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Genetic Separation of Third and Fourth Whorl Functions of *AGAMOUS*

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***AGAMOUS* (*AG*) is an Arabidopsis MADS box gene required for normal development of the third and fourth whorls of the flower. In previously described *ag* mutants, the third whorl stamens are replaced by petals, and the fourth whorl is replaced by another (mutant) flower. We describe two new *ag* alleles, *ag-4* and *AG-Met205*, retaining partial *AG* activity. Both produce flowers with stamens in the third whorl and indeterminate floral meristems; however, *ag-4* flowers contain sepals in the fourth whorl, and *AG-Met205* produces carpels. The *ag-4* mutation results in partial loss of the C terminus of the K domain, a putative coiled coil, and *AG-Met205* contains a site-directed mutation that causes a single amino acid change in this same region of the K box. Two models that might explain how these changes in *AG* result in the separation of different *AG* activities are discussed.**

INTRODUCTION

A common theme in development is that individual genes are able to affect multiple developmental processes. For instance, the Drosophila genes *wingless* and *hedgehog* are required for segment polarity early in embryogenesis. *wingless* is also required for proper differentiation of imaginal discs during the later stages of development (Couso et al., 1993), and *hedgehog* is required for movement of the morphogenetic furrow in eye development (Ma et al., 1993). In Arabidopsis, seedling viability, photomorphogenesis, and anthocyanin pigmentation patterns are all affected by mutations in a series of genes termed *FUSCA* (reviewed in Chasan, 1994). Mutations in another gene, *TRANSPARENT TESTA, GLABRA* (*TTG*), result in a loss of trichomes, a loss of anthocyanin pigments, and aberrations in the seed coat (Koornneef, 1981). In Arabidopsis flowers, a group of homeotic genes responsible for specification of multiple organ types has been described. The group includes *APETALA1* (*AP1*), *APETALA2* (*AP2*), *APETALA3* (*AP3*), *PISTILLATA*, and *AGAMOUS* (*AG*). *AG* is essential for the specification of stamens in the third whorl, specification of carpels, and conferring determinacy to the floral meristem (Bowman et al., 1989). The *AG* gene was cloned by Yanofsky et al. (1990) and contains a MADS domain, which is a region common to many transcription factors, and has been shown to bind DNA (Mueller and Nordheim, 1991; Huang et al., 1993; Shiraishi et al., 1993). Another potentially important domain, the K box, has also been recognized in plant MADS box genes (Ma et al., 1991). This region of the gene product is predicted to form coiled coils and might be important in protein–protein interactions.

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A model that describes the roles of floral homeotic genes in specification of floral organ identity places these genes into three classes that have activities spanning two adjacent whorls. They act either alone or in combination with the activity of another class with a partially overlapping expression domain to specify the identity of the flower organs that arise in each whorl (reviewed in Coen and Meyerowitz, 1991; Jack et al., 1993). Class A gene activity is required in the outer (first and second) two whorls of the flower, class B genes act in the second and third whorls, and the class C gene (*AG*) functions in the inner two (third and fourth) whorls. One important question ensuing from this model is how the activity of a homeotic gene in two adjacent whorls specifies organs of two different identities. For instance, *AG*, a class C gene, is required for normal development of third and fourth whorl floral organs. In the third whorl, *AG* is required for specification of stamens; the fourth whorl *AG* functions can be described either as suppression of a fourth whorl flower or as a specification of carpels and provision of determinacy to the floral meristem. One possibility is that each of the floral organ identity genes performs the same function in each whorl, for example, activating the same set of genes, and the combination of activated genes results in the appropriate organ being specified. Alternatively, the homeotic genes could have different activities in each whorl of their expression domains. For instance, interactions with different cofactors in the two whorls could result in activation of different sets of genes, resulting in the production of different organs.

We investigated the separability of whorl-specific functions in *AG* through analysis of a partial loss-of-function allele, *ag-4*, and an allele created by in vitro mutagenesis. The strong *ag* mutant phenotype is an indeterminate reiteration of sepals–

petals–petals (Bowman et al., 1989). Flowers from *ag-4* plants show only a subset of this phenotype. In place of the fourth whorl carpels, there are four sepals, and the floral meristem continues to proliferate, just as in strong loss-of-function alleles; however, unlike other *ag* alleles, stamens arise in the third whorl, as occurs in the wild type. We examined other *AG* functions in *ag-4* to determine which functions are intact and which are separable. The *ag-4* mutation affects splicing of an exon that contains the coding region for the C terminus of the K domain. An additional test of this region was performed by constructing transgenic plants containing a single amino acid change in the same region. Possible interpretations of how lesions in the K domain differentially affect the activities of *AG* are discussed.

RESULTS

Genetic Dissection of *AGAMOUS* Functions

Flowers from wild-type Arabidopsis *ag-3*, *ag-4*, and *ag-3*/*ag-4* *trans* heterozygotes are shown in Figures 1A to 1D and Figures 2A to 2F. Wild-type flowers have been described previously by Smyth et al. (1990). Floral organs are organized in four whorls, typically with four sepals in the first (outer) whorl, four petals in the second whorl, six stamens in the third whorl, and two fused carpels in the fourth whorl (Figure 1A). The *ag-3* flower has also been described previously by Bowman et al. (1991a). *ag-3* is a strong allele: flowers show homeotic conversions of the organs in the third whorl of the flower; petals replace the third whorl stamens that arise in the wild type. The fourth whorl can be interpreted as either replacement of the fourth whorl organs with a new (*ag*) flower or as four sepals replacing the two carpels along with continued proliferation of the floral meristem (Figures 1B and 2D). Flowers from *ag-4* plants display a subset of the strong *ag-3* phenotype (Figures 1C, 2B, and 2C). The third whorl organs differentiate into stamens, as occurs in the wild type, but as in the strong *ag* alleles, the fourth whorl appears as an internal flower. Both *ag-3* and *ag-4* flowers typically produce eight to 10 internal flowers. The *ag-4* third whorl stamens are not entirely wild type; for pollen-producing stamens, the plants must be grown at 16°C.

