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Functional Domains of the Floral Regulator AGAMOUS: Characterization of the DNA Binding Domain and Analysis of Dominant Negative Mutations

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The Arabidopsis MADS box gene *AGAMOUS* (*AG*) controls reproductive organ identity and floral meristem determinacy. The AG protein binds in vitro to DNA sequences similar to the targets of known MADS domain transcription factors. Whereas most plant MADS domain proteins begin with the MADS domain, AG and its orthologs contain a region N-terminal to the MADS domain. All plant MADS domain proteins share another region with moderate sequence similarity called the K domain. Neither the region (I region) that lies between the MADS and K domains nor the C-terminal region is conserved. We show here that the AG MADS domain and the I region are necessary and sufficient for DNA binding in vitro and that AG binds to DNA as a dimer. To investigate the in vivo function of the regions of AG not required for in vitro DNA binding, we introduced several *AG* constructs into wild-type plants and characterized their floral phenotypes. We show that transgenic Arabidopsis plants with a 35S-*AG* construct encoding an AG protein lacking the N-terminal region produced *apetala2* (*ap2*)-like flowers similar to those ectopically expressing AG proteins retaining the N-terminal region. This result suggests that the N-terminal region is not required to produce the *ap2*-like phenotype. In addition, transformants with a 35S-*AG* construct encoding an AG protein lacking the C-terminal region produced *ag*-like flowers, indicating that this truncated AG protein inhibits normal AG function. Finally, transformants with a 35S-*AG* construct encoding an AG protein lacking both K and C regions produced flowers with more stamens and carpels. The phenotypes of the *AG* transformants demonstrate that both the K domain and the C-terminal region have important and distinct in vivo functions. We discuss possible mechanisms through which AG may regulate downstream genes.

INTRODUCTION

Genetic studies have uncovered many genes that control flower development (for reviews, see Coen and Meyerowitz, 1991; Coen and Carpenter, 1993; Ma, 1994; Weigel and Meyerowitz, 1994; Yanofsky, 1995). In Arabidopsis, *LEAFY* (*LFY*), *APETALA1* (*AP1*), *APETALA2* (*AP2*), and *CAULIFLOWER* (*CAL*) regulate floral meristem initiation, the *CLAVATA* (*CLV*) genes determine meristem size, *SUPERMAN* (*SUP*) and *LEUNIG* (*LEU*) control the expression patterns of other homeotic genes, and *AP1*, *AP2*, *APETALA3* (*AP3*), *PISTILLATA* (*PI*), and *AGAMOUS* (*AG*) specify floral organ identity. In particular, flowers of *ag* mutants exhibit the conversion of the reproductive organs (stamens and carpels) to perianth organs (petals and sepals) and indeterminate growth (Bowman et al., 1989), indicating that *AG* regulates the reproductive organ identity and floral meristem determinacy. *AG* was one of the first floral genes to be characterized molecularly (Yanofsky et al., 1990), and its product is similar in sequence to two transcription factors from humans (SRF) and yeast (MCM1) (Norman et al., 1988; Passmore et al., 1988, 1989) and to the product of the Antirrhinum floral homeotic gene *DEFICIENS* (*DEFA*) (Sommer et al., 1990). The conserved region was named the MADS box, for MCM1, AG, DEFA, and SRF (Schwarz-Sommer et al., 1990). More recently, the term MADS box has been used to designate the conserved DNA region, and the protein motif of 56 residues encoded by the MADS box has been referred to as the MADS domain (Mandel et al., 1992b; Huang et al., 1996). Several other Arabidopsis MADS box genes are known to regulate flower development (Jack et al., 1992; Mandel et al., 1992b; Goto and Meyerowitz, 1994; Kempin et al., 1995). Additional MADS box genes have been isolated from Arabidopsis and other plants, including homologs of the Arabidopsis floral regulatory genes (Ma, 1994; Purugganan et al., 1995; Yanofsky, 1995).

The AG protein can be divided into five regions based on sequence similarities with other plant MADS domain proteins (Ma et al., 1991), as shown in Figure 1: an N-terminal region (N); the highly conserved MADS domain (M); the moderately conserved K domain (K), which has sequence similarity to

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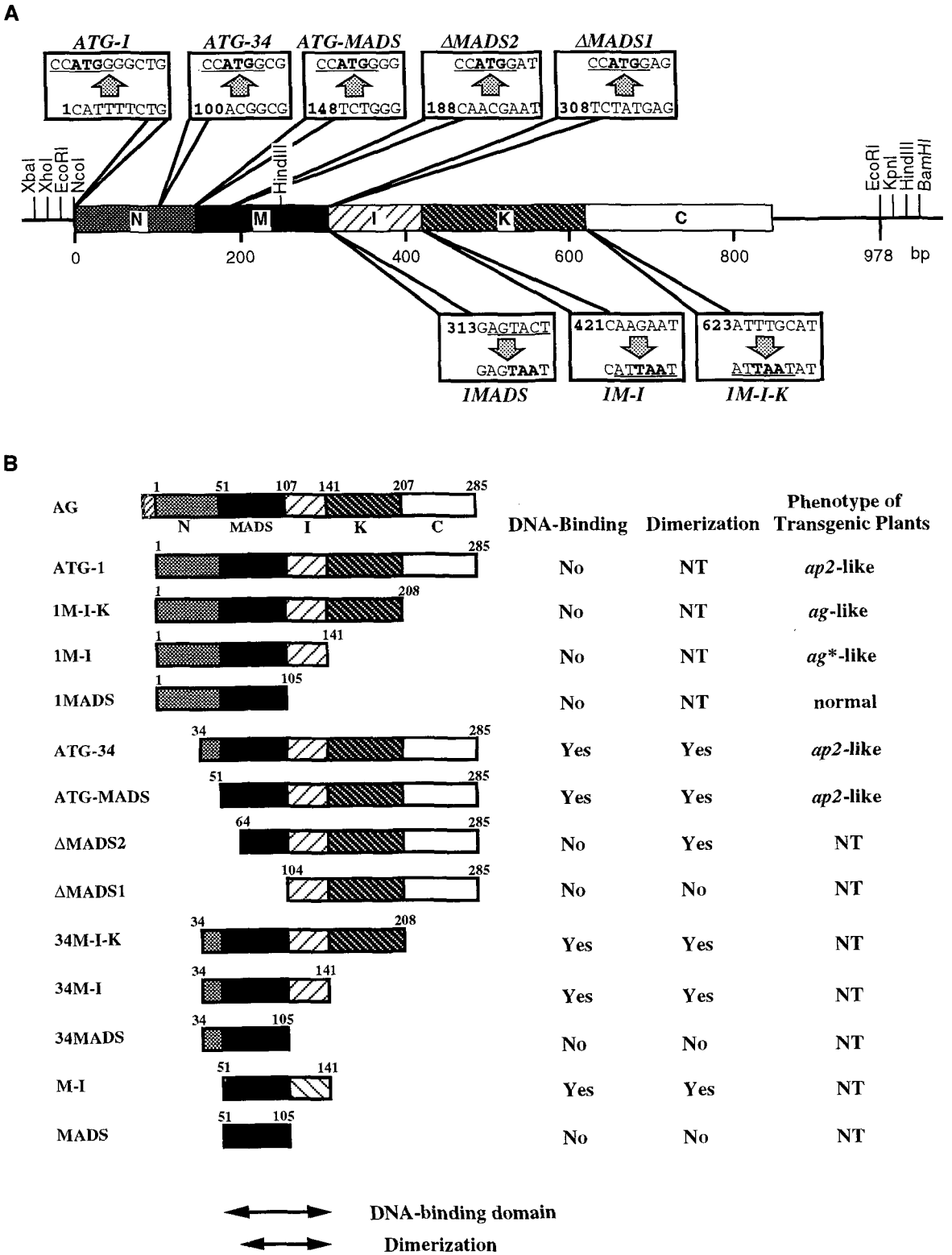

Figure 1. Schematic Representations of *ag* Mutations and Truncated AG Proteins.

keratins; a nonconserved intervening region (I region) between the MADS and K domains; and the nonconserved C-terminal region (C). Because the native AG N terminus has not been identified (Yanofsky et al., 1990), it is likely that the endogenous AG N-terminal region is slightly longer than that shown here (Figure 1). An N-terminal region is present in AG orthologs and in two other Arabidopsis MADS domain proteins, AGL1 and AGL5, which are similar to AG in amino acid sequence (Ma et al., 1991; Mandel et al., 1992a; Bradley et al., 1993; Kempin et al., 1993; Schmidt et al., 1993; Pnueli et al., 1994), suggesting that this region has been retained through evolution. In contrast, all other reported plant MADS domain proteins begin at the MADS domain (Purugganan et al., 1995). In SRF, the MADS domain is a part of the DNA binding domain, which also includes N-terminal (10 residues) and C-terminal (~25 residues) extensions (Norman et al., 1988). All previous DNA binding studies with AG used proteins containing at least a part of the N region, the MADS domain, and much of the I region (Mueller and Nordheim, 1991; Huang et al., 1993; Shiraishi et al., 1993). Therefore, it is not known what regions are essential for AG DNA binding. Also, it has not been demonstrated that AG binds to DNA as a dimer.

