards carpels is evident in floral organs on older plants even in wildtype Landsberg *erecta* lines. Very late flowers of wildtype plants occasionally consist mostly of carpel-like structures as do late flowers in several mutant lines such as *pistillata* (*pi*) and *apetala-3* (*ap-3*). In addition, the extent of transformation of stamens to carpel-like structures in *ap-3* increases with the age of the plant (J.L. Bowman, unpublished). Several mutations (Röbbelen, 1965; Haughn and Somerville, 1987) have been isolated in which the entire inflorescence is composed of carpel-like structures. These observations suggest that carpels may be a default or "ground state" of the developing floral meristem. An obvious, but most likely too simplistic scenario to explain these observations would be as follows: A gene product which is normally only expressed early in the developmental process is necessary for differentiation into floral organs other than the "ground state". This gene product is either not present in the mutant plant or its presence in older plants is reduced due to degradation or instability, thus causing the default state of differentiation to occur.

The temperature at which development occurs influences the observed phenotype of some mutations. *Clavata-1* mutants, when grown at 25°C, have an ovary consisting of four rather than two carpels with other whorls appearing normal, while at 16°C additional organs in other whorls are seen. Flowers with six petals and eight stamens, in addition to four carpels, have been observed. However, the numbers of particular organs other than carpels are not consistent on flowers


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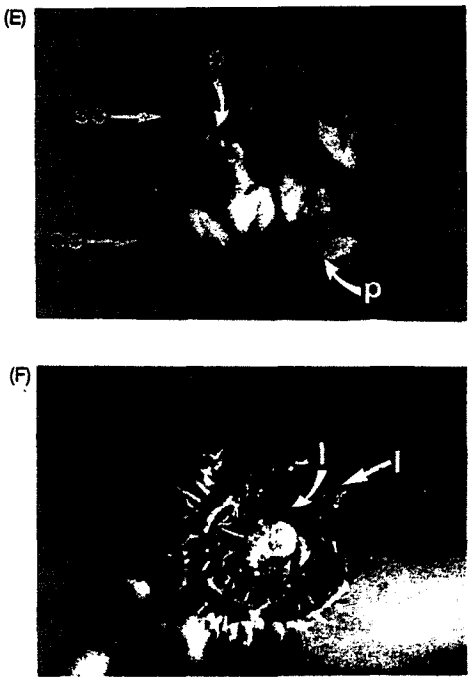

Figure 2. Photographs of wildtype and mutant flowers of Arabidopsis thaliana. *(A) Wildtype flowers at various stages of development: 1) maturing fruit; 2) pre-anthesis; and 3) post-anthesis. Abbreviations indicate relevent floral organs: se=sepal, p=petal, st=stamen, g=gynoecium. (B) Flower of homozygous* apetala-2 *mutant showing the conversion of sepals to leaves (l) with stellate trichomes (t) and petals to stamen-like structures (s). (C) Flower of homozygous* apetala-3 *mutant showing the conversion of petals to sepals (se) and stamen to carpel-like structures (c) with external ovules (o). (D) Flower of homozygous* pistillata *mutant showing the conversion of petals to sepals (se); stamens are absent. (E) Flower of homozygous* agamous *mutant displaying nested whorls of sepals (se) and petals (p); all reproductive structures are absent. (F) Flower of plant homozygous for both the* apetala-2 *and* pistillata *mutations consisting of two whorls of four leaves (l) each and a wildtype gynoecium; no male reproductive structures are present.*

even on the same plant. Apetala-2 is also temperature sensitive, as morphologically wildtype petals are produced at 16°C, while at 25°C the petals are replaced by stamens. At either temperature the sepals are transformed into leaves. This temperature sensitivity can be exploited to determine when the gene product is necessary during development. Preliminary temperature shift experiments indicate that the *ap-2* gene product is required before floral organ primordia have appeared (Bowman unpublished). This is consistent with this gene product having a role in decisions concerning differentiation of the meristem into the precursor cells of floral organs.

A line homozygous for both the *ap-2* and *pi* mutations (Figure 2F) has a phenotype of two whorls of four leaves, each replacing the normal sepal and petal whorls and surrounding a wildtype gynoecium. No stamens or filaments are present (Pruitt et al., 1987). This is consistent with *pi* being epistatic to *ap-2* in the determination of the fate of the meristematic cells that would normally become petal primordia. Double mutant lines constructed between all combinations of the known homeotic mutations and those mutations that cause meristematic variation, could lead to a hierarchal model of regulation of the genes responsible for the initiation and maintenance of floral organ differentiation.

