

Determination of Floral Organ Identity by *Arabidopsis* MADS Domain Homeotic Proteins AP1, AP3, PI, and AG Is Independent of Their DNA-binding Specificity

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The MADS domain homeotic proteins APETALA1 (AP1), APETALA3 (AP3), PISTILLATA (PI), and AGAMOUS (AG) combinatorially specify the identity of *Arabidopsis* floral organs. AP1/AP1, AG/AG, and AP3/PI dimers bind to similar CArG box sequences; thus, differences in DNA-binding specificity among these proteins do not seem to be the origin of their distinct organ identity properties. To assess the overall contribution that specific DNA binding could make to their biological specificity, we have generated chimeric genes in which the amino-terminal half of the MADS domain of AP1, AP3, PI, and AG was substituted by the corresponding sequences of human SRF and MEF2A proteins. In vitro DNA-binding assays reveal that the chimeric proteins acquired the respective, and distinct, DNA-binding specificity of SRF or MEF2A. However, ectopic expression of the chimeric genes reproduces the dominant gain-of-function phenotypes exhibited by plants ectopically expressing the corresponding *Arabidopsis* wild-type genes. In addition, both the SRF and MEF2 chimeric genes can complement the pertinent *ap1-1*, *ap3-3*, *pi-1*, or *ag-3* mutations to a degree similar to that of AP1, AP3, PI, and AG when expressed under the control of the same promoter. These results indicate that determination of floral organ identity by the MADS domain homeotic proteins AP1, AP3, PI, and AG is independent of their DNA-binding specificity. In addition, the DNA-binding experiments show that either one of the two MADS domains of a dimer can be sufficient to confer a particular DNA-binding specificity to the complex and that sequences outside the amino-terminal basic region of the MADS domain can, in some cases, contribute to the DNA-binding specificity of the proteins.

INTRODUCTION

The identification of homeotic gene products as transcription factors that belong to multigene families pointed to the central role that differential regulation of transcription plays in the processes of development and differentiation and also posed the question of how proteins that are highly similar can possess extremely diverse functional specificities. The molecular mechanisms by which the homeotic proteins achieve their regulatory activities are not yet fully understood, for both the homeodomain-containing homeotic proteins of animals and the MADS domain homeotic proteins of plants. APETALA1 (AP1; Mandel *et al.*, 1992a), APETALA3 (AP3;

Jack *et al.*, 1992), PISTILLATA (PI; Goto and Meyerowitz, 1994), and AGAMOUS (AG; Yanofsky *et al.*, 1990) are homeotic genes that function combinatorially to determine the identity of the different types of organs that, arranged in four concentric whorls, make up the *Arabidopsis* flower: four sepals, four petals, six stamens, and two fused carpels. The mode of action of these organ identity genes was explained in genetic terms by the ABC model, which has been validated by an abundance of experimental data in *Arabidopsis thaliana* and in other plant species (Bowman *et al.* 1991; Coen and Meyerowitz, 1991; reviewed in Ma, 1994; Weigel and Meyerowitz, 1994; Yanofsky, 1995).

AP1, AP3, PI, and AG belong to the MADS-domain family of proteins that, in addition to containing a large number of members in plants (more than 20 in *Arabidopsis* and *Antirrhinum*; Ma *et al.*, 1991; Purugga-

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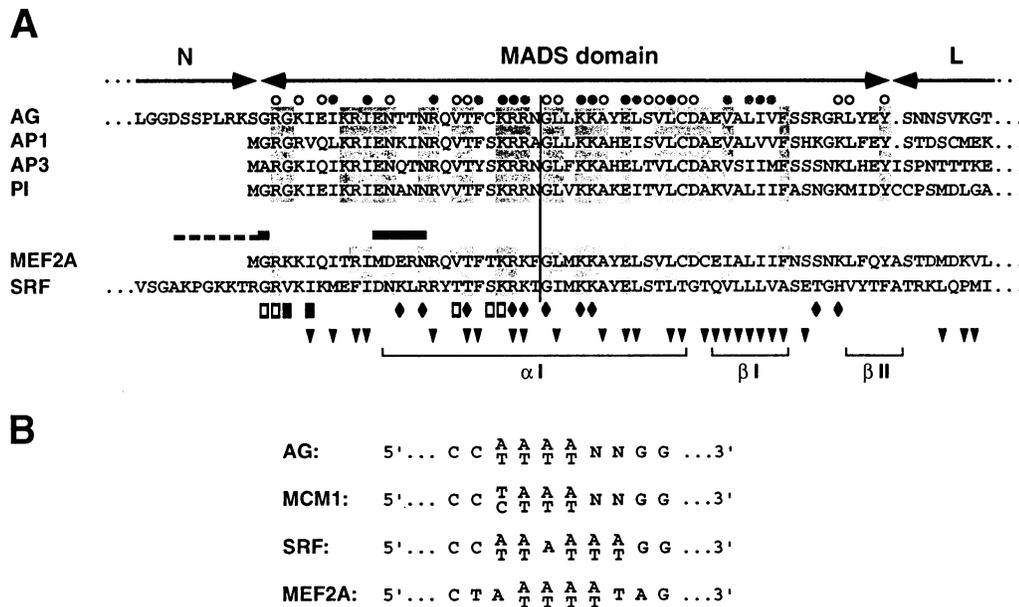


Figure 1. (A) Sequences of the MADS domains of AG, AP1, AP3, PI, MEF2A, and SRF. The MADS domain, preceded by an amino-terminal extension in AG and SRF and followed by the "L" region (also known as "I"), is indicated; amino acids identical among AG, AP1, AP3, and PI are shaded. Amino acids identical among all the proteins belonging to the MADS box family are noted with a solid circle above the AG sequence, those that are highly conserved are noted with a shaded circle, and positions where nonconservative changes are very rare are noted with an open circle (after Theißen *et al.*, 1996). SRF residues that are involved in DNA-

binding, making base contacts (open rectangles), deoxyribose contacts (solid rectangles), or contacts with the phosphate DNA backbone (diamonds) and residues involved in dimerization (triangles) are indicated (Pellegrini *et al.*, 1995). αI , βI , and βII are secondary structure elements observed in the crystal structure of core SRF (Pellegrini *et al.*, 1995). Sequences that have been found to represent DNA-binding specificity determinants in SRF, MCM1, and MEF2A are identified by thick lines above the MEF2A sequence (Nurrish and Treisman, 1995). The site at which the sequences from AG, AP1, AP3, and PI, and MEF2A or SRF were fused to make the chimeric MADS box genes is indicated by a vertical line. In the SRF chimeric proteins, the initiation methionine immediately precedes the SRF sequence shown. (B) Consensus sequences of DNA-binding sites of AG (Huang *et al.*, 1993; Shiraishi *et al.*, 1993), MCM1 (Wynne and Treisman, 1992), SRF (Pollock and Treisman, 1990), and MEF2A (Pollock and Treisman, 1991), as deduced by *in vitro* sequence-selection experiments from pools of random oligonucleotides. N, any nucleotide.

nan *et al.*, 1995; Rounsley *et al.*, 1995; Theißen *et al.*, 1996), includes transcription factors from animals (serum response factor [SRF] and the MEF2 proteins) and yeast (MCM1 and ARG80; reviewed in Shore and Sharrocks, 1995). Sequence similarity among the MADS domain proteins of different kingdoms is mostly limited to the MADS domain itself: a 56-amino acid region involved in dimerization and DNA binding (Shore and Sharrocks, 1995). The crystal structure of core SRF bound to DNA has revealed that the basic amino-terminal half of the MADS domain contributes most of the DNA-contacting amino acids, and the hydrophobic carboxyl region forms part of the dimerization surface (Figure 1A; Pellegrini *et al.*, 1995). Formation of DNA-binding dimers, however, requires additional sequences located immediately carboxyl terminal to the MADS domain (Norman *et al.*, 1988; Pollock and Treisman, 1991; Pellegrini *et al.*, 1995). For AP1, AP3, PI, and AG, these carboxyl terminal sequences contain most of the molecular determinants for the dimerization specificity exhibited by these four proteins (Riechmann *et al.*, 1996a).