We previously reported that the ectopic expression of *AG* in transgenic plants resulted in the homeotic conversion of the outer whorl perianth organs to reproductive organs, as in flowers of *ap2* mutants (Mizukami and Ma, 1992). To study the in vivo function of the regions of AG that are not required for in vitro DNA binding, we introduced constructs expressing the relevant truncated AG proteins into *AG* wild-type plants and analyzed the function of these transgenes using the ectopic formation of reproductive organs as an in vivo assay. It is known that for proteins that are parts of a complex, it is possible to generate dominant negative forms such that an altered protein can inhibit the normal function of the coexisting endogenous one (Herskowitz, 1987). Because some of our altered AG proteins still retained DNA binding activity in vitro, and may do so in vivo, they may inhibit the function of the wild-type AG protein.

We present here results from our in vitro analysis of the AG DNA binding and dimerization domains. In addition, we describe the floral phenotypes of transgenic Arabidopsis plants carrying constructs encoding N- or C-terminally truncated AG proteins. We discuss the likely function of different regions of the AG protein and possible mechanisms of AG action.

RESULTS

AG Constructs

To investigate the function of the structural regions of the AG protein, we generated a number of mutations by using polymerase chain reaction (PCR) and site-specific mutagenesis, as shown in Figure 1A. We previously generated two *AG* constructs by PCR (Mizukami and Ma, 1992): *ATG-1*, which has an initiation ATG codon within an NcoI site engineered at the beginning of our longest *AG* cDNA; and *ATG-34*, which has an introduced NcoI site, with the ATG codon replacing an ACG codon for threonine at the position corresponding to the initiation codon in several *AG* orthologs (Mandel et al., 1992a; Bradley et al., 1993; Kempin et al., 1993; Pnueli et al., 1994). To examine the function of the remaining part of the N region, we used PCR to generate *ATG-MADS*, which has the ATG codon at the beginning of the MADS domain. To test the function of the AG MADS domain in DNA binding and dimerization, we made two additional N-terminal deletions, both of which affected the MADS box: Δ*MADS1*, which starts at the beginning of the I region, and Δ*MADS2*, which starts at the 14th residue of the MADS domain. In addition, to investigate the function of the I, K, and C regions, we introduced into the *AG* cDNA three nonsense mutations, resulting in premature translational terminations: *1MADS*, *1M-I*, and *1M-I-K*, which terminate at the end of the MADS, I, and K regions, respectively. These three mutant *AG* cDNAs are as long as the *ATG-1 AG* cDNA, and the corresponding transcripts are very close in length to

Figure 1. (continued).

(A) Site-directed *AG* mutations. A schematic representation of the *AG* cDNA, with the translated portion represented by boxes, one for each region: N, N-terminal region; M, MADS domain; I, I region; K, K domain; and C, C-terminal region. The 3′ nontranslated region is represented by a line. The flanking polylinker restriction sites, the internal HindIII site, and the number of base pairs are indicated. Each mutation is shown with a box containing the wild-type and mutated sequences, with the arrows indicating the direction of the change; the number preceding each wild-type sequence is the number for the first nucleotide shown here, as it was numbered previously (Yanofsky et al., 1990). For N-terminal deletions, shown above the *AG* cDNA, the NcoI site is underlined, and the ATG initiation codon is in boldface. Each of these constructs lacks the DNA sequences upstream of the corresponding engineered NcoI site. For the C-terminal premature terminations, shown below the *AG* cDNA, the lost (ScaI for *1MADS*) or gained sites (AseI for *1M-I-K* and *1M-I*) due to the mutations are underlined, and the nonsense codons are in boldface. The cDNAs carrying these nonsense mutations have the same length as the ATG-1 (transgenic plants) or ATG-34 (*E. coli*) cDNAs. The orientation of the *AG* cDNA shown here with respect to polylinker sites flanking the EcoRI sites was used for constructs for *E. coli* expression.

(B) Truncated AG proteins. The numbers indicate the boundaries of the regions and the positions of the AG truncations. The double-headed arrows indicate the AG regions for DNA binding and dimerization. DNA binding reactions were performed with the 1M-I-K, 1M-I, and 1MADS proteins, but no binding was detected (data not shown). See Table 1 and the text for a description of *ap2*-like and *ag*-like phenotypes; *ag**-like represents the phenotype of an increased number of stamens and unfused carpels. NT, not tested.

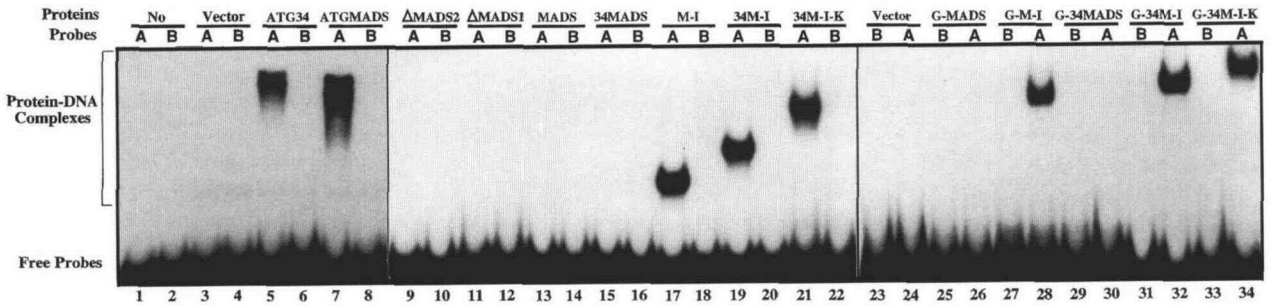

Figure 2. The AG DNA Binding Domain.

Autoradiography of a gel mobility shift assay with truncated AG proteins. Probe A is oligo(A), and probe B is oligo(B). The extracts used are as follows: lanes 1 and 2, no protein; lanes 3 and 4, an extract from the cells carrying only the pET9d vector; lanes 5 to 22, extracts containing one of the AG proteins; lanes 23 and 24, an extract containing only GST from cells carrying the pET19b vector; and lanes 25 to 34, extracts containing one of the GST-to-AG fusion proteins (G represents GST). On the basis of band intensity on protein gels, the approximate molar concentrations of the AG truncated proteins relative to that of M-I were estimated as follows: ATG-34, 0.5; ATG-MADS, 1.5; ΔMADS2, 5; ΔMADS1, 5; MADS, 5; 34MADS, 5; M-I, 1; 34M-I, 2; and 34M-I-K, 5. Similarly, the approximate molar concentrations of the GST fusion proteins relative to that of GST-34M-I were estimated as follows: GST-MADS, 20; GST-M-I, 5; GST-34MADS, 5; GST-34M-I, 1; and GST-34M-I-K, 10. The slightly lower bands present in lanes with ATG-34 and ATG-MADS (lanes 5 and 7) are due to partially degraded proteins as detected using an antiserum (data not shown).

the endogenous *AG* mRNA. Constructs encoding AG proteins with both N- and C-terminal truncations (Figure 1B) were generated by combining portions of *AG* cDNAs carrying appropriate mutations: *34M-I-K* was generated from *ATG-34* and *1M-I-K*, *34M-I* from *ATG-34* and *1M-I*, *34MADS* from *ATG-34* and *1MADS*, *M-I* from *ATG-MADS* and *1M-I*, and *MADS* from *ATG-MADS* and *1MADS*.

AG DNA Binding Domain

Previous studies (Mueller and Nordheim, 1991; Shiraishi et al., 1993) have revealed that AG proteins without K and C regions can bind to DNA in vitro. However, whether the N and I regions are required for AG DNA binding is not known. To investigate which regions are necessary for DNA binding activity, we prepared *Escherichia coli* cell extracts containing various truncated AG proteins and used them to perform gel mobility shift experiments. We have shown previously that the ATG-34 AG protein can bind in vitro to specific nucleotide sequences, including a synthetic oligonucleotide, oligo(A) (Huang et al., 1993). Oligo(A) contains a CArG box (CCATTAATGG; see Methods) that matches the deduced AG binding consensus sequence and is a representative AG binding sequence (Huang et al., 1993). However, the longest available AG protein, ATG-1, could not bind to oligo(A) in vitro (H. Huang and H. Ma, unpublished data), although it probably binds to DNA in vivo because *ATG-1* transformants exhibited the same phenotype as did *ATG-34* transformants (Mizukami and Ma, 1992). There are a number of possible explanations for this result, including improper folding in vitro or an unnatural inhibitory region, because the native N terminus has not been determined (Yanofsky et al., 1990). Therefore, the significance of this in vitro result is not clear. Because in most *AG* orthologs the predicted initiation ATG codon is at the same position as that in *ATG-34*, we tested DNA binding with AG proteins that have the same N termini as ATG-34 and are truncated from the C terminus.