Transformation in the floral organs of *Arabidopsis* may occur in two directions: transformation of the organ of one whorl into those of either an interior whorl (an inward homeosis), or a whorl towards the exterior (an outward homeosis) (Bateson 1894). In *Drosophila* (For a review, see Gehring and Hiromi, 1986), homeotic recessive loss of function mutants usually causes an anterior transformation while dominant gain of function mutants generally lead to posterior transformations. In contrast, homeosis in either or both directions is observed in the recessive homeotic floral mutants identified in *Arabidopsis*. The homeotic loci of *Drosophila* consist primarily of large gene clusters that are expressed in the organism in the same manner that they are arranged on the chromosome. Elucidation of the arrangement of homeotic flower genes in *Arabidopsis*, which are widely dispersed throughout the genome, and comparison with those in *Drosophila*, should provide an interesting evolutionary study of the mechanisms by which two widely divergent organisms have solved certain problems of development.

CHARACTERIZATION OF CLONED GENES

A variety of methods are available for cloning genes from *Arabidopsis*. The most widely used method to date involves the use of a heterologous probe derived from a cloned gene from another organism. An alternative approach uses differential screening of genomic clones with cDNA synthesized from RNA isolated from various tissues. Chromosome walking is feasible in *Arabidopsis* for the isolation of any gene for which a mutant phenotype exists and will be

discussed in detail later in this review. Finally, shotgun cloning is an alternative that may prove successful in some cases for which a dominant selection is available at the tissue culture stage.

Using a heterologous probe derived from the maize *Adh1* gene, the single copy *Arabidopsis alcohol dehydrogenase* (*Adh*) gene has been isolated (Chang and Meyerowitz, 1986). Nucleotide sequence analysis demonstrates that the predicted *Arabidopsis* Adh enzyme is roughly 80% homologous to and the same length as the maize Adh1 and Adh2 enzymes. The open reading frame for the *Arabidopsis* gene is interrupted by six introns whose positions are identical to six of the nine introns present in each of the maize genes. *Adh* should prove useful as both a biochemical and selectable marker, since *Adh* null mutants of *Arabidopsis* have been isolated (Dolferus and Jacobs, 1984). Perhaps the greatest utility of *Adh* stems from the ability to select against its expression. This is achieved in the presence of allyl alcohol where the Adh enzyme converts this alcohol to the toxic compound acrolein. Recently the cloned *Arabidopsis Adh* gene was reintroduced into the genome of an *Adh* null mutant line of *Arabidopsis* and *Adh* function was restored (C. Chang and E.M. Meyerowitz, in preparation). Progeny of the regenerated plants segregated for one or more unlinked copies of the *Arabidopsis Adh* gene inserted into the genome. Among the progeny of the transformed plants were Adh^+ plants in which the Adh activity was shown to be present in the correct tissues and stage of development. Adh^+ plants were correspondingly sensitive to allyl alcohol. This is the first direct demonstration of complementation of a mutant phenotype in transgenic plants. Such complementation is a valuable aid to the method of cloning genes by chromosome walking.

Another class of genes isolated from *Arabidopsis*, using a heterologous probe from *Lemna*, codes for the chlorophyll a/b (*cab*) light harvesting proteins (Leutwiler et al., 1986). Three copies of this gene were identified in *Arabidopsis*, all of which occur as a tightly linked cluster, and all of which code for the identical gene product. This arrangment is much simpler than that which has been observed for *cab* genes in other plant species. By fusing the upstream regulatory sequences from each of these genes to an easily assayable marker, An (1987) demonstrated that all three copies are expressed and properly regulated when integrated into transgenic tobacco plants.

Another gene family isolated from *Arabidopsis* using a heterologous gene probe, derived from a pea cDNA clone, are the genes encoding the small subunit of ribulose-1,5-bisphosphate carboxylase (*rbcS*) (M. Timko, personal communication). This gene is present in four copies in the Columbia ecotype of *Arabidopsis*, in contrast to the two to twelve copies present in a variety of other plants. At least three copies are present within a single tightly linked cluster in *Arabidopsis*. Preliminary analysis of upstream sequences required for regulated expression of one copy of the *Arabidopsis rbcS* gene have been performed using gene fusions in tobacco. These results indicate that approximately 1.5 kb of upstream sequences are sufficient for tissue-specific expression and light inducibility, in agreement with similar studies on cloned *rbcS* genes from other plant species. These studies form the basis for future dissection of the regulatory

mechanisms involved in *rbcS* gene expression.