Selective dimerization seems to be part of the mechanism by which AP1, AP3, PI, and AG achieve their functional specificity, i.e., their distinct organ identity activities (Krizek and Meyerowitz, 1996a; Riechmann

et al., 1996a); yet, the DNA-binding properties of AP1/AP1, AP3/PI, and AG/AG dimers are very similar. The three complexes can bind to the same CArG box [consensus CC(A/T)₆GG] sequences with comparable affinities and induce similar distortions on the DNA (DNA bending) upon binding (Riechmann *et al.*, 1996b). These observations are consistent with the high level of amino acid sequence identity existent among the MADS domains of the four proteins (Figure 1A). In addition, *in vivo* analyses of the activity of hybrid genes formed by swapping regions between AP1, AP3, PI, and AG have shown that, at least in some cases, the MADS domains, or their amino-terminal part, can be interchanged without them determining the specific functions of the resulting hybrid proteins (Krizek and Meyerowitz, 1996a; Krizek, Riechmann, and Meyerowitz, unpublished results). Thus, these *in vitro* and *in vivo* data suggested that AP1, AP3, PI, and AG do not obtain their presumably distinct properties of transcriptional regulation from differences in their intrinsic DNA-binding specificities. However, since the MADS domains and the DNA-binding properties of AP1, AP3, PI, and AG are so similar, exchanging regions among these four proteins did not make it possible to assess the overall contribution that specific DNA binding could make to the

particular organ identity activity of each of these proteins. In addition, the assumption that all the DNA-binding specificity determinants of the plant MADS domain proteins are localized in the amino-terminal half of the MADS domain, which allowed the interpretation of the domain-swapping experiments, was based on the results obtained with SRF, MCM1, and MEF2A (Sharrocks *et al.*, 1993a,b; Nurrish and Treisman, 1995; Pellegrini *et al.*, 1995) and, therefore needed to be validated by experimental data.

To elucidate these aspects of the activity of the MADS domain floral organ-identity proteins, we have generated AP1-, AP3-, PI-, and AG-derived chimeric genes in which the sequences coding for the amino-terminal half of the MADS domain were substituted with the corresponding human SRF (Norman *et al.*, 1988) or MEF2A (Pollock and Treisman, 1991) sequences (Figure 1A). The rationale behind those constructs was that, although different MADS domain family members recognize A+T-rich consensus sequences, they can possess distinct DNA-binding specificities (Nurrish and Treisman, 1995; Figure 1B). In particular, SRF binds the consensus sequence CC(A/T)₂A(A/T)₃GG (Pollock and Treisman, 1990), distinct from the consensus sequences defined for MCM1, CC(C/T)(A/T)₃NNGG (Wynne and Treisman, 1992), and AG, CC(A/T)₄NNGG (Huang *et al.*, 1993; Shiraishi *et al.*, 1993). A more divergent DNA-binding specificity was described for MEF2A, which recognizes CTA(T/A)₄TAG and does not bind to CC(A/T)₆GG sequences (Pollock and Treisman, 1991). From the crystal structure of the core SRF-DNA complex, the region replaced to generate the chimeric proteins contains all the amino acids that are involved in base specific contacts (Pellegrini *et al.*, 1995; Figure 1A). The activity of the SRF- and MEF2-AP1, -AP3, -PI, and -AG chimeric proteins was characterized by *in vitro* DNA-binding assays and *in vivo* ectopic expression experiments. *In vivo* experiments can be used as an assay of AP1, AP3, PI, and AG activity because the ectopic expression of each one of the organ identity genes, under the control of the constitutive cauliflower mosaic virus 35S promoter, causes a distinct dominant gain-of-function phenotype that is in agreement with their respective wild-type functions and with the postulates of the ABC model and that does not depend on the protein expression levels (Mandel *et al.*, 1992b; Mizukami and Ma, 1992; Jack *et al.*, 1994; Mandel and Yanofsky, 1995; Krizek and Meyerowitz, 1996b; Figure 2, A and B; see Figure 4, A and F; and see Figure 5, A-C). In addition, the dominant gain-of-function phenotypes do not depend on the corresponding endogenous genes being functional (Jack *et al.*, 1994; Mandel and Yanofsky, 1995; Krizek and Meyerowitz, 1996b; Figure 2C; see Figure 5D).

Herein, we show that ectopic expression of SRF-AP1, -AP3, -PI, and -AG and of MEF2-AP1, -AP3, -PI,

and -AG completely reproduces the phenotypes exhibited by 35S::AP1, 35S::AP3, 35S::PI, and 35S::AG plants, respectively. *In vitro* DNA-binding assays revealed that the chimeric proteins acquired the respective, and distinct, DNA-binding specificity of SRF or MEF2. Yet, both the SRF and MEF2 chimeric genes can complement the corresponding *ap1-1*, *ap3-3*, *pi-1*, and *ag-3* mutations to a degree similar to that of the wild-type AP1, AP3, PI, and AG. From these results we conclude that determination of floral organ identity by the *Arabidopsis* MADS domain homeotic proteins AP1, AP3, PI, and AG is independent of their DNA-binding specificity. In addition, the DNA-binding experiments show that either one of the two MADS domains of a dimer can be sufficient to confer a particular DNA-binding specificity to the complex and that, although the DNA-binding specificity determinants are localized in the amino-terminal half of the MADS domain, sequences outside this region can in some instances affect the interaction between a particular protein and binding site and, therefore, contribute to binding specificity.

MATERIALS AND METHODS

Construction of Chimeric Genes

The chimeric genes (SRF-AP1, SRF-AP3, SRF-PI, SRF-AG, MEF2-AP1, MEF2-AP3, MEF2-PI, and MEF2-AG) were constructed by fusing the corresponding sequences by a two-round polymerase chain reaction (PCR) method (overlap extension PCR; Horton *et al.*, 1990). In the first round of PCR, sequences of each of the two genes were amplified with flanking sequences on one side that were homologous to the other gene. These two overlapping products were then used together as templates and fused in a second round of PCR using the outer nonhomologous primers. The point at which sequences were fused immediately precedes the codon for the highly conserved glycine residue at amino acid position 26 of the MADS domain (Figure 1A). The chimeric PCR fragments were used to construct the full-length chimeric genes using unique restriction sites present in the cDNAs of AP1, AP3, PI, and AG. The sequence of the regions amplified by PCR was verified. In MEF2A, as well as in AP1, AP3, and PI, the MADS domain is located at the amino terminus of the protein (Figure 1A). Therefore, in the chimeric constructs that included the MEF2A amino-terminal half of the MADS domain (MEF2-AP1, -AP3, -PI, and -AG), the MEF2A initiation codon was used. On the contrary, the SRF MADS domain begins at amino acid 142 of the protein. Part of this amino-terminal extension was maintained in the chimeric genes. An initiation codon was artificially generated with the SRF outer primer at the position occupied by nucleotides 746-748 of the SRF cDNA sequence (Norman *et al.*, 1988). As a result, the chimeric genes that contain SRF sequences (SRF-AP1, -AP3, -PI, and -AG) code for proteins that start with the sequence MVSGA . . . (an amino-terminal extension of 12 amino acids preceding the MADS domain; Figure 1A). The chimeric genes were joined to an 842-bp cauliflower mosaic virus 35S promoter, and the 35S::chimeric MADS box gene constructs were transferred to a modified pCGN1547 plant transformation vector containing 3'-NOS sequences (McBride and Summerfelt, 1989; Krizek *et al.*, 1996a).

Agrobacterium-mediated Plant Transformation and Plant Growth

The chimeric genes in the pCGN1547 vector were transformed into *Agrobacterium* strain ASE. *Arabidopsis thaliana* (Landsberg *erecta*; *Ler*) plants were transformed by vacuum infiltration (Bechtold *et al.*, 1993), and transformants were selected by plating the seeds on kanamycin plates. Plants were grown on a 1:1:1 mixture of soil:perlite:vermiculite under constant cool-white fluorescent light at 23°C.

Strain Constructions

Plants transgenic for the chimeric genes were crossed to homozygous *ap1-1*, *ap3-3*, and *pi-1* and heterozygous *ag-3* plants by manual cross-pollination. F₁ plants were allowed to self-fertilize or crossed again to mutant strains. F₂ progeny plants were selected for kanamycin resistance, and the presence of the mutation was determined by PCR-primer introduced restriction analysis (Jacobson and Moskovits, 1991) of the products of reactions that amplified the relevant gene sequences from leaf tissue (Klimyuk *et al.*, 1993). The 35S::AP1, 35S::AP3, 35S::PI, and 35S::AG strains used for comparison with the 35S::SRF and 35S::MEF2 lines are also in the *Ler* background. Previously described lines carrying these transgenes were in the following genetic backgrounds: Columbia (35S::AP1; Mandel and Yanofsky, 1995), Nossen (No-0, 35S::AP3; Jack *et al.*, 1994; and 35S::PI; Krizek and Meyerowitz, 1996b), and *Ler* (35S::AG; Mizukami and Ma, 1992). The AGL5::GUS strain (carrying a 2.1-kb AGL5 promoter:: β -glucuronidase [GUS] fusion construct; Savidge *et al.*, 1995) is in the No-0 ecotype.

GUS Staining

GUS histochemical staining was performed as described previously (Jefferson *et al.*, 1987; Krizek *et al.*, 1996b). Tissues were cleared of chlorophyll by doing an ethanol series.