In addition to characterizing the identity of the organs that arise in *ag-4* flowers, in Table 1 we present a quantified analysis of these organs. These data show another effect of *ag-4* on stamens; there is a slight reduction in stamen number in *ag-4* (mean of 5.4) compared with the wild type (mean of 5.8). The *ag-3*/*ag-4* *trans* heterozygote has floral organs with identities similar to *ag-4* (Figure 1D). Table 1 also includes data on the number and organ types that arise in the next internal flower. In the *ag-4* mutants, organs arising from internal flowers are reduced in number relative to the first three whorls of wild-type flowers (3.4 sepals and 4.5 stamens/petals in the first internal *ag-4* flower versus 14 organs for whorls 1 to 3 of the wild type) and relative to the first internal flower of *ag-3* mutant flowers (3.5 sepals and 8.7 petals). Most *ag-4* interior floral organs are stamens or petaloid stamens and sepals or petaloid sepals, although patches of stigmatic tissue and ovules also occasionally appear.

ag-4 ap2 Double Mutants

We also addressed the question of whether the *ag-4* gene product retains the potential to affect first and second whorl organs when ectopically expressed in *ap2* mutants. Strong *ap2* mutants, such as *ap2-2*, produce flowers with first whorl organs that have features of carpels and with second whorl organ primordia that fail to initiate, as shown in Figures 1E and 2J (Bowman et al., 1991a). In contrast, a weak *ap2* allele, *ap2-1*, produces leaflike first whorl organs and has only a slight reduction in the number of second whorl organs, which are staminoid (Figures 1H and 2G; Bowman et al., 1989). Ectopic *AG* expression in the outer whorls of *ap2-2* but not in *ap2-1* has been shown by in situ hybridization (Drews et al., 1991). That this ectopic expression accounts for the major phenotypic differences between these two alleles was shown genetically; both the *ag-3 ap2-1* (Figure 1J) and the *ag-3 ap2-2* (Figure 1G) double mutants produce leaflike first whorl organs and staminoid petals in the second whorl (Bowman et al., 1991a).

To test whether the *ag-4* gene product retains the potential to affect first and second whorl organs, we constructed *ag-4 ap2-2* and *ag-4 ap2-1* double mutants. If the *ag-4* gene product is not able to affect the first and second whorls when ectopically expressed, then we would expect these two double mutant phenotypes to be similar. Flowers from *ag-4 ap2-2* plants are shown in Figures 1F, and 2K and 2L, and flowers from *ag-4 ap2-1* plants are shown in Figures 1I, and 2H and 2I. In both mutant backgrounds, the first whorl organs are mostly leaflike, similar to the first whorl organs of *ag-3 ap2-2* flowers. However, in contrast with the *ag-3 ap2-2* double mutant, no second whorl organs are produced in the *ag-4 ap2-2* double mutant. Partially dissected young *ag-4 ap2-2* and *ag-4 ap2-1* flowers are shown in Figures 2L and 2I, respectively. A petaloid organ is visible in the *ag-4 ap2-1* flower, but none could be detected in the *ag-4 ap2-2* doubles. Of 39 flowers counted, an average of 5.4 stamens in total are visible in the second and third whorls in *ag-4 ap2-2*. Thus, in an *ap2-2* background, the *ag-4* gene product retains the ability to suppress initiation of second whorl organ primordia; however, it is largely defective in specifying carpels in the first whorl. Another characteristic of the *ag-4 ap2* double mutants is an increased carpelloid character of the fourth whorl organs, which are occasionally fused along their margins (data not shown) and show continued proliferation within the fused structure.

AP1 Expression in *ag-4*

One of the functions of *AG* is to regulate *AP1* negatively in the inner two whorls of the flower (Gustafson-Brown et al., 1994).