Genes encoding the α and β tubulin proteins were recently cloned from the Columbia ecotype of *Arabidopsis* using heterologous probes derived from *Chlamydomonas reinhardtii* (Ludwig et al., 1987; D.P. Snustad, personal communication). The α and β tubulins are present in at least four and seven copies, respectively. Preliminary data from two of the α tubulin clones, designated *α-1* and *α-3*, display different patterns of expression. The *α-1* gene transcript accumulates primarily in flowers, whereas the *α-3* RNA accumulates in a variety of tissues and may therefore be constitutively expressed. The gene encoding the β-1 RNA is expressed predominantly in roots, weakly in flowers and is barely detectable in leaves. The two α tubulin genes are very similar to one another (92% amino acid identities) and also share significant homology to tubulins from *C. reinhardtii, Stylonichia lemnae* and *Homo sapiens*. Similarly, the β tubulins are highly homologous to polypeptides from other organisms. It will be of considerable interest to fully characterize the complex patterns of expression of the tubulin gene families.

The three copies of a heat shock gene were cloned from *Arabidopsis* using a probe derived from a *Drosophila melanogaster HSP70* gene (Wu et al., 1987). At least two of these copies are tightly linked, and are separated by only 2 kb. One of these genes is induced about four-fold after heat shock, whereas another is apparently constitutively expressed. The *Arabidopsis* heat shock genes are highly homologous to the *Drosophila* gene at the amino acid sequence level.

A variety of other genes have been cloned using heterologous probes. The wild type and mutant forms of *acetolactate synthase* genes have been cloned (Haughn and Somerville, 1986). The mutation results in resistance to high levels of sulphonylurea herbicides and is the result of a single base change. The mutant form of this gene should provide a useful addition to selection strategies for higher plant transformation. Other recently cloned genes include *nitrate reductase* (Crawford et al., 1986), *phytochrome* (P. Quail, personal communication), *glycollate oxidase* (C. Somerville, personal communication), an *ADP glucose pyrophosphorylase* (J. Preiss and C. Somerville, personal communication), *RNA polymerase II* (H. Klee, personal communication) and *5-enolpyruvylshikimate-3-phosphate synthase* (H. Klee, personal communication).

A different method for cloning genes was used to isolate genes specifically expressed during the latter half of embryogenesis (P. Pang et al. in preparation). cDNA synthesized from seed RNA was used as a probe to isolate clones from a genomic library and two different classes of genes were isolated. The first class consists of sequences which share considerable homology to 12S seed storage protein genes isolated from other plant species. The 1700 nucleotide RNA is expressed only in the seed and not in the surrounding pod tissue, leaves, stems, or roots. The second class encodes a 650 nucleotide RNA which is not homologous to any previously characterized gene. This RNA is also expressed only in the developing seed. The time of expression of the 1700 and 650 nucleotide RNAs differs (P. Pang et al., in preparation). RNA homologous to the 12S gene is detectable in

7-8 day old seeds and is present at abundant levels throughout seed maturation (16-17 days). In contrast, the 650 nucleotide RNA is detectable in 4-5 day old seeds but is entirely absent in seeds older than 12 days. Since the 650 nucleotide RNA is not present in late maturing seeds, it is unclear whether its gene product is a seed storage protein or plays some other role in the developing seed. Experiments are in progress to identify and characterize the *cis*-acting regulatory sequences for both classes of genes and to isolate mutations in putative *trans*-acting factor genes required for their expression.

There are four copies of 12S globulin related genes in the Landsberg *erecta* strain of *Arabidopsis*. Two of these are present as a tandem repeat and are nearly identical to one another, whereas the other two copies are quite divergent from the tandem pair and from each other (approximately 75% conserved) and display only weak hybridization even under reduced hybridization stringency (P. Pang et al., in preparation). In all other plant species analyzed, the 12S gene family is more complex than it is in *Arabidopsis*. The gene encoding the 650 nucleotide RNA is present in three copies in Landsberg *erecta*, two of which are present as a tandem repeat, and all of which share considerable homology. The third copy has been mapped to within a few centimorgans from the tandem copies.

An *Arabidopsis* element family similar to *Copia* retrotransposons from *Drosophila melanogaster* was recently identified (Voytas and Ausubel, personal communication). A 100 kb region of DNA was analyzed among 16 geographical races in order to identify insertional polymorphisms which may have arisen by transposition. A 2.3 kb element common to 12 of these races was found which contains a long open reading frame that shares 32% amino acid sequence homology with the *Drosophila Copia*-element protein. Significant homology within this reading frame was also observed to retroviral reverse transcriptases. The element is flanked by 5 bp direct repeats, similar to target duplications caused by other transposable elements, and is bounded by two long terminal direct repeats of approximately 500 bp in length. These data all imply that retrotransposons are common to both animals and plants and probably share a common ancestral origin. It is not known if this element is capable of transposition. Characterization of plant transposable elements such as this may lead to yet another method of cloning genes: transposon tagging.