Our results shown in Figure 2 indicate that, in addition to ATG-34 (lane 5), an AG protein lacking the entire N region, ATG-MADS (lane 7), can bind to oligo(A), but ΔMADS2 (lane 9) and ΔMADS1 (lane 11), which lack part or all of the MADS domain, respectively, cannot. For truncations affecting the C-terminal regions, 34M-I-K (lane 21) and 34M-I (lane 19) can bind to oligo(A), but 34MADS (lane 15) and MADS (lane 13), which lack the I region, cannot. The smallest truncated protein that still binds to oligo(A) is M-I (lane 17). As controls, none of the proteins bind to oligo(B), an oligonucleotide with mutations in the CArG box (GGATGCATCC [Huang et al., 1993]; see Methods), and extract from *E. coli* cells carrying only the pET9d vector does not bind to either nucleotide probe. It is formally possible that potential very weak binding by 34MADS and MADS-truncated AG proteins may have been masked by the faint nonspecific bands, or, due to their small size, the shifts could be masked by the signal from the free probe. Therefore, we generated glutathione *S*-transferase (GST) fusion proteins with several truncated AG proteins and tested them for DNA binding (Figure 2). Our results show that GST-M-I (lane 28), GST-34M-I (lane 32), and GST-34M-I-K (lane 34) were all able to form complexes with oligo(A), but GST-MADS (lane 26) and GST-34-MADS (lane 30) could not form such complexes. These results indicate that the AG MADS domain and I region together are necessary and sufficient for DNA binding.

AG Dimerization Domain

It is known that SRF and MCM1 each bind to DNA as dimers. The mobility of the AG–DNA complex relative to those of SRF and MCM1 (Norman et al., 1988; Passmore et al., 1989; Mueller and Nordheim, 1991), when similarly sized proteins were used, suggests that AG also binds to DNA as dimers. Because dimer formation can be directly demonstrated by the detection of a heterodimer between two AG proteins of different lengths, we tested AG dimer formation in vitro using our truncated AG proteins. As shown in Figure 3A, when 34M-I-K was mixed with M-I (lane 8) or 34M-I (lane 9), a complex of intermediate mobility was observed between the complexes containing bound homodimers (compare lane 8 with lanes 3 and 5, and lane 9 with lanes 4 and 5). As expected, no binding was detected using oligo(B). These results indicate that the intermediate band in each case represents the heterodimer of different truncated AG proteins and that AG can bind to DNA as a homodimer in vitro. We have observed that the AG heterodimers were formed when two AG proteins were mixed while denatured but were not formed when the proteins were in soluble form, presumably as homodimers (data not shown). These observations suggest that AG exists in solution as a stable dimer.

We found that the N-terminal truncated proteins ΔMADS2 and ΔMADS1 did not bind to DNA individually in our gel mobility shift assays. However, it is known that the N-terminal half of the SRF MADS domain is not required for dimerization (Norman et al., 1988). Therefore, it is possible that the heterodimers involving ΔMADS2 or even ΔMADS1 could form in solution, and the heterodimers may or may not bind to DNA. To test this, we mixed increasing amounts of ΔMADS1 or ΔMADS2 with 34M-I, then used the mixtures in DNA binding reactions. We found that excess amounts of ΔMADS2, but not of ΔMADS1, competed in the reaction and reduced the amount of 34M-I–DNA complexes (Figure 3B). These results indicate that ΔMADS2 could still form heterodimers with 34M-I but that ΔMADS1 could not. Furthermore, no new complexes were formed when ΔMADS2 was added, indicating that the ΔMADS2/34M-I heterodimer could not bind to DNA. Therefore, the N-terminal quarter of the MADS domain is not required for dimerization in vitro, but at least part of the remainder of the MADS domain is necessary. Because the N-terminal quarter of the MADS domain is required for DNA binding, it probably contacts DNA.

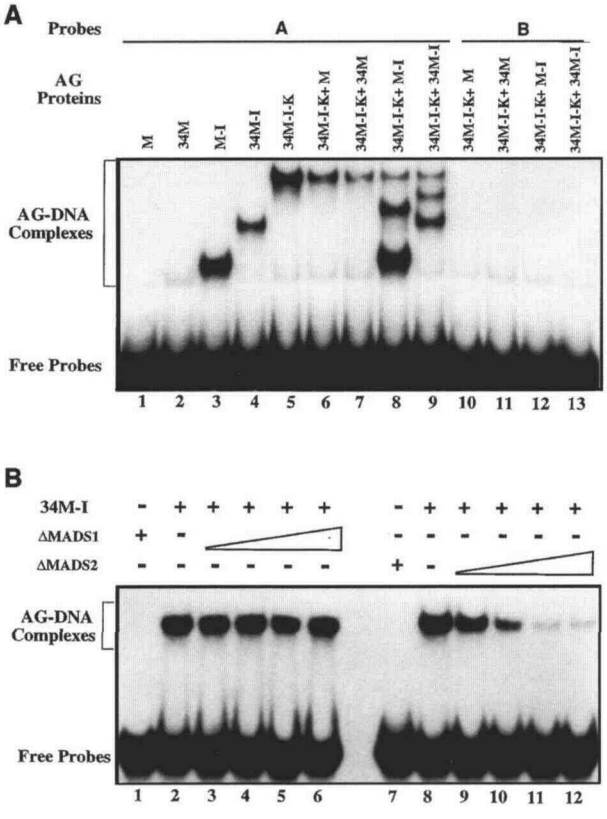

Figure 3. The AG Dimerization Domain: Autoradiography of Gel Mobility Shift Assays.

(A) Heterodimers between different AG truncated proteins detected as band shifts. The very faint bands represent nonspecific binding. Probes A and B are oligo(A) and oligo(B), respectively. The faint bands most likely represent nonspecific binding.

(B) Analysis of competition with DNA for 34M-I by ΔMADS1 or ΔMADS2. The plus and minus signs indicate the presence and absence of the protein, respectively. The open triangles indicate increasing amounts of the proteins as added from left to right. From protein gels, the estimated molar ratios of 34M-I to ΔMADS1 or ΔMADS2 are 1:1 (lanes 3 and 9), 1:3 (lanes 4 and 10), 1:6 (lanes 5 and 11), and 1:12 (lanes 6 and 12).

In Vivo Analysis of AG Domains in Transgenic Plants

Our in vitro analyses indicated that the AG MADS domain and I region are sufficient for DNA binding. However, the functions of the K domain and N and C regions, which are not required for in vitro DNA binding, have not been investigated. To learn about the in vivo functions of these regions, we have introduced fusions of the cauliflower mosaic virus (CaMV) 35S promoter to the mutant *AG* cDNAs into plants via Agrobacterium-mediated transformations and examined their floral phenotypes based on our previous study with transgenic plants carrying the 35S-*ATG-1* and 35S-*ATG-34* constructs (see below).