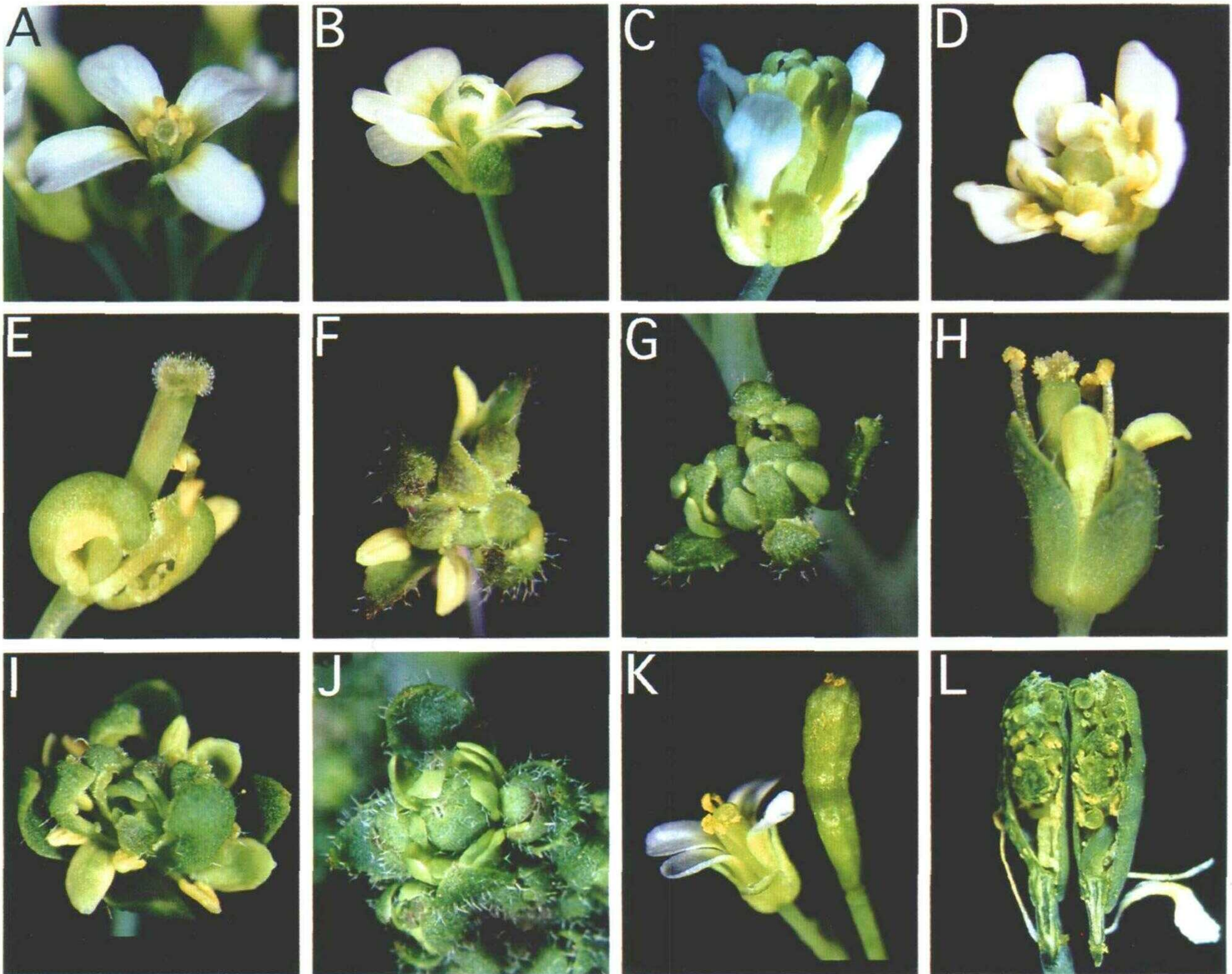

Figure 1. Phenotypes of Flowers from the Wild Type and Single and Double Mutants of Arabidopsis.

(A) Wild-type flower.
(B) *ag-3.*
(C) *ag-4.* Note third whorl stamens and fourth whorl sepals.
(D) *ag-3/ag-4 trans* heterozygote.
(E) *ap2-2.*
(F) *ap2-2 ag-4.*
(G) *ap2-2 ag-3.*
(H) *ap2-1.*
(I) *ap2-1 ag-4.*
(J) *ap2-1 ag-3.*
(K) *AG-Met205 ag-3.*
(L) *AG-Met205 ag-3.* Silique opened up to show stamens and carpels interior to the first whorl of carpels.

We assessed the distribution of *AP1* RNA in *ag-4* mutants using in situ hybridization, as shown in Figures 3A to 3H. In wild-type plants, *AP1* expression can be detected early in flower development (stage 2 and 4 flowers, Figures 3A to 3D) in the flower primordia but not in the inflorescence meristem. In the stage 2 flower, *AP1* RNA is uniformly distributed. During stage 4, *AP1* expression declines in the central region of the floral meristem, resulting in an expression pattern restricted to the first and second whorls of the flower; this pattern is complementary to that of *AG* expression (Mandel et al., 1992). In contrast, in *ag-4* flowers, the RNA remains in the central region of the flower (Figures 3E to 3H). Figures 3E and 3F show stage 2