DNA-binding Assays

The chimeric genes were cloned into pSPUTK (Stratagene, La Jolla, CA) to produce the chimeric proteins in *in vitro* transcription/translation reactions. Derivatives of *SRF-AG* and *MEF2-AG* sequences were synthesized by PCR and cloned into pSPUTK to make the carboxyl terminally truncated proteins *SRF-AGNML* and *MEF2-AGML*, which comprise, respectively: SRF amino-terminal amino acids, the SRF-AG chimeric MADS domain, and the AG L (also referred to as "I") region; and the MEF2-AG chimeric MADS domain and the AG L region. pSPUTK-derived plasmids to produce AP1, AP3, PI, and AGNML have been described previously (Riechmann *et al.*, 1996a,b). The truncated proteins AGNML, SRF-AGNML, and MEF2-AGML were used in the DNA-binding assays because it has been previously observed that AGNML gives a better signal in those experiments than full-length AG, while maintaining intact its DNA-binding properties (Riechmann *et al.*, 1996b; a similar result was obtained when comparing SRF-AG and SRF-AGNML [our unpublished results]). Proteins were synthesized with the TNT-coupled transcription/translation reticulocyte lysate system (Promega, Madison, WI). The DNA-binding activity of the proteins was tested in electrophoretic mobility shift assays (EMSAs), which were performed as described (Riechmann *et al.*, 1996a). Protein-DNA complexes were resolved on 5% (except when indicated otherwise) polyacrylamide:bisacrylamide (60:1) gels in 1× TBE run at 4°C.

DNA-binding site probes A, B, and D are derived from the promoters of *Arabidopsis* AP3, *SUPERMAN* (*SUP*), and *AGL5* genes, respectively, and contain the following CarG boxes: CCATTTTATG (probe A), CCATTTTGG (probe B), and CCAAAAAGG (probe D; Riechmann *et al.*, 1996a,b). Probes S1 and ME contain CarG boxes that are specific for SRF and MEF2A, respectively (Treisman, 1987;

Pollock and Treisman, 1991; Nurrish and Treisman, 1995). Probe S1, 5'-AATTCTCATGGATTAGGCAATACTGCCCATATATGGGCAC-TCAAGTGGACCCCTTACTCTAG-3'; probe ME, 5'-AATTCAG-GAAAAC TATTATAGATCAAATCTAG-3'. Binding probes were prepared as described (Riechmann *et al.*, 1996a), and all probes were labeled to the same specific activity, to allow direct comparison between the reactions containing the same protein(s).

RESULTS

Ectopic Expression of SRF-AG and MEF2-AG Has the Same Phenotypic Effects as Ectopic Expression of AG

Most of the *Arabidopsis* transgenic lines (10/14) ectopically expressing the chimeric gene *SRF-AG* under the control of the constitutive 35S promoter (35S::SRF-AG plants) showed the characteristic 35S::AG phenotype previously described (Mizukami and Ma, 1992; Figure 2, A and B), including small plants with curled leaves; flowers with carpelloid sepals or carpels in the first whorl and in the second whorl staminoid petals, or the absence of organs; and termination of the inflorescence in a carpelloid structure (Figure 2, D and E). Similar phenotypic changes were observed when the chimeric gene *MEF2-AG* was ectopically expressed: 12 of the 15 35S::MEF2-AG lines analyzed exhibited the typical 35S::AG phenotype (Figure 2, G and H). In both cases, the flowers of some of the transgenic lines resembled those of *ag* mutant plants (1/14 and 2/15, respectively), which may be indicative of cosuppression (silencing or inactivation of the endogenous gene; Flavell, 1994; Matzke and Matzke, 1995), whereas some lines had wild-type flowers (3/14 and 1/15 lines). In both *SRF-AG* and *MEF2-AG* expressing lines, the strength of the gain-of-function phenotype increased acropetally: the conversion of first whorl sepals to carpels and of petals to stamens was more complete in later flowers. In these later flowers, but not in the early ones, second whorl organs could be absent.

35S::SRF-AG *ag-3* and 35S::MEF2-AG *ag-3* strains were generated to assess whether the chimeric genes could complement the *ag-3* mutation. Plants homozygous for the strong *ag-3* allele have flowers in which the third whorl stamens are converted to petals whereas another flower, that will reiterate the same organ pattern, develops in place of the fourth whorl carpels (Bowman *et al.*, 1991). 35S::SRF-AG *ag-3* and 35S::MEF2-AG *ag-3* strains showed a partial rescue of the *ag-3* mutant phenotype: petals of those flowers were partially converted to stamens, and sepals were of carpelloid nature (Figure 2, F and I). Ovules or ovule-like organs were eventually seen in those carpelloid sepals. The indeterminacy defect typical of *ag-3* mutant flowers was not rescued, however, and therefore, 35S::SRF-AG *ag-3* and 35S::MEF2-AG *ag-3* flowers repeated the pattern (sepal, carpelloid sepal, sepaloid carpel, or carpel/staminoid petal/staminoid

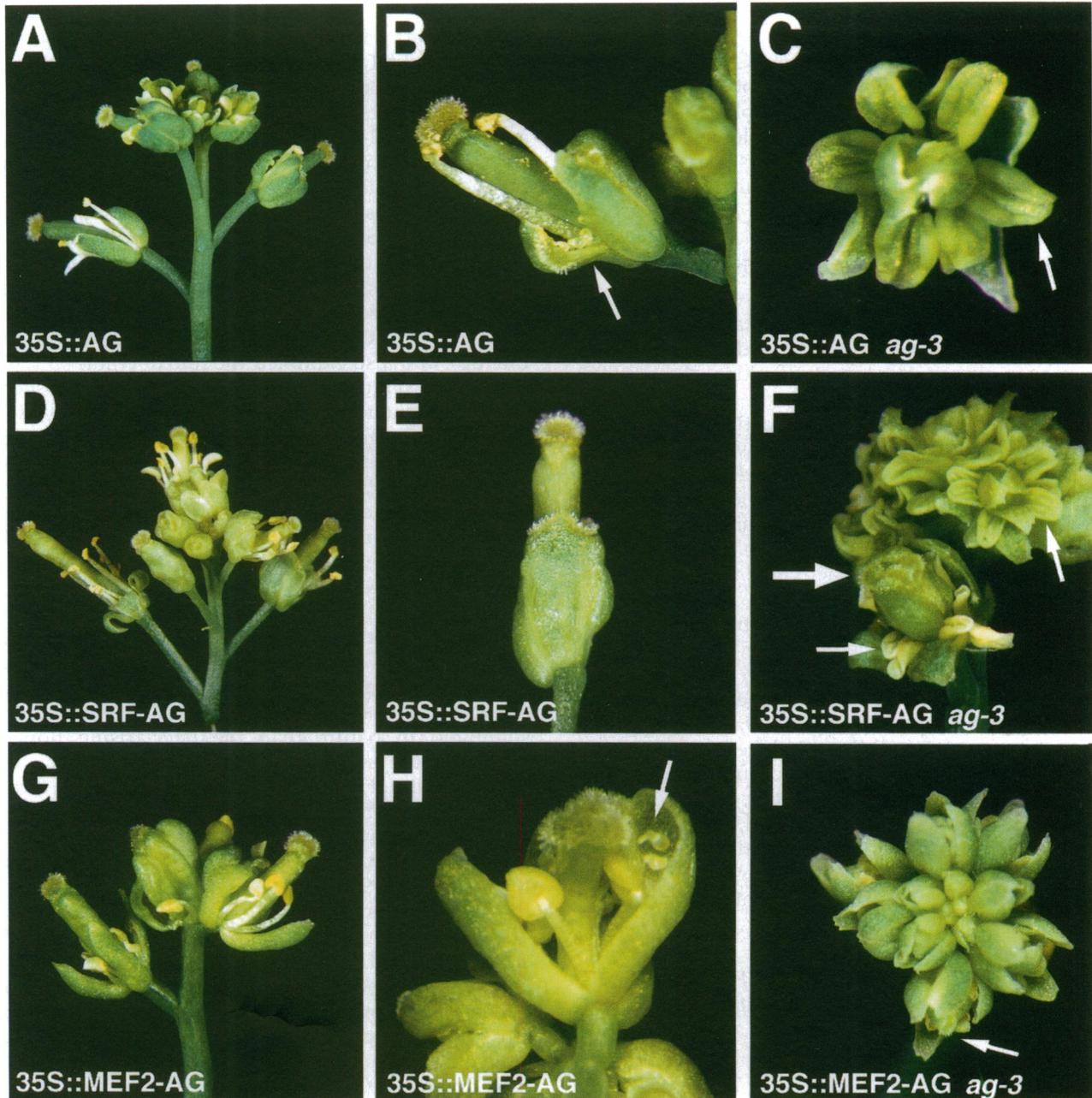


Figure 2. Ectopic expression of *SRF-AG* and *MEF2-AG* causes the same phenotypic changes as ectopic expression of *AG*. (A) 35S::*AG* inflorescence. (B) 35S::*AG* flower. The first whorl organs have been converted to carpels and are partially fused. Ovules are formed on the inner side of these ectopic carpels (indicated by an arrow), and stigmatic tissue is visible. (C) 35S::*AG ag-3* flower, showing petaloid/staminoid organs (arrow). (D) 35S::*SRF-AG* inflorescence. (E) 35S::*SRF-AG* flower. The first whorl organs have been converted to carpels and are fused. (F) 35S::*SRF-AG ag-3* inflorescence. Petals (thin arrows) have staminoid characteristics and sepals show carpelloid features (thick arrow). (G) 35S::*MEF2-AG* inflorescence. (H) 35S::*MEF2-AG* flower. Ovules are formed on the inner side of the first whorl organs (indicated by an arrow). (I) 35S::*MEF2-AG ag-3* inflorescence. Petals have staminoid characteristics and sepals show carpelloid features (indicated by an arrow).