METHODS OF TRANSFORMATION

An essential feature for most molecular genetic studies includes the ability to transform genetically and regenerate plants. Two systems have recently been employed to introduce foreign DNA into *Arabidopsis* and both are dependent on the natural ability of *Agrobacterium tumefaciens* to transform certain higher plants.

Agrobacterium tumefaciens is a soil organism that induces tumourous growths on susceptible host plants (For reviews see Nester et al., 1984; Koukolikova-Nicola et al., 1987). During the initial stages of tumourigenesis, a defined region of *Agrobacterium* DNA, the T-DNA, is physically transferred from the bacterium into the nuclear genome of the infected plant cell. The T-DNA contains genes which direct the synthesis of the phytohormones auxin and cytokinin and it is the overproduction of these compounds within the transformed plant cell that results in the uncontrolled cell division characteristic of a tumour. Within the bacterium, the T-DNA is flanked by 24 bp imperfect direct repeats which are required in *cis* for transfer (Yadav et al., 1982; Wang et al., 1984). None of the genes within the T-DNA are required for transfer and thus the entire internal portion of the T-DNA can be deleted without affecting transfer (Ream et al. 1983). *Agrobacterium* strains which have had these T-DNA sequences deleted are often referred to as disarmed strains since they are no longer tumourigenic but are still fully transfer proficient. Since the 24 bp repeats serve to define the region of DNA to be transferred, any DNA inserted between these sequences can be directed to integrate into the plant genome. A set of genes outside the T-DNA, called the virulence (*vir*) genes, act in *trans* to promote T-DNA transfer (Klee et al., 1983; Stachel et al., 1986).

Although the precise mechanism by which *Agrobacterium* genetically transforms plant cells is not completely understood, our knowledge of this process has greatly facilitated the development of efficient vector systems (for a review see Klee et al., 1987b). Many plant transformation vectors exist as relatively small plasmids capable of replicating in both *E. coli* and *Agrobacterium*. These vectors contain the *cis*-acting repeat sequences required for DNA transfer and a selectable marker gene which confers resistance to specific antibiotics upon transformed plant cells. The most widely used antibiotic resistance gene product, used to select transformed plant cells, is the bacterial neomycin phosphotransferase type II (NPTII) enzyme which detoxifies compounds such as Kanamycin and G418 by phosphorylation (Beck et al., 1982). Other studies have shown that the hygromycin phosphotransferase enzyme is useful as a selectable marker in plants and may be the gene product of choice for transformation of *Arabidopsis* (Lloyd et al., 1986). Other markers are also suitable for selection of transformed plant cells but have yet to be incorporated into existing vectors. These include resistance to chlorsulfuron (encoded by the mutant form of acetolactate synthase) (Haughn and Somerville, 1986) and to the antibiotic gentamycin (Klee et al., in preparation).

The most common procedure for transformation and regeneration of *Arabidopsis* is based on the leaf disc method developed by Horsch et al. (1985). *Arabidopsis* leaves are first surface sterilized, cut into small slices and cultured on regeneration medium for 3-8 days (Lloyd et al., 1986; Chang and Meyerowitz in preparation). The leaf slices, which have begun to callus, are then co-cultured for two days with the appropriate *Agrobacterium* strain in the presence of a tobacco nurse culture. The nurse culture, although not essential, has been shown to increase greatly transformation efficiency. The leaf slices are then transferred onto regeneration/selection medium to kill the

Agrobacterium and any untransformed plant cells. During the next few weeks the transformed callus proliferates and differentiates into shoots. These shoots are excised, transferred to rooting medium and eventually planted into soil. The major advantages of this transformation procedure are its simplicity and reliability. It is also a versatile procedure which has proven successful for a variety of higher plants. However, this process requires the use of tissue culture techniques which are somewhat laborious and can introduce new mutations unlinked to the T-DNA insertion site.

A recent method of transformation involves a non-tissue culture approach where germinating seeds of *Arabidopsis* are co-cultured with *Agrobacterium* (Feldmann and Marks, 1987). For this method, seeds are imbibed for 12 hours before being exposed to *Agrobacterium* for 24 hours. The transformed T_1 seedlings are allowed to grow into mature plants and the resulting T_2 seeds are then germinated in the presence of selection (for example, kanamycin). This procedure is highly variable and results in transformation frequencies from 0.32% transformed T_2 plants to values which are considerably lower. The primary advantage of this method of transformation is that it obviates the need for tissue culture and thus might eventually be applicable to other plants for which regeneration is a problem. However, much work is still needed before it will be possible to assess fully the utility of this method.