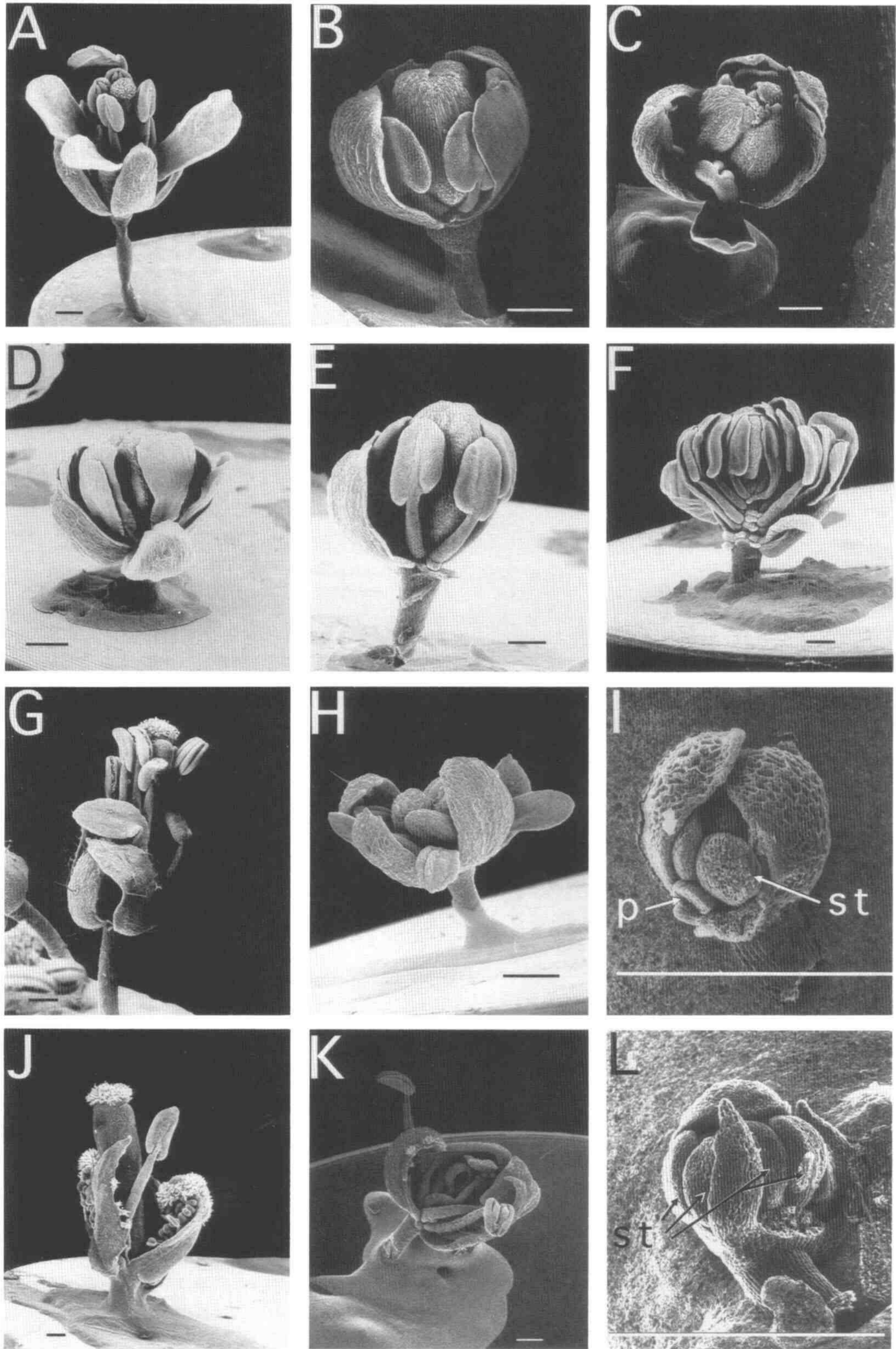

Figure 2. Scanning Electron Microscopy of the Arabidopsis Wild Type and *ag*, *ap2*, and Double *ag ap2* Mutants.

Table 1. Organ Counts in *ag-4*, *ag-3*, and the Wild Type

Genotype[a]	First Whorl	Second Whorl	Third Whorl	Next Internal Flower
Wild type (Landsberg *erecta*)	4 sepals	4 petals	5.8 stamens	
ag-4	4 sepals	4 petals	5.4 stamens	3.4 sepals[b]; 4.5 stamen/petals
ag-3	4 sepals	9.76 petals		3.5 sepals; 8.7 petals

[a] For wild-type flowers (Landsberg *erecta*), 52 flowers were counted from 12 plants between the fifth and fifteenth flowers on the plant. For the *ag-4* flowers, 56 flowers were counted from 11 plants, ranging from the fifth to fifteenth flowers. Thirty flowers were counted on eight *ag-3* plants, ranging from the fourth to the sixteenth flowers.

[b] On the *ag-4* flowers, 17 of 40 had stigmatic tissue on the fourth whorl organs, and 11 of 40 contained ovules along the margins of the fourth whorl organs.

and stage 5 flowers, and in both, *AP1* RNA is uniformly distributed. In an older *ag-4* flower (Figures 3G and 3H), *AP1* RNA can be detected in the proliferating meristem. Older organs, however, show a more variable pattern of *AP1* transcript abundance (data not shown). The absence of a phenotypic effect of *AP1* transcription in developing stamens indicates that *AP1* expression alone is insufficient to alter stamen identity. This finding is also shown by the absence of stamen alterations in plants transgenic for gene constructs constitutively expressing *AP1* (B.A. Krizek and E.M. Meyerowitz, unpublished data).

AG Expression in *ag-4* Mutants

The *ag-4* phenotype, which consists of indeterminate flowers with stamens in the third whorl and sepals in the fourth whorl, suggests that in mutants homozygous for this allele, third whorl functions are retained but fourth whorl functions are disrupted. Two alternative explanations for the *ag-4* phenotype are that either the *ag* expression domain is restricted to the third whorl or the *ag-4* gene product has been altered in such a way that only fourth whorl functions are affected. To distinguish between these two alternatives, the distribution of *ag* RNA within *ag-4* and wild-type flowers was assessed by in situ hybridization (Figures 3I to 3P). In the wild type, *AG* expression appears during stage 3 of flower development; expression occurs in the central region of the floral meristem that will give rise to the stamens and carpels (Drews et al., 1991). *AG* expression is maintained in the third and fourth whorls until late in development, when it is restricted to specific cell types (Bowman et al., 1991b). In *ag-4* mutant flowers, *ag* transcripts can also be detected in the third and inner whorls of the flower (Figures 3M to 3P). Later in development, *ag-4* transcripts are not always maintained, especially in organs that differentiate as sepals (Figure 3P). These results indicate that in *ag-4* mutants, failure to specify carpels and lack of floral meristem determinacy do not result from a lack of *ag* RNA accumulation in the appropriate pattern. *ag* RNA has also been detected in third and inner whorls of *ag-1* flowers (Gustafson-Brown et al., 1994).

Identification of the *ag-4* Lesion

Because the in situ hybridization pattern of *ag* RNA in *ag-4* flowers suggested that the mutation does not affect the *ag* expression pattern, we investigated the possibility that the *ag-4* mutation alters a domain that is required for a subset of the *AG* functions. We identified the *ag-4* mutation by sequencing both genomic and cDNA clones. The position of the mutation is shown in Figure 4B. The mutation is a change in the 3′ splice site from an AG to an AA in the fifth intron. cDNA clones were isolated and sequenced, and we found two forms of the *ag-4* cDNA (Figure 4C). The first one, as predicted from the genomic sequence, is the complete omission of the sixth exon. A second form was also found; it contains 6 bp in the place of exon 6. This variant might arise due to recognition of a cryptic 5′ splice site within exon 6. The affected region corresponds to

Figure 2. (continued).