petal)_n (Figure 2, F and I). 35S::*AG ag-3* plants have similar indeterminate flowers that show only a partial rescue of the organ identity defects characteristic of the *ag* mutations (Figure 2C). In some cases, however,

the few first early-arising flowers of 35S::*AG ag-3* plants (but not the later arising ones) were determinate and had almost normal carpels in the fourth whorl. This acropetal decrease in the degree of rescue

of the *ag-3* mutant phenotype was also observed in 35S::SRF-AG *ag-3* and 35S::MEF2-AG *ag-3* plants. Thus, ectopic expression of SRF-AG and MEF2-AG both causes a gain-of-function phenotype that is identical to the one shown by 35S::AG plants and, in addition, the two chimeric genes complement by ectopic expression the organ-identity defects characteristic of the *ag-3* mutation to a degree similar to that of wild-type AG.

AGL5 Is Ectopically Expressed in 35S::SRF-AG and 35S::MEF2-AG Plants

AGL5 (for AG-like) is a gene specifically expressed in carpels that requires AG for its induction and that could be a direct AG target (Savidge *et al.*, 1995). Ectopic expression of the *Brassica* ortholog of AG (*BAG1*) has been shown to activate an *AGL5* promoter::GUS fusion in cauline leaves, where *AGL5* is normally not expressed (Savidge *et al.*, 1995). As an additional criterion to establish the identity of the *in vivo* activities of the chimeric proteins SRF-AG and MEF2-AG with wild-type AG, we tested whether *AGL5*::GUS was ectopically activated in cauline leaves of 35S::SRF-AG and 35S::MEF2-AG plants. 35S::AG, 35S::SRF-AG, and 35S::MEF2-AG plants were crossed to the transgenic *AGL5*::GUS strain (Savidge *et al.*, 1995). In all three cases, a similar pattern of GUS activity was detected in the cauline leaves of the doubly transgenic F₁ plants (Figure 3), indicating that ectopic expression of all three induces ectopic activation of expression from the *AGL5* promoter in a similar manner. GUS staining was detected, however, in the leaf base and some of the veins and, occasionally, in the tip of the leaves, in contrast with the previously described uniform distribution throughout the cauline leaves of *AGL5*::GUS 35S::BAG1 plants (Savidge *et al.*, 1995).

Ectopic Expression of SRF-AP3, MEF2-AP3, SRF-PI, and MEF2-PI Has the Same Phenotypic Effects as Ectopic Expression of AP3 or PI

Ectopic expression of the chimeric genes *SRF-AP3* (35S::SRF-AP3 plants; Figure 4B) and *MEF2-AP3* (35S::MEF2-AP3 plants; Figure 4C) caused a transformation, partial or complete, of the fourth whorl carpels into stamens, resulting in flowers that in that whorl had additional stamens, staminoid/carpelloid mosaic organs, or extra carpels (10/21 35S::SRF-AP3 and 7/16 35S::MEF2-AP3 transgenic lines). This gain-of-function phenotype is identical to that of 35S::AP3 plants (Jack *et al.*, 1994; Figure 4A). The flowers of several 35S::SRF-AP3 (8/21) and 35S::MEF2-AP3 (5/16) transgenic lines had sepaloid petals and stamens that did not elongate, a phenotype that may be indicative of cosuppression, because these flowers somewhat resemble those of *ap3* mutant plants. The remain-

ing transgenic lines (3/21 and 4/16 lines) had wild-type flowers. These observations suggest that the amino-terminal halves of both the SRF and MEF2A MADS domains can substitute for that region of AP3. To further test this conclusion, 35S::SRF-AP3 *ap3-3* and 35S::MEF2-AP3 *ap3-3* strains were generated. *ap3-3* is a strong (null) allele of *AP3* that shows homeotic conversions of petals to sepals (second whorl) and of stamens to carpels (third whorl; Jack *et al.*, 1992). Both 35S::SRF-AP3 *ap3-3* and 35S::MEF2-AP3 *ap3-3* strains showed a partial complementation of the *ap3-3* mutation: flowers of these plants had sepaloid petals in the second whorl and stamens in the third whorl (with anthers often bearing stigmatic tissue on the top), and also exhibited the fourth whorl 35S::AP3 phenotype (Figure 4, D and E). For some transgenic lines, this phenotypic rescue could be improved by increasing the gene dosage (by selfing plants hemizygous for the transgene to generate homozygous plants). 35S::AP3 *ap3-3* flowers of No-0 or *Ler* genetic backgrounds have a similar phenotype (Jack *et al.*, 1994; and our unpublished results). Therefore, SRF-AP3 and MEF-AP3 complement the *ap3-3* mutation to the same degree that wild-type AP3 does by ectopic expression. The strength of the gain-of-function phenotype exhibited by 35S::SRF-AP3 and 35S::MEF2-AP3 lines, as well as by 35S::AP3 plants, decreases acropetally: the early-arising flowers show a higher number of extra stamens and/or mosaic organs that are more staminoid in nature than the later-arising flowers. Similarly, the stamens of early 35S::SRF-AP3 *ap3-3* flowers, but not those of the late flowers, were fertile.

Flowers of 35S::PI plants have the first whorl sepals partially converted to petals, resulting in mosaic organs with both sepal and petal tissue (Krizek and Meyerowitz, 1996b; Figure 4F). Ectopic expression of the chimeric genes *SRF-PI* (35S::SRF-PI plants; Figure 4G) and *MEF2-PI* (35S::MEF2-PI plants; Figure 4H) caused an identical phenotype (4/10 and 8/13 transgenic lines, respectively). Some of the transgenic lines had either a cosuppression-like phenotype (sepaloid petals in the second whorl and stamens that did not elongate in the third; 4/10 and 3/13 lines) or wild-type flowers (2/10 and 2/13 lines). That the chimeric proteins retained wild-type PI activity was further tested in phenotypic rescue assays. Flowers homozygous for the strong *pi-1* allele show a homeotic conversion of the second whorl petals to sepals, the third whorl organs are either absent or of a filamentous nature, and the central gynoecium is large and usually composed of more than two carpels (Bowman *et al.*, 1989). 35S::SRF-PI *pi-1* and 35S::MEF2-PI *pi-1* strains showed partial complementation of the *pi-1* mutation by the chimeric genes, although it decreases acropetally: flowers of these plants have petals in the second whorl and have, in the third whorl, fertile stamens (in the early flowers) or staminoid/carpelloid organs (in