FUTURE PROSPECTS

Thus far, genes isolated from *Arabidopsis* have been obtained largely through the use of either heterologous probes or differential screening with cDNA probes. However, these approaches are easily applied in any plant system and do not directly take advantage of the strengths of *Arabidopsis*. For many genes of interest only a mutant phenotype and a genetic map position are known and this information is sufficient for a method of molecular cloning for which *Arabidopsis* is particularly suited; i.e. chromosome walking. Chromosome walking has been employed to clone genes in other organisms such as *Drosophila* (Bender et al., 1983); *Arabidopsis*, however, is the only known plant system in which chromosome walking is relatively easy, due both to the small genome and the near absence of middle repetitive DNA (Leutwiler et al., 1984; Pruitt and Meyerowitz, 1986).

The first step in obtaining a molecular probe closely linked to the desired gene can be accomplished by constructing a restriction fragment length polymorphism (RFLP) map (Botstein et al., 1980). RFLPs are produced by alterations in DNA structure (such as base changes, insertions, or deletions) in allelic sequences of different individuals and are detected by genomic DNA blot procedures. They segregate as Mendelian co-dominant markers. An RFLP map of the *Arabidopsis thaliana* genome has been constructed consisting of over

85 single-copy molecular markers in five linkage groups (Chang et al. 1988). The map is based on two crosses: one was between the wildtype Niederzenz ecotype (Nd-0) and a strain of the Columbia ecotype carrying three recessive visible mutations, *angustifolia* (*an*), *glabra-1* (*gl-1*) and thiazole requiring (*tz*) on chromosomes 1, 3 and 5 respectively; the other cross was between Nd-0 and a line of the Landsberg ecotype carrying the *er* mutation, on chromosome 2 and two floral mutations, *apetala-2* on chromosome 4 and *clavata-1* on chromosome 1. All three ecotypes are rapid flowering strains originally isolated in Europe and show an estimated 1 to 2 % divergence in their respective DNA sequences. Separate digestion of genomic DNA with three different enzymes and subsequent probing with randomly-chosen low-copy sequences with an average size of 12.5 kb, revealed polymorphisms between ecotypes 40-50% of the time depending on the strains (Chang et al. 1988).

An internally consistent map of approximately 470 centimorgans and consisting of five linkage groups has been established by analysis of the segregation of polymorphic molecular and visible markers in meiosis. The five linkage groups have been assigned to their respective chromosomes and the orientation of chromosomes 1 and 5 have been determined relative to the visible map, with the most probable orientations of the other chromosomes shown in Figure 3 (Chang et al. 1988).

Using the estimated haploid genomic size of *Arabidopsis* of 70,000 kb (Leutwiler et al., 1984), the average density of RFLPs is currently more than one RFLP every megabase pair and the average distance from any locus to the nearest RFLP clone is a few hundred kb. Actual correlation between physical and genetic distance has not yet been established for any genomic region, but a rough conversion would be the genome size in kb divided by the total genetic length in centimorgans, or approximately 150 kb per centimorgan.

Once a molecular probe closely linked to a gene of interest has been identified (preferably linked by less than two centimorgans), a walk to the desired gene can proceed via isolation of successive overlapping clones from a genomic library with large inserts. *Arabidopsis* is the only known higher plant suited to this cloning strategy for two major reasons. Firstly, the relation of genetic and physical distance makes it feasible to construct an RFLP map with markers of sufficient density to allow the average distance to any locus to be less than a few hundred kb (about 120 markers). Other plants in which RFLP maps have been constructed, such as maize (Helentjaris, 1987) and tomato (Bernatzky and Tanksley, 1986), would need over 6000 and 1500 RFLPs respectively, to achieve the same density per kb (using Feulgen densitometry for estimated DNA content, Bennett and Smith, 1976). Secondly, and more importantly, *Arabidopsis* is the only known higher plant in which successive overlapping clones may be isolated without extreme technical difficulty. This is because of the scarcity of middle repetitive sequences in its genome. The mean length of single copy DNA in *Arabidopsis* has been estimated to be 120 kb (Pruitt and Meyerowitz, 1986), whereas in pea and tobacco the figures are 0.3 kb (Murray et al., 1978) and 1.4 kb (Zimmerman and Goldberg, 1977) respectively