(A) Wild type.
(B) and **(C)** *ag-4*. One sepal and one petal have been removed from the flower in **(B)** to show the organs of interior whorls.
(D) *ag-3*.
(E) and **(F)** *ag-3/ag-4 trans* heterozygotes. The flower in **(F)** has had organs removed from one side to show the reiteration of organ types.
(G) *ap2-1*.
(H) and **(I)** *ag-4 ap2-1*. The flower in **(I)** has been dissected to reveal the developing stamens (st) and petals (p).
(J) *ap2-2*.
(K) and **(L)** *ag-4 ap2-2*. The flower in **(L)** has been dissected to reveal the developing stamens (st) and the absence of second whorl organ primordia.
Bars = 300 μm.

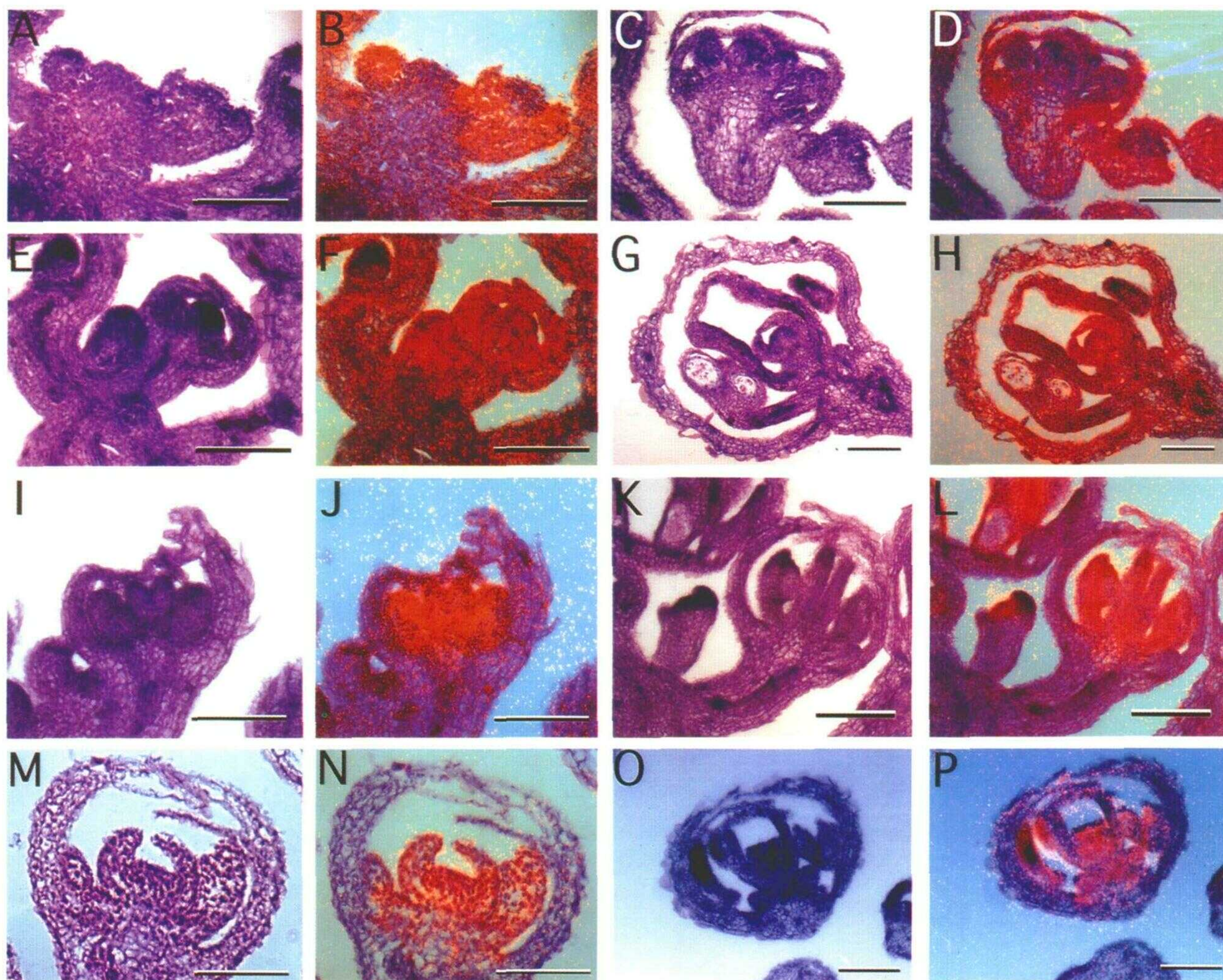

Figure 3. Distribution of *AP1* and *AG* RNA in the Flowers of the Wild Type and *ag-4* Mutant.

(A) to **(H)** In situ hybridizations using an *AP1* antisense RNA probe. In **(A)** to **(D)**, sections of wild-type tissue are shown. An inflorescence with stage 2 and stage 4 flowers is shown in **(A)** and **(B)**, and a stage 6 flower is shown in **(C)** and **(D)**. Sections of *ag-4* inflorescences are shown in **(E)** to **(H)**. An inflorescence with a stage 2 and stage 5 flower is shown in **(E)** and **(F)**, and an older *ag-4* flower showing internal flowers is shown in **(G)** and **(H)**.

(I) to **(P)** In situ hybridizations using an *AG* antisense RNA probe. Wild-type flower sections are shown in **(I)** to **(L)**. A stage 6 flower is shown in **(I)** and **(J)**, and a stage 8 flower is shown in **(K)** and **(L)**. Sections of *ag-4* flowers are shown in **(M)** to **(P)**. A stage 6 flower is shown in **(M)** and **(N)**, and **(O)** and **(P)** show an older flower that contains internal flowers.

(A), **(C)**, **(E)**, **(G)**, **(I)**, **(K)**, **(M)**, and **(O)** are bright-field images; **(B)**, **(D)**, **(F)**, **(H)**, **(J)**, **(L)**, **(N)**, and **(P)** are bright-field/dark-field double exposures using a red filter for the dark-field exposures. Bars = 100 μm.

the C-terminal end of the K box (Figure 4A), a motif conserved in plant MADS box genes and proposed to function in protein–protein interactions (Ma et al., 1991).