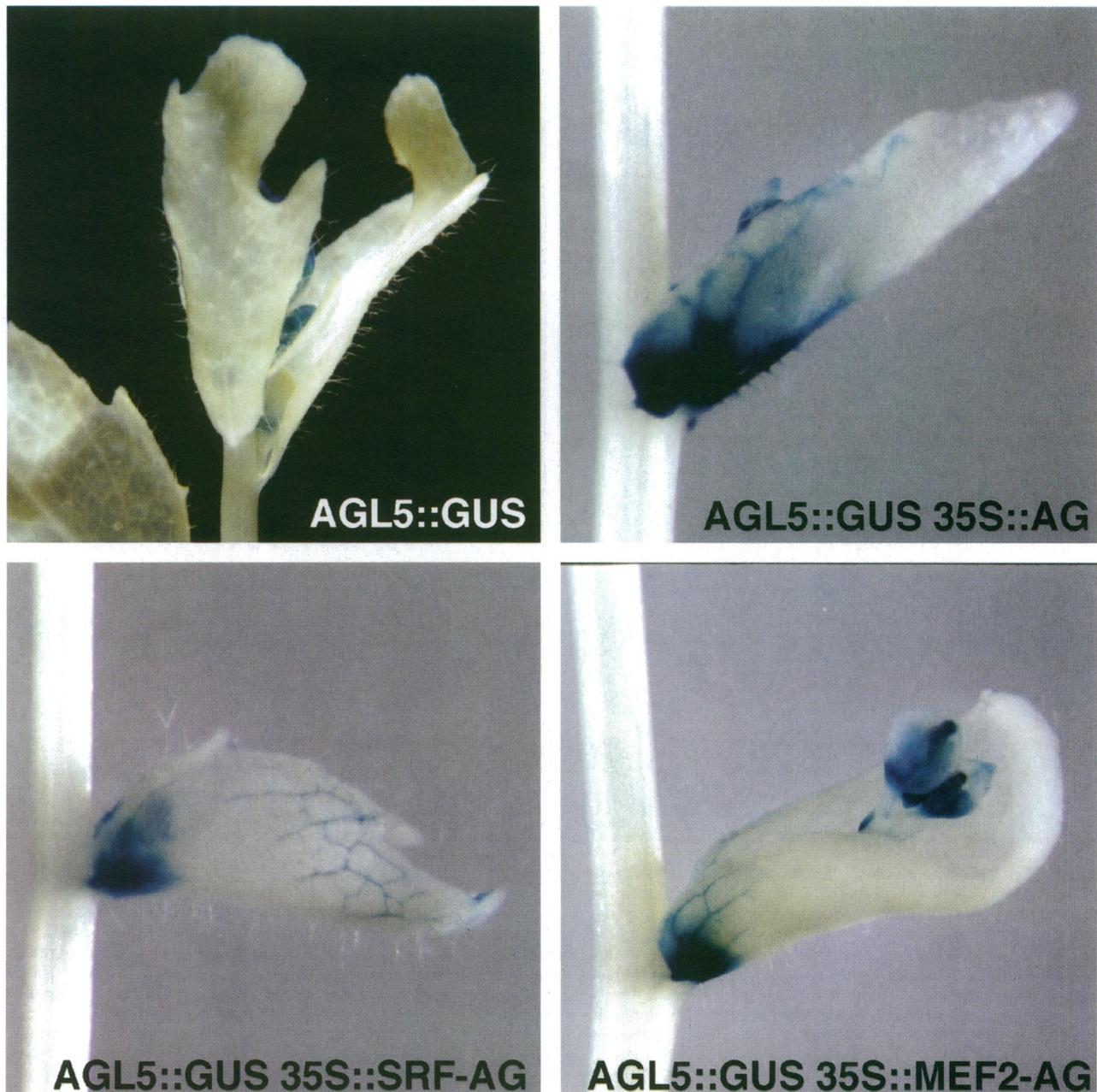


Figure 3. Ectopic activation of *AGL5* expression in 35S::SRF-AG and 35S::MEF2-AG plants. GUS staining of cauline leaves of *AGL5::GUS*, *AGL5::GUS 35S::AG*, *AGL5::GUS 35S::SRF-AG*, and *AGL5::GUS 35S::MEF2-AG* plants. No GUS staining is detected at the base of the leaves of *AGL5::GUS* plants, but it is apparent in the three doubly transgenic lines.

the late flowers), in addition to exhibiting the first whorl 35S::PI phenotype (Figure 4, I and J). The degree of rescue of the *pi-1* mutant phenotype in those strains is in all ways comparable to that obtained by ectopic expression of the wild-type PI protein (35S::PI *pi-1* strain; our unpublished results).

Flowers of plants in which both AP3 and PI are ectopically expressed (35S::AP3 35S::PI strain) have two

outer whorls of petals and multiple stamens interior to those (Krizek and Meyerowitz, 1996b). Similar strains were generated by crossing the relevant lines ectopically expressing the chimeric genes. Both 35S::SRF-AP3 35S::SRF-PI and 35S::MEF2-AP3 35S::MEF2-PI plants showed identical transformations of the first and fourth whorls (Figure 4, K and L). In all doubly transgenic lines, the development of stamens at the expense of fourth

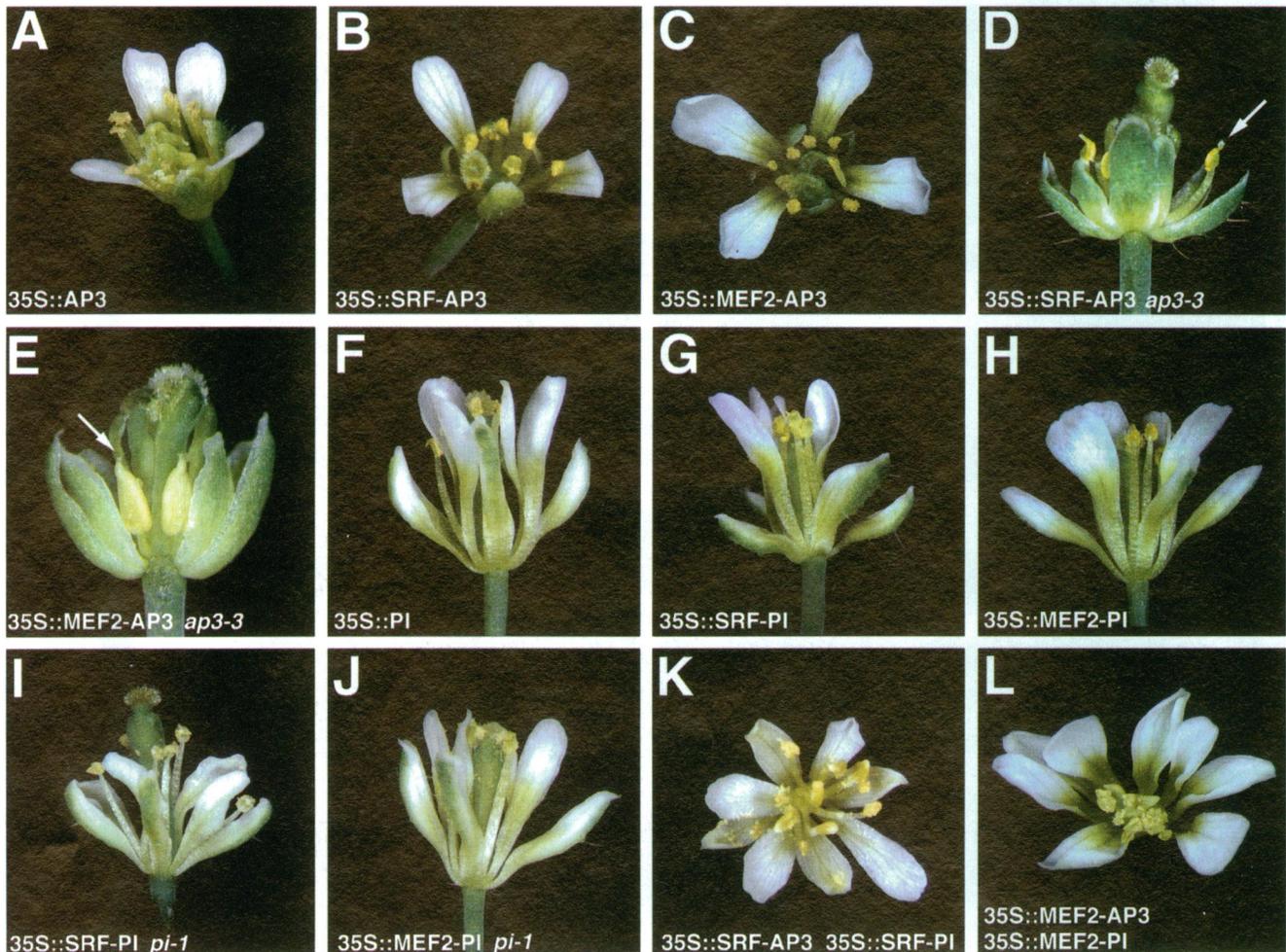


Figure 4. Ectopic expression of *SRF-AP3*, *SRF-PI*, *MEF2-AP3*, and *MEF2-PI* causes the same phenotypic changes as ectopic expression of *AP3* or *PI*. (A) 35S::*AP3* flower with extra stamens and staminoid/carpelloid mosaic organs (stamen-like organs topped with stigmatic tissue) in the inner whorls. (B) 35S::*SRF-AP3* flower with seven stamens. (C) 35S::*MEF2-AP3* flower with extra stamens and staminoid/carpelloid mosaic organs. (D) 35S::*SRF-AP3 ap3-3* flower with sepaloid petals in the second whorl, stamens (with anthers topped with stigmatic tissue; indicated by an arrow) in the third whorl, and extra carpels in the fourth whorl. (E) 35S::*MEF2-AP3 ap3-3* flower. One sepal has been removed to show the inner organs: sepaloid organs in the second whorl, stamens (with anthers bearing stigmatic tissue on the top; arrow) in the third whorl, and extra carpels in the fourth whorl. (F) 35S::*PI* flower with petaloid sepals in the first whorl. (G) 35S::*SRF-PI* flower with petaloid sepals in the first whorl. (H) 35S::*MEF2-PI* flower with petaloid sepals in the first whorl. (I) 35S::*SRF-PI pi-1* flower with petaloid sepals in the first whorl, petals in the second whorl, and stamens in the third whorl. (J) 35S::*MEF2-PI pi-1* flower with petaloid sepals in the first whorl, petals in the second whorl, and stamens in the third whorl. (K) 35S::*SRF-AP3 35S::SRF-PI* flower with petals in whorls one and two and stamens interior to those. (L) 35S::*MEF2-AP3 35S::MEF2-PI* flower with petals in whorls one and two and stamens interior to those.

whorl carpels decreased in an acropetal manner. The vegetative phenotype exhibited by 35S::*AP3 35S::PI* plants, smaller leaves curled around the midrib (Krizek and Meyerowitz, 1996b), was also shown by 35S::*SRF-AP3 35S::SRF-PI* and 35S::*MEF2-AP3 35S::MEF2-PI* plants (our unpublished results).