As shown in Figures 4A and 5A, a wild-type Arabidopsis flower consists of four concentric whorls of floral organs: four sepals, four petals, six stamens, and a gynoecium with two fused carpels. Normal *AG* expression is restricted to the inner two whorls (Yanofsky et al., 1990; Drews et al., 1991). In *ap2* mutant flowers, sepals and petals are transformed into carpels and stamens, respectively (Figures 4B, 4C, and 5C) (Komaki et al., 1988; Bowman et al., 1989; Kunst et al., 1989),

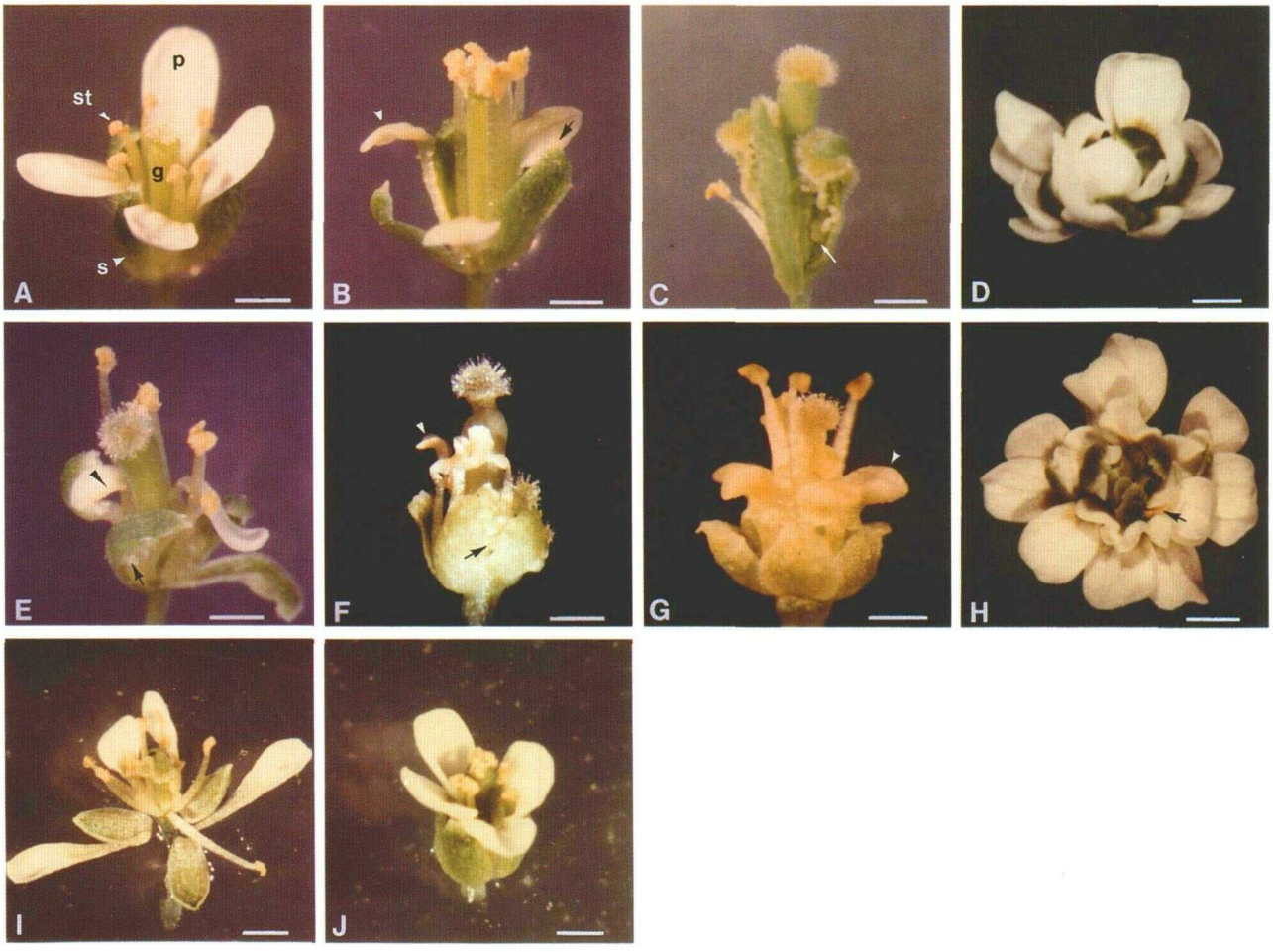

Figure 4. Flowers of the Wild Type, *AG* Transformants, and Mutants.

(A) A wild-type flower showing first whorl sepals (s), second whorl petals (p), third whorl stamens (st), and a central gynoecium (g) with fused carpels.
(B) A flower from the weak *ap2-1* mutant. The petals (arrowhead) are yellowish and narrower than those of wild-type flowers, and the sepals have stigmatic papillae at the tips (arrow).
(C) A flower from the severe *ap2-2* mutant. The medial first whorl organs are carpelloid organs with ovules (arrow). The second whorl petals are absent, and only one third whorl stamen is seen.
(D) An *ag-1* flower.
(E) A flower from the *ATG-1W1* transformant with a weak *ap2*-like phenotype. The sepals have carpelloid features such as stigmatic papillae (arrow), and petals are reduced in their number and size. An arrowhead points to a staminoid region of the abnormal petal.
(F) A flower from the *ATG-34S1* T_0 (primary) transformant with a severe *ap2*-like phenotype. The flower has carpels with ovules (arrow) in the first whorl and staminoid petals (arrowhead) in the second whorl. The *ATG-34S1* flowers were sterile.
(G) A flower from the *ATG-MADS* T_0 transformant 5502-11-2. The petals (arrowhead) are narrower than normal.
(H) A flower from the *1M-I-K* transformant 144-3 with a severe *ag*-like phenotype. A staminoid petal is indicated (arrow).
(I) A flower from the *1M-I* transformant 53-3. The flower has seven stamens, instead of the normal six, and partially unfused carpels.
(J) A flower from the *1MADS* transformant 198-1.
Bars = 500 mm.

and *AG* expression expands to the outer two whorls (Drews et al., 1991). These observations suggested that AP2 is a negative regulator of *AG* mRNA accumulation in the outer two whorls and that ectopic *AG* expression is responsible for the formation of reproductive organs in the outer whorls of *ap2* mutant flowers (Drews et al., 1991).

We have previously reported that transgenic plants carrying fusions of the 35S promoter with the *ATG-1* and *ATG-34 AG* cDNAs exhibited *ap2*-like homeotic conversion of perianth organs to reproductive organs in the outer floral whorls (Mizukami and Ma, 1992). Because the flowers of these *AG* transformants also produced stamens and carpels in the third

and fourth whorls, respectively, the ATG-1 or ATG-34 AG proteins did not interfere with the specification of reproductive organ identity in the inner floral whorls by the endogenous *AG* function. To probe the in vivo function of different structural domains or regions of the AG protein, we characterized transgenic plants with different 35S-*AG* constructs encoding truncated AG proteins for their floral phenotypes; in particular, we examined (1) whether the altered AG proteins retained the ability to convert organ identity in the first and second whorls, and (2) whether they interfered with endogenous *AG* function in the inner whorls.

For comparison, we briefly summarize here our previous results from *ATG-1* and *ATG-34* transformants. As shown in Table 1, we obtained 22 and eight independent transformants with 35S-*ATG-1* and 35S-*ATG-34* constructs, respectively. Fourteen of the *ATG-1* transformants and seven of the *ATG-34* transformants exhibited *ap2*-like floral phenotypes with conversion of sepals and petals into carpelloid and staminoid organs, respectively (Figures 4E, 4F, and 5G) (Mizukami and Ma, 1992). These transformants exhibited a range of *ap2*-like floral phenotypes that correlated with the levels of *AG* mRNA (Mizukami and Ma, 1992). A few transformants with the 35S-*ATG-1* or 35S-*ATG-34* constructs produced *ag*-like flowers, which were most likely the result of cosuppression (Table 1).

Table 1. Floral Phenotypes of *AG*-Transformed Plants

AG Construct	T_0 Transformants[a]	T_0 Transformants in Different Phenotypic Classes			
		ap2-like[b]	*ag*-like[c]	*ag**-like[d]	Nearly Normal[e]
ATG-1	22	14	3[f]	0	5
ATG-34	8	7	1[f]	0	0
ATG-MADS	5	4	0	0	1
1M-I-K	23	0	11	0	12
1M-I	9	0	0	3	6
1MADS	6	0	0	0	6
Control[g]	7	0	0	0	7

[a] The number of different T_0 transformants that were examined for floral phenotype.

[b] These plants produced flowers with conversion of perianth to reproductive organs. Five of the *ATG-1* plants, two of the *ATG-34* plants, and all of the *ATG-MADS* plants died before producing seeds.

[c] These plants produced flowers with conversions of reproductive to perianth organs and floral meristem indeterminacy. Four of the *1M-I-K* lines were completely sterile, similar to *ag-1* mutants; these *1M-I-K* lines could not be maintained.

[d] These plants had more stamens and carpels than normal. This phenotype is designated *ag**-like.

[e] These flowers were indistinguishable from wild-type flowers without dissection.

[f] This phenotype probably resulted from cosuppression of the *AG* gene and *AG* transgene, as is known to occur for transgenic plants (Napoli et al., 1990). The frequency of cosuppression here is similar to those of previously reported cases (e.g., Angenent et al., 1994).

[g] These plants carry the pMON530 vector.