Arg-205 Mutation in *AGAMOUS*

We performed an additional test of the C-terminal region of the *AG* K domain. The design of this experiment was based on the temperature-sensitive *ap3-1* allele, a replacement of Lys by Met at position 153 at the end of the K box (Jack et al., 1992). We constructed transgenic lines (designated *AG-Met205*) containing an *AG* construct in which the Arg at position 205 was changed to a Met (the residue changed is indicated in Figures 4 and 5). These constructs also contained ~6 kb of upstream sequences, genomic sequences through the large second intron, cDNA sequences through the coding region, and ~600 bp of 3′ sequences. This mutant construct

and a wild-type version were transformed into wild-type plants and crossed to *ag-3* and *ag-4* mutants to test for each construct's ability to rescue the mutant phenotype. All five *AG-Met205* lines characterized in the *ag-3* background produce flowers with wild-type organ identity; however, instead of two carpels in the fourth whorl, there are four, as shown in Figure 1K (on rare occasions, some flowers fail to fuse the fourth whorl organs; data not shown). These carpels develop into misshapen siliques, that is, they are shorter than the wild type and irregularly thickened. A dissected silique is shown

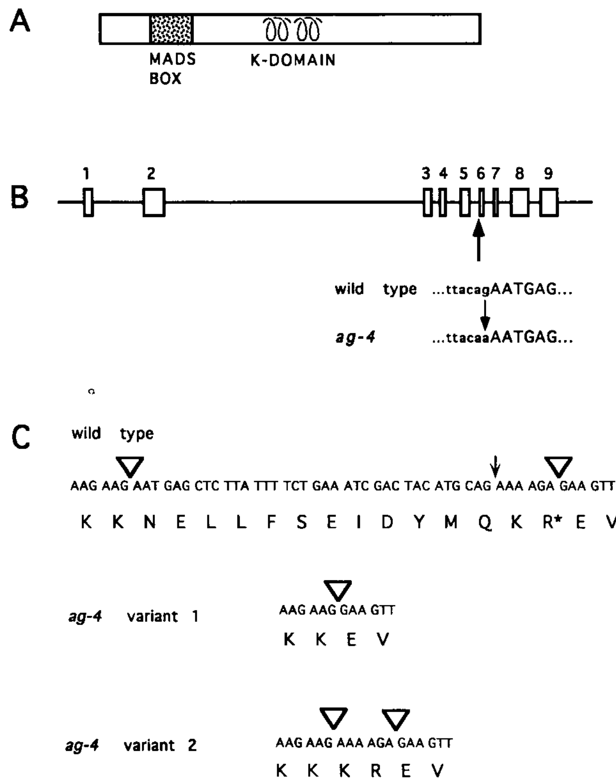

Figure 4. Identification of the Lesion in the *ag-4* Allele.

(A) Representation of the *AG* gene product. The MADS domain is indicated by a textured box, and the two putative coiled coils of the K domain are represented by curved lines.

(B) Map of the genomic region containing the *AG* gene. Numbered boxes indicate the characterized exons (Yanofsky et al., 1990), and the arrow indicates the location of the *ag-4* lesion. Below, intron sequences are indicated in lowercase letters, and exon sequences are in capital and boldface letters; the arrow indicates the G-to-A mutation in *ag-4*.

(C) cDNAs from the region around the lesion. Triangles indicate the positions of the splice sites. The wild-type sequence (top) shows all of exon 6 and the adjacent sections of exons 5 and 7. The arrow shows the position of the cryptic splice site presumably used in *ag-4* variant 2. The R* residue indicates the position of the amino acid changed to methionine in *AG-Met205*. The two *ag-4* variants (below) show the same parts of the region of exon 5; *ag-4* variant 1 retains none of exon 6, and variant 2 contains 6 bp between exons 5 and 7.

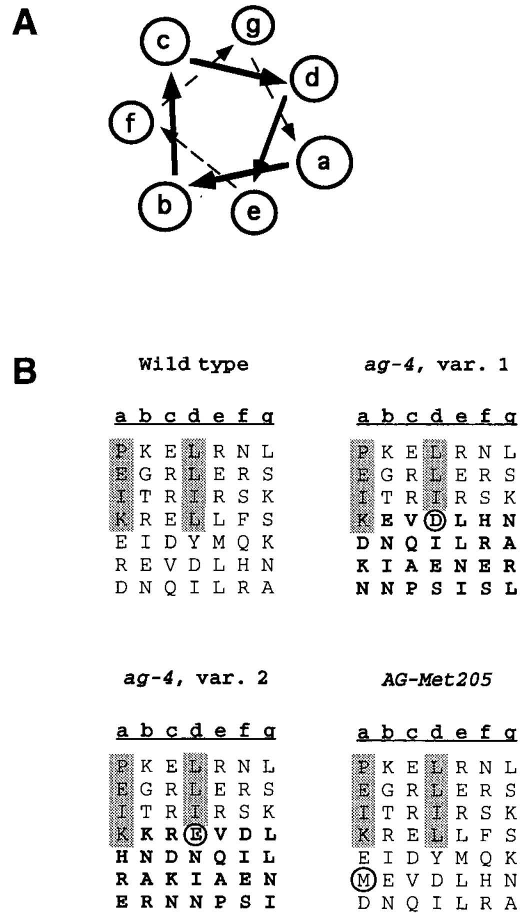

Figure 5. Possible Coiled-Coil Region Affected in *ag-4* Mutants.

(A) Heptad repeat configuration in coiled-coil motifs indicating positions of residues designated a to g as shown in **(B)**.