In summary, *SRF-AP3* and *MEF2-AP3* or *SRF-PI* and *MEF2-PI* are indistinguishable from *AP3* or *PI* in the gain-of-function phenotypes (both in singly and doubly transgenic lines) and in their capability to rescue the corresponding *ap3-3* or *pi-1* mutant phenotypes by ectopic expression.

Ectopic Expression of SRF-AP1 and MEF2-AP1 Has the Same Phenotypic Effects as Ectopic Expression of AP1

Features characteristic of *AP1* ectopic expression (35S::*AP1* plants) include early flowering, premature differentiation of the main inflorescence in a terminal floral structure (composed of several incomplete flowers), and the conversion of lateral inflorescences into solitary flowers (Mandel and Yanofsky, 1995; Figure 5, A–C). When ectopically expressed in a *Ler* genetic background, *AP1* also causes reduced stamen fertility and alterations in gynoecium shape. An identical phe-

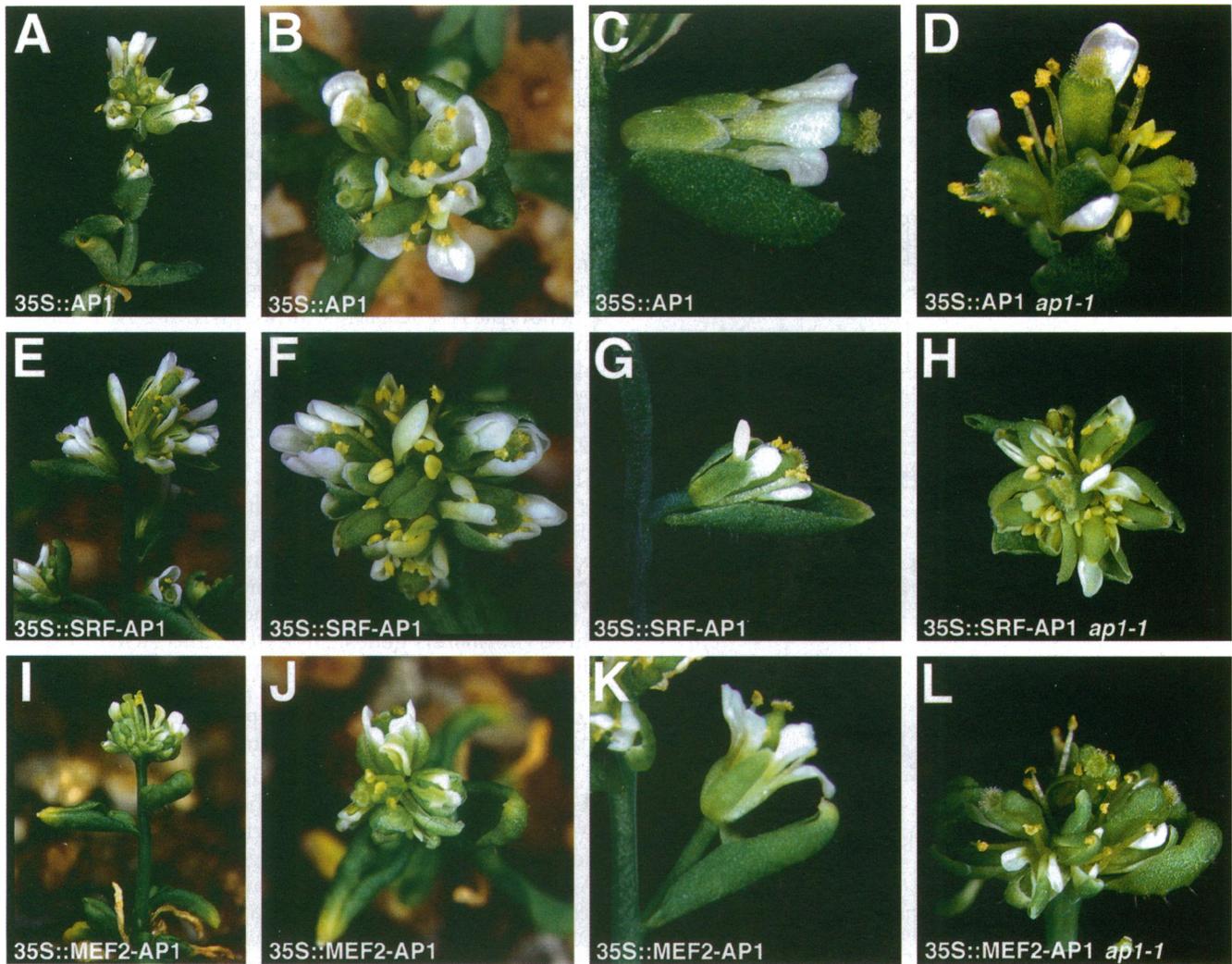


Figure 5. Ectopic expression of *SRF-AP1* and *MEF2-AP1* causes the same phenotypic changes as ectopic expression of *AP1*. (A) 35S::*AP1* plant. (B) 35S::*AP1* terminal flower. (C) Conversion of a secondary inflorescence of a 35S::*AP1* plant into a solitary flower. (D) 35S::*AP1 ap1-1* terminal flower. Sepals and petals are present, although in reduced number. (E) 35S::*SRF-AP1* plant. (F) 35S::*SRF-AP1* terminal flower. (G) Conversion of a secondary inflorescence of a 35S::*SRF-AP1* plant into a solitary flower. (H) 35S::*SRF-AP1 ap1-1* terminal flower. Sepals and petals are present, although in reduced number. (I) 35S::*MEF2-AP1* plant. (J) 35S::*MEF2-AP1* terminal flower. (K) Conversion of a secondary inflorescence of a 35S::*MEF2-AP1* plant into a solitary flower. (L) 35S::*MEF2-AP1 ap1-1* terminal flower. Sepals and petals are present in these flowers, although their number is reduced.

nototype was observed in transgenic lines ectopically expressing the chimeric gene *SRF-AP1* (5/9 different 35S::*SRF-AP1* strains; Figure 5, E–G). Three lines showed a wild-type phenotype, and one line had curled leaves and crinkled carpels but was otherwise normal (see below). In flowers of plants homozygous for the strong *ap1-1* allele, sepals are converted to bract-like organs, in the axils of which secondary flowers develop, but second whorl petals are absent (Bowman *et al.*, 1993). 35S::*SRF-AP1 ap1-1* plants showed that the chimeric gene can largely, although not completely, complement the *ap1-1* mutation: flowers of these plants have sepals and petals, although in re-

duced number compared with wild-type (Figure 5H). A similar degree of rescue of the *ap1-1* mutant phenotype is observed in 35S::*AP1 ap1-1* plants (Figure 5D).