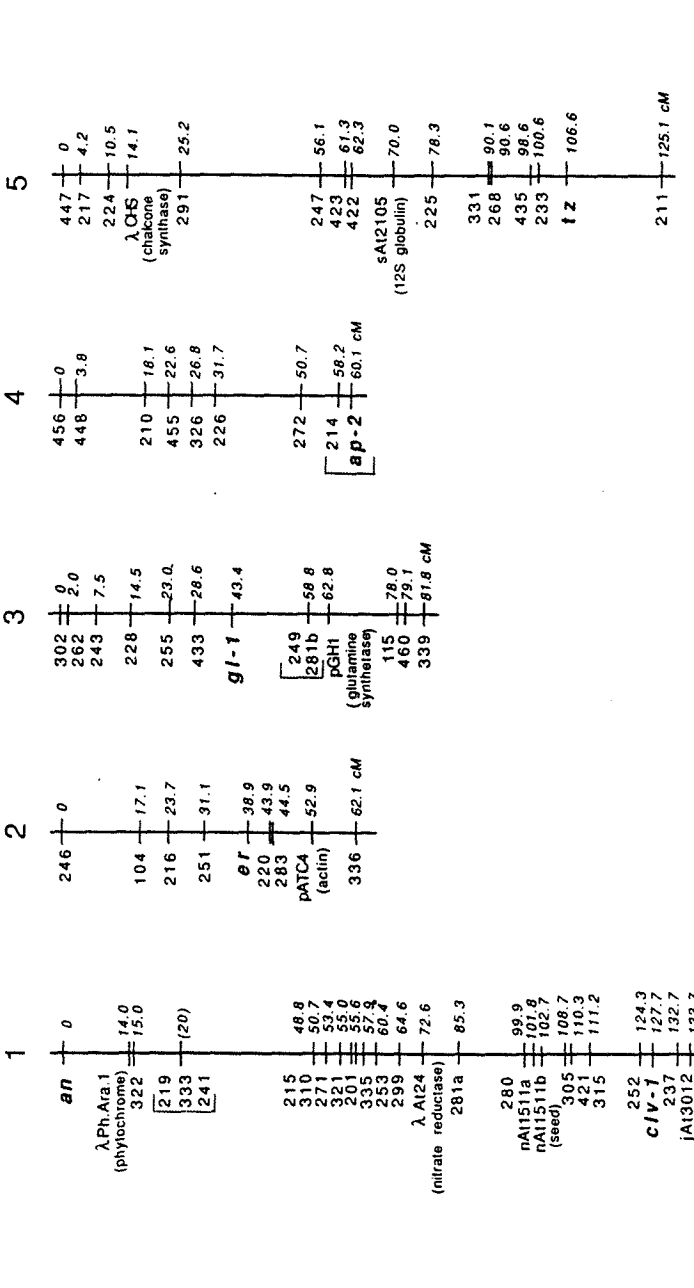

Figure 3. RFLP map of Arabidopsis thaliana *genome. Markers are designated by the number or name of the genomic clone that detects the RFLP at the indicated position. Visible markers were placed using segregation data of the crosses discussed in the text. Known (chromosomes 1 and 5) and probable (chromosomes 2, 3 and 4) orientation of the chromosomes with respect to the visible map are shown. All distances are in centimorgans. Note: many additional markers are currently being added to this RFLP map (Chang et al. in preparation).*

(Table 1). Furthermore, the small size of the *Arabidopsis* genome greatly facilitates the construction and repetitive screening of genomic libraries.

The clone that contains the gene of interest can be identified by complementation of the mutant phenotype through *Agrobacterium* mediated DNA transformation (see methods of transformation). As discussed earlier, transformation of an *alcohol dehydrogenase* (*Adh*) null mutant with a cloned copy of the *Arabidopsis Adh* gene resulted the complementation of the mutant phenotype (Chang and Meyerowitz in preparation), thus demonstrating the feasibility of this approach.

Obtaining these genes more quickly and with less labour may be facilitated by the construction of a physical map of the *Arabidopsis* genome. Random clones from a genomic library can be ordered into contiguous regions by "fingerprinting" restriction enzyme digests of large numbers of clones and identifying overlaps with the aid of a computer. A nearly complete set of ordered clones representing the entire genome could then be aligned with the visible map via alignment with the RFLP map. A complete set of ordered clones may never be obtained, but even a partial set will allow relatively large jumps during the walking steps. Two organisms for which partial sets of ordered clones have been isolated are the yeast *Saccharomyces cerevisiae* (Olsen et al., 1986) and the nematode *Caenorhabditis elegans* (Coulson et al., 1986), whose genome size is similar to that of *Arabidopsis thaliana*. The construction of an ordered library in *Arabidopsis* is currently in progress (Hauge et al., 1987).