(B) Amino acid sequence from the second putative coil of the coiled-coil region through the area affected in *ag-4* mutants. The shaded boxes indicate possible alignments of residues designated a and d in the wild type. For the two *ag-4* variants, the altered sequences are indicated in boldface, and the residue change that occurs within the d position of the coiled coil is circled. For *AG-Met205*, the residue that has been changed is circled.

in Figure 1L. Interior to the four fourth whorl carpels, additional whorls of stamens and carpels arise. Three of these *AG-Met205* lines were also characterized in an *ag-4* background; flowers from these plants show the same increase in carpel number and floral indeterminacy as was observed for *AG-Met205 ag-3* flowers (data not shown). In contrast with this result, most transgenic lines carrying the wild-type construct fully rescued the *ag-3* mutant phenotype (five of seven fully rescued); however, in two lines carrying the wild-type construct in *ag-3/ag-3* homozygous plants, flowers are similar to the *AG-Met205* lines in that the flowers show an increase in carpel number and a lack of floral meristem determinacy. In contrast with the *ap3-1*

allele upon which the mutation was based, the *AG-Met205* lines did not show temperature sensitivity of *AG* function.

DISCUSSION

The goal of the studies described here was to determine whether the functions of *AG* are the result of a single activity and, if not, which aspects of the *ag* phenotype could be separated. Phenotypic analysis of *ag-4*, *AG-Met205 ag-3*, and *AG-Met205 ag-4* showed that stamen specification, carpel specification, and determinacy can be separated genetically, which suggests that they are the result of different *AG* activities. Furthermore, the temperature sensitivity of stamen development in *ag-4* mutants suggests that the late *AG* expression seen in this organ (Bowman et al., 1991b) might play a role in stamen or pollen development. These studies also revealed that the ability of *AG* to suppress initiation of second whorl organ primordia when ectopically expressed is the result of a function other than determinacy; *ap2-2 ag-4* double mutants retain second whorl organ suppression and are indeterminate. Thus, the functions of *AG* include potential suppression of second whorl organ primordia, specification (along with B function genes) of stamens, specification of carpels, and specification of a determinate floral meristem.

Models for *AG* Function

We can envision two general models to explain the partial loss-of-function phenotypes exhibited by *ag-4* and *AG-Met205*. First, it is possible that the loss of 12 and 14 amino acids in *ag-4* and the replacement of methionine for arginine at position 205 result in less stable *ag* gene products. This quantitative model suggests that the different *AG* functions vary with respect to the amount of gene product required. The *ag* phenotypes allow the functions to be ordered, from the highest amount required to the least, as follows: determinacy > carpel specification > stamen specification = second whorl primordium suppression. The quantitative model can be tested by constructing allelic combinations that would, in theory, either increase or decrease the amount of *ag* gene product and by examining the flowers for enhancement or suppression of specific mutant defects. We first tried reducing the amount of the *ag-4* gene product by constructing the *ag-4/ag-3 trans* heterozygote. If the quantitative model is correct, the reduced level of *ag-4* gene product might affect the identity of the third whorl organs. The *trans* heterozygote (Figures 1D, and 2E and 2F) produces flowers with stamens in the third whorl, indicating that a single copy of the *ag-4* gene is sufficient. This does not necessarily dispute quantitative models of gene action; rather, it might indicate that only very low levels of *ag-4* are required for stamen specification. Another allelic combination, *ag-4/ag-4 AG-Met205*, should have a higher level of the mutant gene products; flowers from these plants still show an indeterminate floral meristem. Again, the result does not necessarily dispute the quantitative model but might rather indicate that floral determinacy requires a high level of *AG*. Support for a quantitative model of *AG* activity comes from observations of transgenic plants containing a wild-type *AG* gene construct crossed to the strong *ag-3* mutant. Many lines completely rescued the mutant phenotype; however, two lines produce flowers with indeterminate floral meristems. Although we cannot rule out the possibility that the transgene acquired a spontaneous mutation in both lines, no gross rearrangements were detected; it is more likely that the transgene was inserted into a region of the genome that causes it to be expressed at a reduced level. Recent studies with *AG* antisense constructs have shown a range of flower phenotypes similar to those of *ag-4* and *AG-Met205 ag-3/ag-3*, also supporting the quantitative model (Mizukami and Ma, 1995).

The second model for *AG* function focuses on the proposed role of the K domain in protein–protein interactions. The two *ag-4* variants affect the C terminus of the second helix in the proposed coiled-coil motif called the K box, and *AG-Met205* contains an Arg-to-Met change 12 residues beyond the end of the putative second coil. As shown in Figure 5A, coiled coils are characterized by a heptad repeat in which the first (designated a) and the fourth (designated d) positions are usually occupied by hydrophobic and apolar residues (reviewed in Cohen and Parry, 1994). These motifs are found in a wide array of structural (e.g., keratins [Steinert et al., 1978]) and regulatory proteins (e.g., Myc, Fos, Jun, and GCN4 [Landschulz et al., 1988] and MATa1 and MATα2 [Ho et al., 1994]). The 3.5-residue turn of the coiled coil results in a cluster of apolar residues along one face and provides a domain for interaction with other polypeptides. The second proposed helix of *AG* and the region beyond the helix that is affected in the *ag-4* variants are shown in Figure 5B. Although we do not know that this region forms a coiled coil, we have placed boxes around potential a and d alignments (Figure 5B) of apolar residues. Both of the *ag-4* variants cause a replacement of Leu-194, a hydrophobic residue in a conserved position, with a charged amino acid (Asp or Glu) and change the identity of residues that follow this domain. It is attractive to hypothesize that these changes disrupt the hydrophobic face of the second coil, which in turn prevents interactions with cofactors required for carpel specification and determinacy (suppression of both A function and a fourth whorl flower). The *AG-Met205* transgene has a single amino acid change just beyond the second putative coil of the K domain and results in a disruption of a single *AG* function—specification of floral meristem determinacy. This residue is also within the region affected in *ag-4*; it is possible that the *ag-4* mutation results in disruption of two different cofactor interaction sites. Precedent for the activity of a MADS box gene being regulated by interactions with cofactors is provided by the yeast MCM1 gene (reviewed in Herskowitz, 1989). MCM1 is expressed in all three cell types (**a** and α haploid cells and **a**/α diploid cells) and contributes to specification of different mating types to the **a** and α cell types by interactions with different cofactors.