Early flowering and the formation of a terminal flower was also caused by ectopic expression of *MEF2-AP1* (35S::*MEF2-AP1* plants; 11 of 23 transgenic lines; Figure 5, I–K). For this chimeric gene, however, a greater variability in the strength of the gain-of-function phenotype was observed. Some of the transgenic lines were extremely dwarfed and produced a terminal flower before the stem could elongate; those could be considered as exhibiting a very strong *AP1* gain-

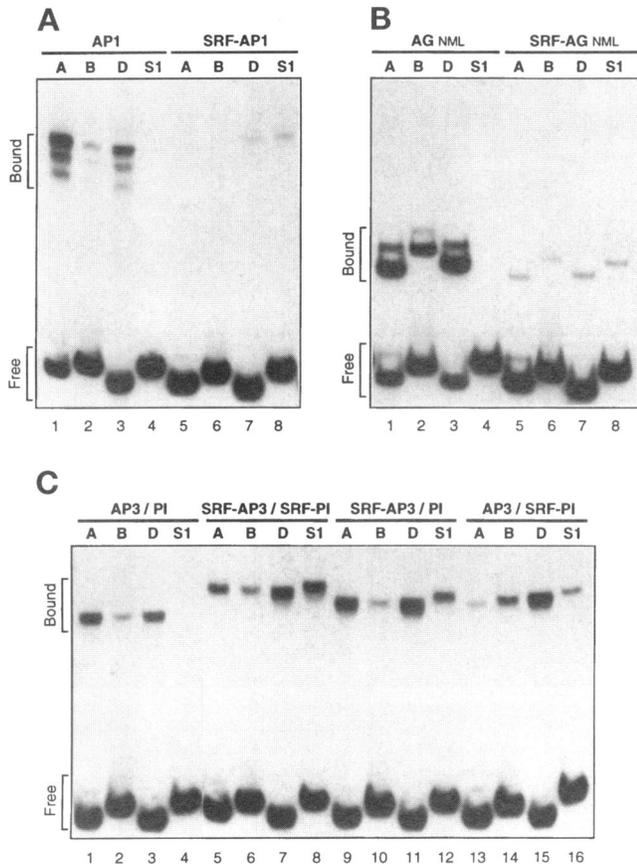


Figure 6. DNA-binding activity of SRF chimeric proteins assayed with CARG box-containing probes A, B, D, and S1. (A) EMSA of in vitro-translated AP1 and SRF-AP1. The various shifted bands present in the reactions with AP1 correspond to protein-DNA complexes formed by the full-length proteins and by truncated proteins also produced in the translation reactions (Riechmann *et al.*, 1996a). (B) EMSA of in vitro-translated AGNML and SRF-AGNML. Reactions with AGNML showed an additional band of lower mobility that could be due to a different conformation or shape of the protein-DNA complexes (Riechmann *et al.*, 1996b). (C) EMSA of AP3, PI, SRF-AP3, and SRF-PI. Proteins were synthesized by cotranslation in the indicated combinations.

of-function phenotype. Some other lines, on the contrary (12/23 lines), consisted of plants that were normal in size but had strongly curled leaves and crinkled carpels. This is apparently a weaker AP1 gain-of-function phenotype because 1) some of these inflorescences eventually formed a terminal flower and 2) the same phenotype was observed in some plants ectopically expressing AP1 and, in particular, among the progeny of a 35S::AP1 line that initially exhibited all the characteristics of the AP1 gain-of-function phenotype. 35S::MEF2-AP1 lines exhibiting the most typical 35S::AP1 phenotype (Figure 5, I-K) were crossed to *ap1-1* mutant plants. The 35S::MEF2-AP1 *ap1-1* strain showed that the chimeric gene can partially complement the *ap1-1* mutation: sepals and petals are present

in the terminal flower of these plants (Figure 5L), although their number is reduced. 35S::MEF2-AP1 lines exhibiting the presumed weaker gain-of-function phenotype were also crossed to *ap1-1* mutant plants. These transgenic lines provided a very weak rescue of the *ap1-1* mutant phenotype: petals were only rarely observed in the flowers of the 35S::MEF2-AP1 *ap1-1* F2 plants (our unpublished results).

In summary, both the SRF-AP1 and MEF2-AP1 chimeric genes retain the activities characteristic of AP1 as manifested in ectopic expression experiments and phenotypic rescue assays, although MEF2-AP1 may be more sensitive to expression level for the achievement of proper AP1 function.

DNA-binding Activity of the Chimeric Proteins

The DNA-binding capabilities of in vitro-translated wild-type AP1, AP3, PI, AG, and the SRF and MEF2 chimeric proteins were compared using several CARG box-containing sequences as binding sites in EMSAs. Probes A, B, and D were chosen because they contain CARG sequences that are found in the promoters of three *Arabidopsis* genes, AP3, SUP, and AGL5, respectively (Riechmann *et al.*, 1996b). Probes S1 and ME contain CARG boxes specific for SRF and MEF2A, respectively (Treisman, 1987; Pollock and Treisman, 1991; Nurrish and Treisman, 1995).

AP1 bound to probes A, B, and D but did not recognize the SRF-specific probe S1 (Figure 6A, lanes 1-4). This result is consistent with the previous observations that the DNA-binding specificities of AP1, AP3/PI, and AG are very similar (Riechmann *et al.*, 1996b), that SRF and MCM1 have related but distinct DNA-binding specificities (Wynne and Treisman, 1992), and that the DNA-binding specificity of AG is more closely related to that of MCM1 than that of SRF (Huang *et al.*, 1993; Shiraishi *et al.*, 1993). The chimeric protein SRF-AP1 bound to probe S1 (Figure 6A, lane 8), indicating that exchanging the amino-terminal half of a plant MADS domain indeed transferred the determinants for DNA-binding specificity. This chimeric protein also recognized probe D but did not bind to probes A or B (Figure 6A, lanes 5-7). Therefore, the substitution of the AP1 amino-terminal half of the MADS domain with that of SRF caused a change in DNA-binding specificity with respect to the wild-type AP1 protein.

Similarly to AP1, the core truncated protein AGNML (which maintains intact the full-length AG DNA-binding properties; Riechmann *et al.*, 1996b) bound to probes A, B, and D and did not recognize probe S1, which was bound by SRF-AGNML (Figure 6B). These results indicate that the DNA-binding specificity of AG was modified as a consequence of the SRF amino acid replacement. SRF-AGNML was capable of binding to probes D and, in contrast to SRF-AP1, also to probes

A and B (Figure 6B, lanes 5–7), indicating that sequences outside the amino-terminal half of the MADS domain (which is shared by SRF-AP1 and SRF-AG-NML) can affect the interaction between a particular protein and binding site(s). Similar results were obtained when comparing the DNA-binding activity of the heterodimers AP3/PI and SRF-AP3/SRF-PI: the latter recognized probe S1, which was not bound by AP3/PI, and also probes A, B, and D (Figure 6C, lanes 1–8). The two heterodimers formed by one wild-type protein and one chimeric protein, SRF-AP3/PI and AP3/SRF-PI, were capable of binding efficiently to probe S1 (Figure 6C, lanes 12 and 16), suggesting that either one of the two MADS domains of the dimer is sufficient to confer to the complex the capability to bind to a particular site in the assay used. The relative affinities of AP3/PI, SRF-AP3/SRF-PI, SRF-AP3/PI, and AP3/SRF-PI for the different probes varied among the complexes. For example, AP3/PI showed the strongest binding to probes A and D, recognizing the probes in the order $A \sim D > B$, whereas the order for AP3/SRF-PI was $D > B > S1 > A$ (Figure 6C).

Similar experiments were performed with the MEF2-chimeric proteins, for which more drastic changes in DNA-binding specificity were expected because MEF2A binds to CArG boxes of the type $CTA(A/T)_4TAG$, instead of $CC(A/T)_6GG$ (Pollock and Treisman, 1991; Nurrish and Treisman, 1995). In contrast to AP1, AGNML, and AP3/PI; MEF2-AP1, MEF2-AGML, and MEF2-AP3/MEF2-PI did not bind to probes B and D, both of which have CArG sequences of the $CC(A/T)_6GG$ -type (Figure 7), therefore, confirming that a drastic change in DNA-binding specificity had been generated (binding of the MEF2 chimeric proteins to these probes was undetectable even after prolonged exposure of the film). MEF2-AP1, MEF2-AGML, and MEF2-AP3/MEF2-PI bound the MEF2A-specific probe ME (CTATTTATAG) and also recognized probe A, which contains a hybrid CArG box, with each one of its half sites of a different type: CCATT-TTTAG (Figure 7). The binding of the MEF2 chimeric proteins to this hybrid probe is in agreement with the suggestion that only one of the two MADS domains of a dimer is required for the complex to achieve a particular DNA-binding specificity. As expected considering this "one-domain-is-sufficient" hypothesis, the heterodimer AGNML/MEF2-AGML could bind to all four probes (Figure 7B, lanes 8–12), and the same result was obtained with the heterodimer AP3/MEF2-PI (Figure 7C, lanes 13–16). MEF2-AP3/PI, on the other hand, exhibited only the MEF2 DNA-binding specificity, because it bound probes A and ME but did not recognize probes B and D and, therefore, represents an exception to the predictions of this hypothesis.