The 35S-ATG-MADS Transgene Causes an ap2-like Phenotype

Because the complete removal of the N region of AG did not affect AG DNA binding activity in vitro, we tested the function of this region in vivo by characterizing the phenotypes of transgenic plants carrying the 35S-*ATG-MADS* construct. We obtained five independent transformants of the 35S-*ATG-MADS* construct, four of which produced *ap2*-like abnormal flowers with the homeotic conversion of perianth to reproductive organs (Figure 4G and Table 1). A nearly mature flower from a T_0 *ATG-MADS* transformant had carpelloid sepals with stigmatic papillae at the tips in the first whorl (Figures 5H and 5I) and narrower than normal petals in the second whorl (Figures 4G and 5H), like those in the weak *ap2-1* mutant (Figure 4B). Furthermore, like the severe *ATG-1* or *ATG-34* transformants, the *ATG-MADS* flowers were sterile. PCR was used to confirm that the *ATG-MADS* transformants carried the appropriate *AG* construct (data not shown). These results indicate that the AG N region is not needed for producing ectopic reproductive organs in the outer two floral whorls in 35S-*AG* transformants.

The 35S-1M-I-K Transgene Results in ag-like Phenotypes

We showed above that the AG C-terminal region is not required for either DNA binding or dimerization in vitro. To investigate the function of the C region in vivo, we examined the floral phenotypes of transgenic plants with the 35S-*1M-I-K* construct, which encodes an AG protein lacking the C region. In contrast to the transgenic plants with the *AG* N-terminal deletions, many of the plants with the 35S-*1M-I-K* construct produced abnormal flowers that exhibited conversion of the reproductive organs to perianth organs and nested flowers, resembling *ag* mutant flowers (Figures 4D and 5D) (Bowman et al., 1989), and the flowers of the 35S-*AG* antisense transformants (Mizukami and Ma, 1995). Eleven of a total of 23 independent *1M-I-K* transformants produced flowers with varying degrees of similarity to *ag-1* mutant flowers (Figures 4H and 5J to 5O, and Table 1), and the other 12 produced normal flowers (Table 1). Four *1M-I-K* transformants were phenocopies of the *ag-1* mutant and thus were completely sterile and could not be propagated. Two of the remaining transformants, 144-3 and 345-3, produced flowers with severe and intermediate *ag*-like phenotypes. Many flowers of these transformants were indeterminate and showed complete conversion of reproductive organs into perianth organs in the third and fourth whorls (Figures 4H, 5J, and 5L). These flowers, however, often produced some staminoid and carpelloid organs in the inner flowers (Figures 4H, 5K, and 5L). Occasionally, the flowers of the 144-3 and 345-3 transformants, after producing extra whorls of petaloid and sepaloid organs, produced a gynoecium at the center (Figure 5M) and a few seeds. A weak transformant, 350-1, had some flowers with petaloid and sepaloid organs in the third and fourth whorls, respectively (data not shown).

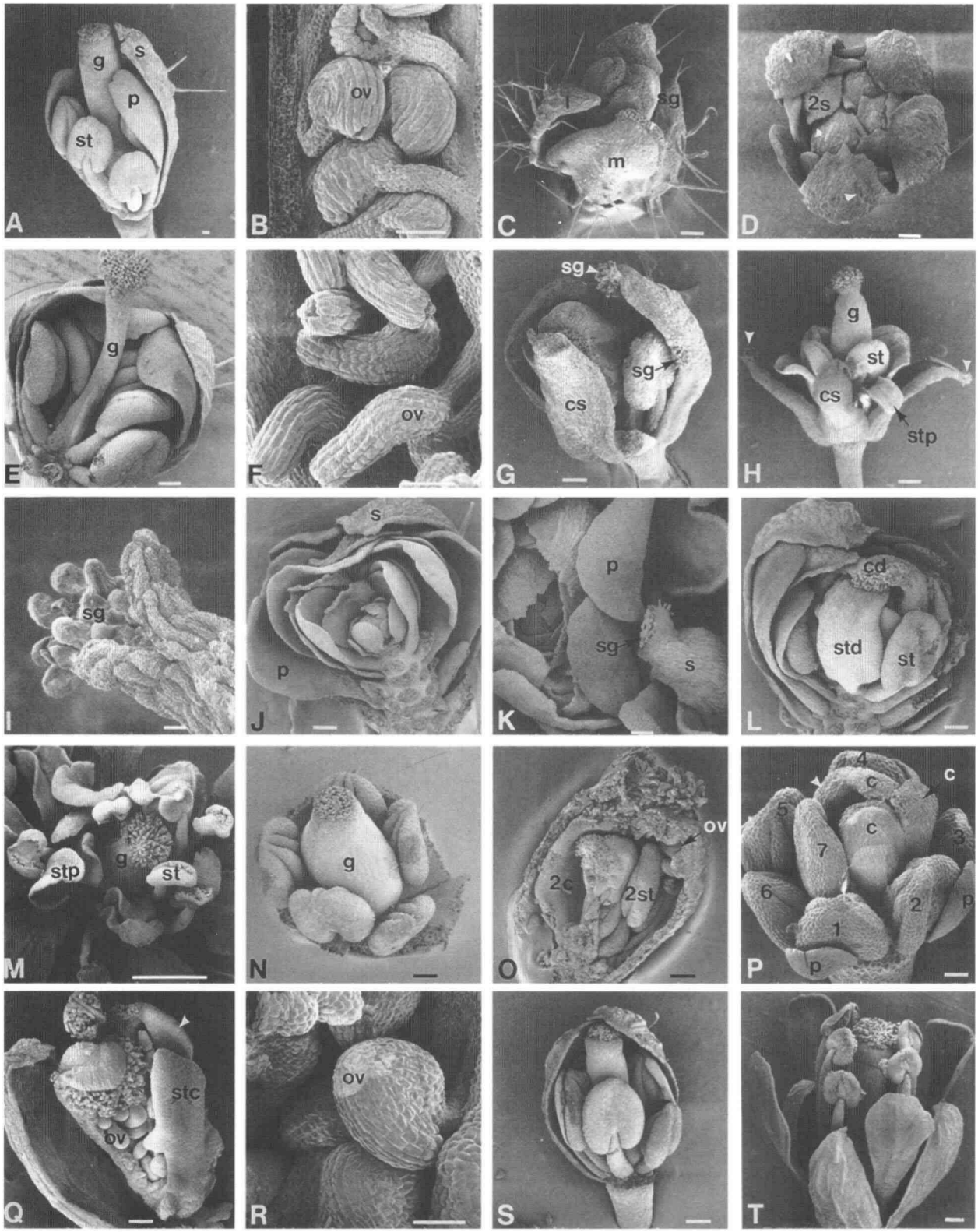

Figure 5. Scanning Electron Microscopy of Flowers and Ovules from the Wild Type, *AG* Transformants, and Mutants.

In addition, this transformant also produced many flowers with normal third whorl stamens and a central gynoecium; however, the gynoecium was much thicker than normal, even though it consisted of two fused carpels (Figure 5N). Upon dissection, in addition to a few ovules, a secondary flower with stamens and carpels was observed inside the gynoecium (Figure 5O). This type of indeterminate flower (the *Matryoshka* flower named after the Russian nested dolls) with normal reproductive organ identity was also observed in our antisense *AG* transformants (Mizukami and Ma, 1995).

It is known that sense constructs can sometimes cause reduced expression, exhibiting the phenomenon of cosuppression and resulting in mutant phenotypes (e.g., Napoli et al., 1990; see also above). However, the *ag*-like phenotypes of the *1M-I-K* transformants cannot be due to cosuppression in all of the transformants for the following reasons. First, in several *1M-I-K* transformants, two of which are shown in Figure 6, the levels of total *AG* mRNA are at least as high as that of wild type. Second, the *1M-I-K AG* transgene was found to be expressed in leaves of the transgenic plants (data not shown). Third, the appearance of *1M-I-K* transformants with *ag*-like phenotypes (48% of total transformants; Table 1) is much more frequent than that of *ATG-1* or *ATG-34* transformants with the *ag*-like phenotype (<15%; Table 1). Together, these results suggest that the C-terminal region is required for the alteration of organ identity of the outer floral organs to reproductive organs and that the 1M-I-K AG protein interferes with the function of the endogenous *AG* gene in the inner floral whorls.

*The 35S-*1M-I *Transgene Results in Increased Numbers of Reproductive Organs*

Transformants with the 35S-*1M-I* construct, which produced a truncated AG protein lacking both the K domain and the C region, produced flowers that had determinate flowers with normal stamens and unfused carpels and an increased number of reproductive organs (8.0 ± 1.1 stamens and 2.1 ± 0.3 carpels; Figures 4I and 5P to 5R). This floral phenotype was observed in three of nine independent *1M-I* transformants (Table 1), and the transgene was found to be expressed in flowers (Figure 6) and leaves (data not shown). A slightly increased number of stamens and carpels (some partially unfused) was

Figure 5. (continued).

(A) A nearly mature wild-type flower (stage 12; Smyth et al., 1990) consisting of sepals (s), petals (p), stamens (st), and gynoecium (g). Three sepals and a petal were removed to show interior reproductive organs.

(B) Wild-type ovule (ov).