Other recent advances may also greatly facilitate the methods of chromosome walking. Longer genetic distances can be covered in fewer steps than standard chromosome walking precedures by chromosome "jumping" (Collins and Weissman, 1984; Poustka et al., 1987). This technique involves the construction of special "jumping" libraries derived from digestion of genomic DNA with restriction enzymes that cut infrequently (i.e. *Not*I and *Sfi*I). Recently, jumping libraries have been used to map an 850kb region of human chromosome 4 (Poustka et al., 1987).

The cloning of even larger segments of DNA may be made feasible using artificial yeast chromosomes (YACs) (Burke et al., 1987) which consist of telomeric, centromeric and autonomously replicating (ARS) sequences from yeast and the exogenous insert DNA (greater than 400kb). The artificial chromosomes replicate and segregate as endogenous yeast chromosomes. Transformation and screening procedures are currently being worked out so that yeast libraries may approach the convenience of lambda and cosmid libraries. Pulse field gradient gel electrophoresis (Schwartz and Cantor, 1984; Carle et al., 1986; Davis et al., 1987; Smith and Cantor, 1987), which allows the separation and isolation of very large fragments of DNA (up to several megabases), provides a critical technology for analyzing artificial yeast chromosomes, chromosome jumps and genomic digests with restriction enzymes that cut infrequently. Together, these technologies should eventually allow the rapid isolation of any gene for which a mutant phenotype exists.

One further method of cloning, which may eventually be applicable for some genes in *Arabidopsis*, involves gene rescue by shotgun cloning and complementation. This method is probably feasible only for phenotypes which can be selected for or screened at the tissue culture stage since it is at present unwieldy to attempt the regeneration of tens of thousands of transformed plants. Many plant transformation vectors currently exist which are useful for construction of genomic libraries and such a bank from *Arabidopsis* need only contain 5,000-10,000 individual clones in order to have a 99% probability of containing any sequence. This approach to gene cloning was successfully tested recently in a model system (Klee et al., 1987a).

Once a gene has been cloned, many techniques are available to elucidate its function. Often the amino acid sequence of the protein product, predicted from the nucleotide sequence, will provide clues as to its function. The tissue- and temporal- specificity of expression of cloned genes may be determined through *in situ* and Northern blot hybridization to RNA. In addition, antisera directed against cloned gene products may provide a sensitive means to determine sub-cellular localization.

The regulatory sequences of cloned genes provide molecular tools to dissect further gene function and to understand the underlying regulatory mechanisms involved in tissue- and temporally- specific expression. For example, fusing the upstream regulatory sequences of cloned genes to easily assayable markers, such as β-glucuronidase or firefly luciferase, provides a very sensitive assay for expression. These fusions are easily used to identify the *cis*-acting regulatory sequences and may provide a means to select for mutations in *trans*-acting regulatory elements. For example, by fusing *cis*-acting promoter elements to markers such as alcohol dehydrogenase, it is possible to overexpress this enzyme in a particular tissue type. Since the activity of this enzyme is lethal to plants grown in the presence of allyl alcohol, mutant plants can be readily screened for their ability to survive this selection. Once obtained, these mutations are easily characterized, mapped and eventually cloned by chromosome walking.

After patterns of expression are established, functions of the gene product can be postulated. For example, the homeotic floral mutation *apetala-3* causes conversion of petals to sepals and a partial conversion of stamens to carpels. If expression of the gene is limited to presumptive petal and stamen cells, one may infer that possible functions of the gene product are the reception of positional signals, or a step in a biochemical pathway necessary for the formation of specialized structures common to only petals and stamens. If, however, it is expressed in presumptive sepal and/or carpel cells, it is possible that the gene product is part of a positional signaling between cells in the floral meristem. Hypotheses concerning hormonal involvement may be proposed if the expression is limited to organs distant from the floral meristem.

From the examples discussed earlier, it is evident that phytohormones have global effects on development. To separate these

actions it is imperative to have the ability to regulate the production of phytohormones in a tissue- and time- specific manner. One approach to this would involve transforming plants (wildtype and null mutants to control for endogenous production) with cloned phytohormone genes under control of tissue- and/or time-specific promoters. *Agrobacterium* auxin biosynthetic genes have recently been shown to function in transgenic petunia plants producing a phenotype consistent with the overproduction of auxin such as shoot apical dominance, epinastic effects and increased secondary xylem and phloem cell growth (Klee et al., 1987c). Although these types of experiments will undoubtedly provide considerable insight into hormone action, it will eventually be necessary to isolate the plant phytohormone biosynthetic loci. These plant genes will provide a wealth of information simply by determining when and where during development they are expressed.