The mechanism for genetic separation of gene functions observed in the phenotypes of *ag-4* and *AG-Met205* might not be simply a question of quantitative versus qualitative levels; rather, both explanations might apply. That is, floral determinacy seems to require a larger amount of the *AG* gene product, as shown by the failure of some transgenic lines carrying a wild-type construct to rescue the *ag-3* mutant fully. That high level of *AG*, however, might have an absolute requirement for a domain that is C-terminal to the K domain. A full understanding of the nuances of quantitative effects of *AG* and the need for specific functional sites for cofactor interaction require highly specific antibodies to determine the levels of the gene product and to characterize interacting gene products.

METHODS

Plant Material

The wild-type strain used in these experiments was *Arabidopsis thaliana* ecotype Landsberg *erecta*. The *ag-4* allele was a generous gift from J. Alvarez and D. Smyth (Monash University, Clayton, Victoria, Australia). All plants were grown in constant light at 25°C in a 1:1:1 mix of vermiculite, perlite, and potting soil, except when *ag-4* was grown for crosses at 16°C to allow efficient pollen production. The *ap2-2 ag-3* and *ap2-1 ag-3* plants were derived from the stocks used in previous studies (Bowman et al., 1991a), which were designated as *ag-1* double mutants. We sequenced the *ag* allele from the *ap2-2 ag* stocks and determined that these plants actually contain the *ag-3* allele; its phenotype is identical to *ag-1*.

Scanning Electron Microscopy

Tissue for scanning electron microscopy was fixed, prepared, visualized, and photographed as described previously (Bowman et al., 1989; Smyth et al., 1990). Prior to coating the samples shown in Figures 2B, 2E, 2F, 2I, and 2L, the flowers were dissected, and organs were removed to allow visualization of the internal structures.

In Situ Hybridization

In situ hybridizations followed standard protocols (Cox and Goldberg, 1988; Drews et al., 1991), except that the tissue was fixed in FAA (50% ethanol, 5% acetic acid, 3.7% formaldehyde) for 1 hr. The *APETALA1* (*AP1*) and *AGAMOUS* (*AG*) probes have been described previously (Drews et al., 1991; Mandel et al., 1992).

Genomic and cDNA Clones

Our strategy for sequencing the genomic region of *ag-4* was to use the polymerase chain reaction (PCR) to amplify the two exon-containing regions on either side of a large 3-kb intron (Yanofsky et al., 1990). Oligonucleotide 012, 5'-TAGAATTCTTTCTTAAAAACTAT-3', corresponds to the *AG* genomic region just before exon 3, except that the third residue was changed to a G rather than to a T to provide a convenient restriction site for cloning. Oligonucleotide 013, 5'-AAGCATAGAATTCACTGATACAA-3', spans the EcoRI site just past the coding region of exon 9. These two primers result in amplification of the 3' half of the genomic regions containing exons 3 through 9. Oligonucleotide AGPCR14, 5'-TAGAGAGAATTCAAAAGAAAAATAATATTC-3', corresponds to the region just before exon 1, except that the sixth and the eleventh nucleotides were altered to provide a restriction site. Oligonucleotide AGPCR15, 5'-TAAGAATTCAGCACGAGAAGAAGAAGAAACCT-3', corresponds to the region just past the second exon, except that nucleotides 7 through 9 have been altered to provide a restriction site. Clones were obtained from three independent amplifications for each of the *ag-4* genomic regions, and both strands were sequenced by dideoxy methods using a Sequenase II kit (U.S. Biochemical Corp.) according to the manufacturer's recommended protocols. *ag-4* and wild-type flowers were collected and frozen in liquid nitrogen, and poly(A)$^+$ RNA was selected using the FastTrack kit (Invitrogen, San Diego, CA) according to the manufacturer's recommended protocols. *ag-4* transcripts were amplified using the cDNA cycle kit (Invitrogen) and *AG*-specific primers. A PCR product spanning exons 5 through 7 was sequenced directly, and seven clones containing this region were also sequenced. We found three clones corresponding to variant two (Figure 4B) and four clones corresponding to variant one.

Construction of *AG-Met205*

PCR-based mutagenesis was used to change the arginine at position 205 to a methionine. Oligonucleotides containing the mutation and flanking sequences were synthesized (tmut1, 5'-GACTACATGCAGAAAATGGAAGTTG-3'; tmut2, 5'-TCCCATTTTCTGCATGTAGTC-3'). These oligonucleotides were used to construct a transgene that contains 8 kb of upstream sequence and the genomic region through the large intron (intron 2; Yanofsky et al., 1990). The coding region 3' of the large intron was derived from the cDNA, and ~600 bp of the 3' flanking region was added. This construct was inserted into the plant transformation vector pCGN1547 (McBride and Summerfelt, 1990). The construct was transferred to Arabidopsis using root-mediated transformation (Valvekens et al., 1988).

Image Processing

Negatives and slides were scanned and digitized using a Nikon Coolscan (Nikon, Inc., Melville, NY); brightness and contrast were adjusted using Adobe Photoshop 2.5 (Mountain View, CA), and the color balance was similarly adjusted for the in situ hybridization double exposures. Final figures were printed using a Kodak XLS 8300 Digital Printer (Eastman Kodak, Rochester, NY).

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