(C) A nearly mature *ap2-2* flower with leaflike and carpelloid organs in the lateral (l) and medial (m) first whorls, respectively. Stigmatic papillae (sg) are seen at the tip of the carpelloid organ.

(D) A nearly mature *ag-1* flower. The arrowheads indicate large and long cells that are typical of sepal epidermis; the fourth whorl has four sepals (2s).

(E) A nearly mature *sup-1* flower with a reduced gynoecium (g). Two sepals, one petal, and two stamens were removed.

(F) Hair dryer–shaped *sup-1* mature ovules (ov).

(G) A nearly mature *ATG-1W1* flower that was the fourth to last flower of the primary inflorescence before its termination. Its phenotype is as severe as that of the severe *ap2-2* mutant flower **(C)**. cs, carpelloid sepal; sg, stigmatic papillae.

(H) *ATG-MADS* transformant 5502-11-2: a nearly mature flower from a T_0 plant. The medial first whorl organs have stigmatic papillae (arrowheads) at the tip, and the second whorl organs are staminoid petals (stp). Stamens (st) and the gynoecium (g) are found in the inner whorls. cs, carpelloid sepal.

(I) *ATG-MADS* transformant 5502-11-2: a more highly magnified image of stigmatic papillae (sg) at the tip of the first whorl organ.

(J) An *ag*-like indeterminate flower of the *1M-I-K* transformant 144-3. The flower consists of only sepals (s) and petals (p). The floral organs in half of the flower were removed.

(K) Sepals (s) and petals (p) of a flower of the *1M-I-K* transformant 144-3. A secondary sepal (s) is topped with stigmatic papillae (sg).

(L) A young flower from the *1M-I-K* transformant 345-3 with stamens (st) and chimeric organs in the extra inner whorls. The outer whorl perianth organs were removed to show the staminoid (std) and carpelloid (cd) organs.

(M) The center of a mature flower of the *1M-I-K* transformant 345-3. g, gynoecium; st, stamen; stp, staminoid petal.

(N) A young flower of a weak *1M-I-K* transformant 350-1. Although the flower developed normal stamens and carpels, the central gynoecium (g) is thicker than normal. All first whorl sepals, second whorl petals, and one of the third whorl stamens were removed.

(O) A dissected gynoecium of the 350-1 flower. The secondary stamen (2st) and carpels (2c) develop inside the indeterminate gynoecium. ov, ovule.

(P) A young flower of the *1M-I* transformant 53-3 with an increased number of stamens and carpels (c). The seven stamens are numbered; the seventh one occupies a position interior to the third whorl. One unfused carpel has a stamen-like epidermis (arrowhead). All sepals were removed. p, petal.

(Q) A dissected mature flower of the *1M-I* transformant 79-4. The center of the flower consists of two unfused carpels and a staminoid carpel (stc). A part of a carpel (arrowhead) exhibits staminoid features. Morphologically normal ovules (ov) were produced.

(R) Morphologically normal, mature ovules (ov) of the 53-3 transformant.

(S) A nearly mature flower of the *1MADS* transformant 81-1 with normal phenotype.

(T) A mature flower of the *1MADS* transformant 198-1 with normal phenotype.

Bars for **(B)**, **(F)**, and **(R)** = 50 μm; for **(M)** = 500 μm; and for **(A)**, **(C)** to **(E)**, **(G)** to **(L)**, **(N)** to **(Q)**, **(S)**, and **(T)** = 100 μm.

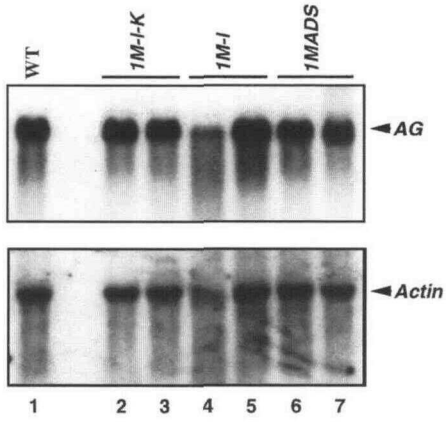

Figure 6. Expression of *AG* in Wild-Type and *AG*-Transformed Flowers.

AG expression in wild-type (WT) flowers (top; lane 1) is shown for comparison. RNAs from two transgenic lines for each of three C-terminal *AG* truncation constructs were analyzed and are as follows: *1M-I-K*, transformants 345-3 (lane 2) and 144-3 (lane 3); *1M-I*, transformants 53-3 (lane 4) and 79-4 (lane 5); and *1MADS*, transformants 81-1 (lane 6) and 198-1 (lane 7). They all show *AG* expression. Approximately 8 μg of total RNA from the wild-type flower was loaded in lane 1, and 5 μg of total RNA was loaded in all other lanes. Due to the similar sizes of the endogenous and transgenic *AG* transcripts, the signals represent the sum of the two. The same filter was hybridized with the *AAC1* actin cDNA (Nairn et al., 1988) at moderate stringency (bottom) as a control.

also observed when the *AG* function was moderately inhibited by antisense *AG* RNA (Mizukami and Ma, 1995); further, a milder phenotype of an increased number of fused carpels was observed in flowers of *ag-1/+* heterozygous plants (Mizukami and Ma, 1995). Therefore, the phenotypes of the *1M-I* transformants suggest that the 1M-I AG protein inhibits the normal AG function slightly, or only in a particular aspect, although it does not affect reproductive organ identity.

It is known that *sup* mutants produce flowers with an increased number of stamens (Figure 5E) (*flo10-1* [*sup-2*], 7.6 stamens [Schultz et al., 1991]; *sup-1*, 14.6 stamens [Bowman et al., 1992]; *sup-5*, 7.3 stamens [Gaiser et al., 1995]). However, if they are present, *sup* mutant ovules have an abnormal shape resembling a hair dryer (Figure 5F) (Gaiser et al., 1995), whereas the ovules of *1M-I* transgenic flowers had normal morphology (Figure 5Q), similar to that of wild-type ovules (Figure 5B). Therefore, the phenotype of the *1M-I* transformants is different from that of *sup* plants.

*Plants with the 35S-*1MADS *Transgene Produce Normal Flowers*

In vitro binding studies demonstrated that the I region is required for DNA binding as well as for dimerization (Figures 2 and 3). We found that transgenic plants with the 35S-*1MADS* construct encoding an AG protein without the I, K, and C regions exhibited no abnormal floral phenotype. All six independent transformants (Table 1) produced determinate flowers, without any homeotic organ conversions or organ number changes (Figures 4J, 5S, and 5T), that are indistinguishable from wild-type flowers (Figure 5A). Therefore, we conclude that the 1MADS protein neither had *AG* function in the outer whorls nor disrupted endogenous *AG* function in the inner floral whorls.

DISCUSSION

The DNA Binding and Dimerization Domains of AG

The AG protein contains a highly conserved sequence motif called the MADS domain. The MADS domain is present in transcription factors, such as SRF and MCM1, and is required for DNA binding by these proteins (Norman et al., 1988; Passmore et al., 1989; Mueller and Nordheim, 1991). In addition, the SRF DNA binding domain includes a small N-terminal region and a larger C-terminal region adjacent to the MADS domain. The AG protein and its orthologs from other plants all have an N-terminal region, the N region. So far, the N region of AG is the longest among those that have been described (Yanofsky et al., 1990; Mandel et al., 1992a; Bradley et al., 1993; Kempin et al., 1993; Schmidt et al., 1993; Pnueli et al., 1994). All previous DNA binding studies with AG involved proteins retaining at least the last 10 amino acid residues of the N region (Mueller and Nordheim, 1991; Huang et al., 1993; Shiraishi et al., 1993); therefore, it was not known whether these 10 residues are necessary for AG DNA binding. We showed here that an AG protein lacking its N region (ATG-MADS) can bind to a sequence with a CArG box, indicating that the N region is likely not necessary for DNA binding in vitro. Indeed, many other plant MADS domain proteins lack an N region (Purugganan et al., 1995). Thus, unlike SRF, plant MADS domain proteins may not require sequences N-terminal to the MADS domain for binding to DNA. Because this region has been maintained through evolution for AG and its orthologs, as well as some other closely related proteins (e.g., AGL1 and AGL5 from Arabidopsis) (Ma et al., 1991; Mandel et al., 1992a; Bradley et al., 1993; Kempin et al., 1993; Schmidt et al., 1993; Pnueli et al., 1994), it may have an in vivo function that is specific to this subfamily of MADS domain proteins.