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APPENDIX FOUR

SUMMARY OF SEGREGATION DATA FROM CROSSES

cross AxB	wild type	A	B	AB	$\chi 2$	p
ap2-1 pi-1	142	48	38	17	1.86	.5-.7
ap2-1 ap3-1	114	28	31	8	3.55	>.3
ap2-2 pi-1	20	8	12	2[a]	2.82	.3-.5
ap2-2 ap3-1[b]	30	9	5	1	3.41	>.3
ap2-2 ag-1	135	52	58	2	1.97	.5-.7
ap2-2 sup-1	62	22	38	7	9.86	.05-.01
pi-1 sup-1[c]	112	115[d]	34	d	51.4	<.001
ap3-1 ap1-1	49	28	20	11	7.85	.05
pi-1 ap1-1	58	18	18	4	0.83	.7-.9
ap1-1 sup-1	18	4	6	2	0.55	>.9
ap3-1 clv1-1	53	15	13	3	2.39	.5
pi-1 clv1-1	44	7	14	5	3.57	>.3

[a]These doubles were progeny tested to be sure of genotype.

[b]Plants with the *ap3-1* phenotype were selfed at 16°C, and ones identified that segregated 3:1, wild type : double mutant phenotype.

[c]Plants with the *pi-1* phenotype were crossed to *sup-1* to identify double mutants. The plants from this cross were then selfed and the progeny will be scored. Since the *sup-1* mutation was only outcrossed once after its induction with EMS, perhaps there is a lethal segregating with *sup-1* causing the apparently odd segregation numbers. It is also possible that *pi-1* has an effect when heterozygous but this is unlikely considering the phenotypes of the two mutations. Further crosses are in progress to check this possibilty.

[d]This class contains both A and AB since they are indistinguishable phenotypes and must be progeny tested to identify genotype.

ap1-1 ap2-9 plants were identified by selfing a plant homozygous for *ap1-1* and heterozygous for *ap2-9*. The progeny segregated 19:7, *ap1-1* : double.

Some of the large $\chi 2$ values in the preceding and following tables may be a reflection of the small number of progeny scored in some crosses.

cross A B C*	wt	A	B	C	AB	BC	AC	ABC	χ2/p
ap2-1 pi-1 ag-1	137	71	47	60	24	20	3	1[a]	1.83/>.95
ap2-1 ap3-1 ag-1	155	74	54	67	20	17	9	4	7.61/.3-.5
ap2-1 pi-2 ag-1	118	25	43	61	9	22	1	3	27.4/<.001
ap2-2 pi-1 ag-1	194	138[b]	96	68	b	33	5	1[a]	20.6/.01-.001
pi-1 ag-1 sup-1	91	46[c]	34	7[d]	9[e]	4	c	e	24.2/<.001

[a]More of these crosses were planted to obtain several triple mutants but the numbers of each phenotype segregating were not recorded.

[b]This number represents A + AB, since they are phenotypically difficult to distinguish.

[c]This number represents A + AC, since they are phenotypically difficult to distinguish.

[d]See c on page A-53.

[e]This number represents AB + ABC, since they are phenotypically difficult to distinguish.

*Another cross between *ag-2* and *sup-1* was performed by Detlef Weigel and the segregation numbers are presently being recorded.

*The *ap2-1 ag-1 sup-1* was identified in the following cross:

$$\frac{pi\text{-}1\ ag\text{-}1\ ap2\text{-}1}{pi\text{-}1\ +\ +} \times \frac{sup\text{-}1}{sup\text{-}1}$$

The segregation numbers were: wt, 30; *sup-1*, 7; *pi-1* + *pi-1 sup-1*, 14; *ag-1*, 4; *ap2-1*, 4; *ag-1 sup-1*, 1; *ag-1 pi-1* + *ag-1 pi-1 sup-1*, 3; *ap2-1 pi-1*, 2; *ag-1 ap2-1*, 3; *ap2-1 ag-1 sup-1*, 1; *ap2-1 ag-1 pi-1* + *ap2-1 ag-1 pi-1 sup-1*, 2. More plants from this cross have been planted out.

*The 'cauliflower' phenotype was identified in a cross between *ap1-1* (Ler) and an *ag-2* heterozygote (Ws). In a family that did not segregate *ag-2* the numbers were:

wt 108 *ap1-1* 29 *ap1-1* 'cauli' 8

None of the *ap1-1* 'cauli' were *erecta*.

*Many more plants of each of the crosses listed in the tables were examined, but numbers of segregants were not recorded in most cases.