It has been shown that the 10 residues N-terminal of the MADS domain of SRF are involved in DNA binding sequence specificity and affinity (Sharrocks et al., 1993; Nurrish and Treisman, 1995). It is possible that the N region of AG plays a similar role in DNA binding sequence specificity in vivo, one that allows it to target specific gene promoters. However, transgenic plants carrying constructs encoding AG proteins with or without the N region display similar phenotypes of organ identity conversion in the outer whorls, suggesting that whatever function the N region may have, it does not seem to be required for altering organ identity in the outer whorls in vivo. Our results with C-terminal truncations indicate that

the K and C regions are not required for DNA binding, in agreement with previous DNA binding studies with other truncated AG proteins (Mueller and Nordheim, 1991; Shiraishi et al., 1993). Our results do indicate, however, that the MADS domain and at least an N-terminal part of the I region are required for DNA binding.

Because SRF and MCM1 bind to CArG boxes as homodimers (Norman et al., 1988; Passmore et al., 1989; Tan and Richmond, 1990; Mueller and Nordheim, 1991), AG is also likely to form dimers. To confirm this hypothesis and determine domains necessary for dimerization, we set up binding reactions with mixtures of truncated AG proteins of different lengths. We found that AG indeed binds to DNA as a homodimer in vitro. In addition, AG can form heterodimers in vitro with AGL1, AGL2, or AGL3, three Arabidopsis MADS domain proteins (Huang et al., 1996). We also found that the first quarter of the MADS domain is not required for homodimer formation in vitro, but at least a part of the remainder of the MADS domain is required. These results are similar to those found with SRF (Norman et al., 1988) and indicate that the AG dimerization domain is smaller that the AG DNA binding domain. Furthermore, these results suggest that the first quarter of the AG MADS domain may be involved in contacting DNA, similar to SRF (Pollock and Treisman, 1991).

The three-dimensional structure of the SRF DNA binding domain complexed with DNA has recently been solved (Pellegrini et al., 1995). This structure reveals that the N-terminal portion of the SRF MADS domain indeed has several contacts with the DNA, and the region C-terminal to the MADS domain is important for dimerization, in agreement with our results for AG. We also observed that heterodimers were formed between different AG truncated proteins only after the proteins were denatured and then renatured, suggesting that the dimers are stable in solution. This concept is supported by the three-dimensional SRF structure, which indicates that the numerous hydrophobic interactions between the subunits are important for dimerization.

The C Region Is Essential for AG Function

We have shown that the ectopic expression of *AG* cDNAs with intact C-terminal regions results in *ap2*-like phenotypes (Mizukami and Ma, 1992). Removal of the C region from the AG protein, however, did not allow transgenic plants to produce an *ap2*-like floral phenotype, indicating that this region is required for *AG* function in specifying stamen and carpel identities in the outer floral whorls. Furthermore, the transgenic plants carrying such a construct (the 35S-*1M-I-K* construct) produced flowers similar to *ag-1* mutant flowers, with indeterminacy and organ conversion in the inner whorls, suggesting that an AG protein lacking its C-terminal region can inhibit the function of endogenous AG protein. In general, it is known that for proteins that function as dimers or in a complex, it is possible to create altered forms that inhibit the function of the endogenous one; such mutations are referred to as dominant negative mutations (Herskowitz, 1987). In plants, the maize *C1-I* allele was shown to contain dominant negative mutations (Paz-Ares et al., 1990; Goff et al., 1991) of the *C1* gene, which encodes a transcriptional activator with a Myb domain (Paz-Ares et al., 1987). Our results with the *1M-I-K* transformants suggest that the transgene behaves as a dominant negative mutation of *AG*. Both the failure of the 35S-*1M-I-K* transgene to convert the outer whorl organs to reproductive organs and its inhibition of normal *AG* functions suggest that the C region may be essential for *AG* function. The ability to create dominant negative mutations of MADS box genes by generating transgenes encoding a protein without the C region provides a useful means of analyzing the functions of these genes, even when recessive mutations are not available.

An AG Protein Lacking the K Domain and C Region Leads to Partial Reduction of AG Function

We found that transgenic plants carrying the construct (35S-*1M-I*) encoding an AG protein lacking the K domain and C region produced flowers with stamens and carpels in the inner whorls, unlike the *ag-1*–like flowers produced by transgenic plants carrying a construct that encodes an AG protein lacking the C region. Therefore, the 1M-I protein does not seem to inhibit *AG* function in specifying reproductive organ identity. However, flowers of *1M-I* transformants are not completely normal; they had a slightly increased number of stamens and carpels. This floral phenotype is similar to that of *AG* antisense transgenic plants with slightly reduced levels of *AG* mRNA and to that of *ag-1/+* heterozygotes (Mizukami and Ma, 1995). These phenotypes together suggest that the 1M-I–truncated AG protein can partially inhibit an *AG* function but not affect the aspect of *AG* function involved in specifying organ identity.

The additional stamens of flowers of the *1M-I* transformants seem to develop in an extra whorl between the third and fourth whorls. Development of extra whorls of stamens has been observed in the *sup-1* mutant, and it has been suggested that the *SUP* gene may regulate floral meristem determinacy (Bowman et al., 1992), whereas the *flo10-1* mutant (*sup-2*) produces flowers with an average of 7.6 stamens, 2.77 unfused carpels or carpelloid organs, and 0.28 fused carpels (Schultz et al., 1991). In addition, the strong *clv1-4* mutant has been observed to produce flowers with additional whorls of carpels interior to the fourth whorl, suggesting a defect in meristem determinacy, whereas weak *clv1* alleles cause only a slight increase in stamen (six to eight) and carpel (three to five) numbers (Clark et al., 1993). Because *AG* controls floral meristem determinacy (Bowman et al., 1989) and weak phenotypes, due to a slight reduction of *AG* expression either in antisense *AG* transgenic plants or in *ag-1/+* heterozygotes (Mizukami and Ma, 1995), cause increased stamen and carpel numbers, it is possible that floral meristem indeterminacy and an increase in reproductive organ number are related, the latter being a weak manifestation of the former. Therefore, the phenotype of slightly increased reproductive organ number in the 1M-I

transformants can be considered to be the result of partial inhibition of *AG* function in floral meristem determinacy.

Possible Roles of the K Domain in AG Action

Our analysis of the floral phenotypes of the transgenic plants carrying the 35S-*1M-I-K* or 35S-*1M-I* constructs shows that although the 1M-I-K protein can inhibit AG function to a level as low as that found in the *ag-1* mutant, 1M-I seems only to be able to affect slightly the *AG* function in floral meristem determinacy. The only structural difference between the proteins encoded by these two constructs is that the 1M-I-K protein contains the K domain but the 1M-I protein does not. Therefore, the K domain seems to be necessary for the complete inhibition of endogenous *AG* function by the dominant negative transgene.

Additional support for the importance of the K domain comes from the deduced sequences of *ag*-mutated proteins. The *ag-1* mutation, one of the strong alleles, is a transition of a G residue to an A residue at the acceptor site of the fourth intron (Yanofsky et al., 1990), leading to the inability of the intron to be spliced out. The slightly longer than normal *ag-1* mutant RNA is expressed at a reduced level (Gustafson-Brown et al., 1994), and the mutated protein, if expressed, is altered starting at the 19th residue of the 66-residue K domain and terminates six codons into the fourth intron. The strong phenotype of the *ag-1* mutant is consistent, with the K domain and/or C region being important for *AG* function, although the reduced expression could also contribute to the mutant defect. A recently described mutant, *ag-4*, produces flowers with morphologically normal stamens, conversion of carpels to sepals, and floral meristem indeterminacy (Sieburth et al., 1995). The *ag-4* mutation also has a G-to-A transition at the acceptor site of the fifth intron; this leads to the loss of 12 or 14 residues near the C terminus of the K domain while leaving the C region intact. Furthermore, when an arginine residue was changed to a methionine near the C terminus of the K domain by a site-directed missense mutation (*ag-Met205*), the mutant flowers exhibited an increased number of sometimes unfused carpels and indeterminacy (Sieburth et al., 1995). These results support the hypothesis that the K domain is important for AG function.

Because the K domain was defined by its moderate sequence similarity to a region of the intermediate filament protein keratin that forms a coil, it has been suggested that the K domain may be involved in a protein–protein interaction(s) (Ma et al., 1991). The K domain of the DEFA protein (the ortholog of AP3) was suggested to be involved in heterodimer formation with GLOBOSA (GLO), the Antirrhinum ortholog of PI, because the temperature-sensitive, mutated DEFA protein lacking a lysine residue near the N terminus of the K domain could not bind to DNA with GLO in vitro (Schwarz-Sommer et al., 1992). We have shown here that, unlike DEFA, AG DNA binding or homodimerization in vitro does not require the K domain.

Even though the AG K domain is not required for dimerization by AG in vitro, it is formally possible that in vivo this domain could still facilitate dimerization of AG and/or stabilize the binding of the AG dimer to DNA. This possibility could account for the difference in floral phenotypes between the *1M-I-K* and *1M-I* transformants in the following way. The 1M-I-K protein could form a stable heterodimer with the endogenous AG protein and inhibit the normal AG function. On the contrary, the 1M-I protein might only form transient heterodimers with the endogenous AG protein. This would result in a slight reduction of the amount of functional AG dimers. Because we have shown that floral meristem determinacy is most sensitive to the reduction of *AG* mRNA (Mizukami and Ma, 1995), a partial reduction of the functional AG dimers by the 35S-*1M-I* transgene could cause a weak degree of indeterminacy manifested as an increase in reproductive organ number. We have also found that the AG DNA binding domain can form heterodimers in vitro with AGL1, AGL2, or AGL3, three Arabidopsis MADS domain proteins (Huang et al., 1996). This raises the possibility that heterodimers of AG and another MADS domain protein may contribute to AG function in vivo. Therefore, the 1M-I-K and 1M-I AG proteins might behave differently in vivo in heterodimerization with other MADS domain proteins, contributing to the different phenotypes.

Alternatively, the different effects of the two dominant negative transgenes can be explained if we can speculate that the K domain may be required for the interaction of the AG protein with an accessory DNA binding factor X. Accordingly, the 1M-I-K–truncated AG protein can interact through the K domain with factor X and compete with the intact AG protein, thereby inhibiting *AG* function. In contrast, the 1M-I protein cannot interact with factor X due to the lack of a K domain. It is known that SRF and MCM1 both associate with other DNA binding proteins through their DNA binding domains (Mueller and Nordheim, 1991; Primig et al., 1991; Grueneberg et al., 1992). If AG indeed interacts with another protein through its K domain, then this is a new mechanism for protein–protein interaction by a MADS domain protein. Because the K domain is present in all known plant MADS domain proteins, these proteins may share a common regulatory mechanism.

Conclusion

We found that the AG MADS domain and I region are sufficient and necessary for DNA binding and for dimerization. Furthermore, our results indicate that both the K domain and C region are indispensable for *AG* function in flower development. Because these four domains are found in all known plant MADS domain proteins, it is reasonable to speculate that each domain of those proteins also plays a role similar to that of the corresponding region of the AG protein.

METHODS

Plasmid Construction

A number of *AGAMOUS* (*AG*) mutant cDNA constructs for truncated AG proteins were generated using polymerase chain reaction (PCR)

and site-specific mutagenesis (Figure 1). For the *ATG-1* and *ATG-34* constructs (see also Mizukami and Ma, 1992), as well as the *ATG-MADS* construct, an NcoI site (italic in the oligonucleotide sequence shown below; same in the later sequences) with an ATG codon (boldface) was introduced by PCR with 5′ primers (EcoRI sites underlined) oMC021, 5′-GGAATTC*CATGG*GGCTGCAACTTCTC-3′; oMC022, 5′-GGAATTC*CATGG*CGTACCAATCGGA-3′; and oMC023, 5′-G*GAATTCCATGG*GGAGAGGAAAGATC-3′, respectively; and 3′ primer (HindIII site underlined) oMC024, GTAAGCTTTCTTGAG. The PCR products were digested with EcoRI and HindIII, and each was ligated with the 0.7-kb HindIII-EcoRI *AG* downstream cDNA fragment (Figure 1A) and EcoRI-digested pGEM7Zf(+) in tripartite reactions, resulting in the three reconstituted *AG* cDNAs inserted into the EcoRI site of pGEM7Zf(+) in either orientation.

The Δ*MADS1* and Δ*MADS2* N-terminal truncation constructs were generated by site-specific mutagenesis on *ATG-1*, using oligonucleotides oMC060 (Δ*MADS1*), 5′-TTAGAGTACTC*CATGG*GACGACCAC-3′, and oMC061 (Δ*MADS2*), 5′-GACTTG ACGATC*CATGG*TGTTCTCG AT-3′, followed by NcoI digestion and religation to remove the sequences between the *ATG-1* NcoI site and the newly generated NcoI sites. The constructs for the three C-terminal truncations were generated by creating premature termination codons using site-specific mutagenesis of *ATG-1* with the following oligonucleotides (in antisense orientation): oMC036 (*1MADS*), 5′-GTTGTTAGATTACTCATAG-3′; oMC037 (*1M-I*), 5′-TTTGGCTGATTAATGTTGATAATAC-3′; and oMC038 (*1M-I-K*), 5′-GTTATCGTTATATTAATCAACTTCT-3′ (newly created AseI sites are underlined, and the bases complementary to the stop codons are in boldface). The constructs were verified by sequencing.

To generate the mutant constructs for N- and C-terminal doubly truncated AG proteins, the XhoI-HindIII fragment carrying the N-terminal truncation was ligated with the HindIII-EcoRI fragment carrying the C-terminal truncation and XhoI-EcoRI–digested pGEM7Zf(+). For example, the XhoI-HindIII fragment of *ATG-34* and the HindIII-EcoRI fragment of *1M-I-K* (Figure 1A) were ligated to pGEM7Zf(+) to generate the *34M-I-K* construct.

For expression in *Escherichia coli* cells, the mutated *AG* cDNAs with the orientation shown in Figure 1A were released with NcoI and BamHI digests and inserted into NcoI-BamHI–digested pET9d (Studier et al., 1990); the resulting constructs were introduced into BL21 (DE3) *E. coli* cells for expression. Expression of the truncated AG proteins was observed as bands of the expected sizes on SDS–polyacrylamide gels (data not shown). For expression in plants, the *AG* cDNAs with the orientation opposite (with respect to sites outside of the EcoRI sites) that shown in Figure 1A were used to make fusions with the cauliflower mosaic virus (CaMV) 35S promoter (Odell et al., 1985). The *AG* cDNAs were released with BamHI and XhoI digests and inserted into BglII-XhoI–digested pMON530 (Rogers et al., 1987) carrying the 35S promoter. Expression of the transgenes was detected by RNA gel blot hybridization analysis (Figure 6; data not shown).

Protein Extraction and Gel Mobility Shift Assay

E. coli cell extracts containing the truncated AG proteins were prepared as previously described (Huang et al., 1993), and the AG proteins were used for DNA binding experiments as described previously (Huang et al., 1993, 1995). For heterodimer formation, cells with one AG truncation were mixed with those of another and lysed with a denaturing buffer (buffer A). The cell lysates were then diluted with a nondenaturing buffer (buffer B) to renature the proteins (Huang et al., 1993). When proteins in buffer B were mixed, no heterodimer was observed (data not shown). The sequences of probes used here are as follows: oligo(A), 5′-ACTCGAGGAATTCGGTACCCCGGGTTCGAAATCGATAAGCTTTACCATTAATGGTAAAGCTTGGATCCGGAGAGCTCCCAACGCGT-3′ (CArG box underlined); and oligo(B), 5′-ACTCGAGGAATTCGGTACCCCGGGTTCGAAATCGATAAGCTTTAGGATGCATCCTAAAGCTTGGATCCGGAGAGCTCCCAACGCGT-3′ (mutated CArG box underlined).

Plant Growth, Transformation, Maintenance, and Characterization

Plants (*Arabidopsis thaliana* ecotype Landsberg) homozygous for the *erecta* mutation were used as the wild type in this study; the *ag-1*, *ap2-1*, and *ap2-2* mutants were isolated in this background (Bowman et al., 1989). Plant growth and maintenance were as previously described (Mizukami and Ma, 1992). Plant transformation was performed as previously described (Valvekens et al., 1988; Huang and Ma, 1992). Floral phenotypes were observed with the aid of a light microscope and a scanning electron microscope. Samples for scanning electron microscopy were prepared according to previously described procedures (Mizukami and Ma, 1992).

Analysis of *AG* Expression

Due to the lack of antibodies that can detect AG protein in plant extracts, we used RNA gel blot hybridization to determine the level of *AG* expression in flowers of wild type and the 35S-*AG* transformants. Total RNA was isolated from various plant tissues essentially as described by Ausubel et al. (1994), with modifications by Huang et al. (1995). For RNA gel blot hybridizations, total RNAs were separated on an agarose–formamide gel and transferred to a nylon filter (Hybond N; Amersham) and hybridized in Church buffer (Church and Gilbert, 1984) with a HincII-EcoRI fragment of the 3′ portion of the *AG* cDNA labeled with phosphorus-32, using a random priming labeling kit. Hybridization with the *AG* probe and washes were performed at 65°C to detect specifically *AG* mRNA. The *AAC1* cDNA encoding an actin from Arabidopsis (Nairn et al., 1988) was used as a control probe with washes at 50°C.

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