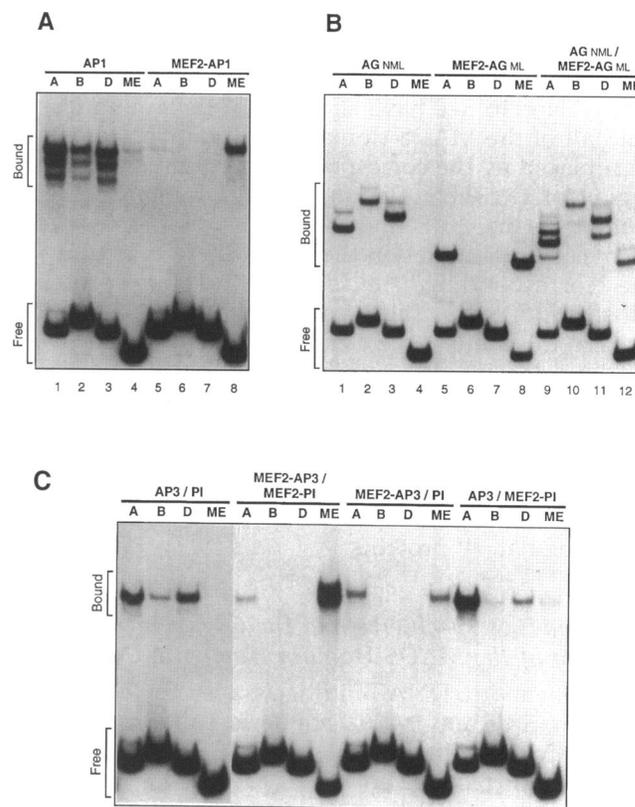


Figure 7. DNA-binding activity of MEF2 chimeric proteins assayed with CArG box-containing probes A, B, D, and ME. (A) EMSA of in vitro-translated AP1 and MEF2-AP1. The various shifted bands present in the reactions correspond to protein–DNA complexes formed by the full-length proteins and by truncated proteins also produced in the translation reactions. (B) EMSA of in vitro-translated AGNML and MEF2-AGML. Reactions with AGNML showed an additional band of lower mobility that could be due to a different conformation or shape of the protein–DNA complexes (Riechmann *et al.*, 1996b). AGNML and MEF2-AGML were also synthesized by cotranslation and assayed together for DNA binding (lanes 9–12). The heterodimer of both proteins could recognize all four probes. Protein–DNA complexes were separated from the free probes on a 8% gel. (C) EMSA of AP3, PI, MEF2-AP3, and MEF2-PI. Proteins were synthesized by cotranslation in the indicated combinations.

DISCUSSION

Previous studies in which hybrid genes were generated by swapping sequences among AP1, AP3, PI, and AG showed that the MADS domains (or their amino-terminal halves) were functionally interchangeable among the four proteins, indicating that this region does not determine their biological specificity, and therefore, suggested that the functional specificity does not arise from differences in the intrinsic DNA-binding properties of AP1, AP3, PI, and AG (Krizek and Meyerowitz, 1996a; Krizek, Riechmann, and Meyerowitz, unpublished results). The DNA-binding characteristics of the four proteins were indeed found to be

very similar in in vitro assays (Riechmann *et al.*, 1996b), in agreement with the high level of amino acid sequence identity existent among their MADS domains. Herein, we have shown that the amino-terminal half of the MADS domain of all four proteins can be replaced by the corresponding and more divergent region of two heterologous MADS domain proteins from humans, SRF and MEF2A, and the resulting chimeric proteins retain the organ identity activity of the corresponding *Arabidopsis* wild-type proteins. Most importantly, the heterologous MADS domains used in this study have clearly distinct intrinsic DNA-binding specificities compared with the *Arabidopsis* MADS domains. This difference has allowed us to address some questions about the overall role that DNA-binding specificity may play in the in vivo activity of AP1, AP3, PI, and AG and about the relationship between this specificity and the dimeric nature of these regulatory proteins.

DNA-binding Specificity and the Organ Identity Activity of the MADS Domain Homeotic Proteins

First, the in vitro DNA-binding assays showed that the different SRF and MEF2 chimeric proteins had the capability of binding to SRF- and MEF2A-specific sites that are not recognized by AP1, AP3/PI, or AG. In addition, the MEF2 chimeric proteins did not bind to *Arabidopsis* CC(A/T)₆GG-type probes that are recognized by AP1, AP3/PI, and AG. Because the amino acid replacement resulted in a change in binding specificity, it can be concluded that the amino-terminal half of the MADS domain contains the determinants of DNA-binding specificity in the context of the plant MADS proteins, in agreement with the results obtained with SRF, MCM1, and MEF2A (Nurrish and Treisman, 1995). However, we also found that sequences carboxyl terminal to this region can affect the interaction between a particular protein and binding site(s). This is exemplified by the fact that SRF-AP1 differed from SRF-AG_{NML} and SRF-AP3/SRF-PI in the binding (or the absence of it) to probes A and B. Also, MEF2-AP1 and MEF2-AP3/MEF2-PI bound probe ME considerably better than probe A, whereas MEF2-AG_{NML} showed similar binding to the two probes. In each case, all three complexes share the same amino-terminal SRF or MEF2 MADS regions. Thus, the assertion that DNA-binding specificity is solely a function of several of the amino acids of the MADS domain amino-terminal basic region and of its amino-terminal flanking sequences (Nurrish and Treisman, 1995) cannot be generalized to the plant MADS domain proteins, because sequences carboxyl terminal to that region can contribute to DNA-binding specificity, at least in particular cases. From the crystal structure of core SRF bound to DNA, the carboxyl terminal half of the MADS domain makes some con-

tacts with the phosphate DNA backbone (Pellegrini *et al.*, 1995; Figure 1A) and, although three of the five residues in that SRF region involved in these contacts are conserved among SRF, MEF2A, AP1, AP3, PI, and AG, it is possible that this type of interaction mediates the observed differences in DNA binding. The importance of phosphate-backbone contacts for binding site recognition has been previously described for a variety of proteins (for example, Pabo *et al.*, 1990; Furukubo-Tokunaga *et al.*, 1992).

In any case, it is clear that the SRF substitution modified, and the MEF2A replacement changed substantially, the DNA-binding specificity of the resulting chimeric proteins with regard to the corresponding *Arabidopsis* wild-type proteins. Hence, the analysis of the in vivo activity of these chimeric proteins should provide an estimate of the dependence of the organ identity activity on the DNA-binding specificity of AP1, AP3, PI, and AG. The A, B, and D probes used in the DNA-binding assays are derived from the *Arabidopsis* genome, and they were of particular interest because the genes from which they are derived have been proposed to be regulated by AP3/PI (probe A, from AP3; Goto and Meyerowitz, 1994; Jack *et al.*, 1994; Krizek and Meyerowitz, 1996b), by AG (probe D, from AGL5; Savidge *et al.*, 1995), and by AP3/PI and AG (probe B, from SUP; Sakai and Meyerowitz, unpublished results), and therefore, contain CA_rG boxes that may be in vivo binding sites for these organ identity proteins. The SRF chimeric proteins acquired new DNA recognition properties (they bound to probe S1) but also retained the capability of binding to probes A, B, and D (with the exception of SRF-AP1, which did not bind to probes A and B). However, the relative affinities with which the binding sites were recognized could vary substantially, as exemplified by the different binding of AP3/PI and AP3/SRF-PI (the complex that would be formed in 35S::SRF-PI *pi-1* plants, in which SRF-PI would be the only dimerization partner for the endogenous AP3 protein) to probes A, B, and D. Those variations in DNA-binding affinities do not affect the organ identity activity of the proteins, because SRF-AP1, SRF-AP3, SRF-PI, and SRF-AG were indistinguishable from AP1, AP3, PI, and AG, respectively, in ectopic expression experiments and phenotypic rescue assays. The MEF2 chimeric proteins lost the capability of binding in vitro to CA_rG boxes of the CC(A/T)₆GG type, which are recognized by AP1, AP3/PI, and AG. In spite of that, MEF2-AP1, MEF2-AP3, MEF2-PI, and MEF2-AG conserved the organ identity activity of AP1, AP3, PI, and AG, respectively, implying that they are capable of regulating target gene expression much in the same way as do the wild-type proteins. MEF2-AG_{NML} did not appreciably bind to the AGL5-derived probe, but expression from the AGL5 promoter was ectopically induced in the cauline leaves of 35S::MEF2-AG plants, as it is in the

cauline leaves of 35S::AG plants (Savidge *et al.*, 1995). This result does not imply that *AGL5* is not a direct target of *AG*. On the contrary, it may be an example of target gene regulation *in vivo* without detectable DNA binding *in vitro* to the site that might mediate that regulation. Interestingly, the cooperative activation of muscle gene expression by MEF2 and the myogenic basic helix-loop-helix proteins in transfected fibroblasts requires direct interactions between the DNA-binding domains of both factors, but only one of them needs to be bound to DNA (Molkentin *et al.*, 1995).

In summary, it can be concluded from the *in vitro* and *in vivo* analyses of the activity of the SRF and MEF2 chimeric proteins that determination of floral organ identity by AP1, AP3, PI, and AG is independent of, or only loosely related to, their DNA-binding specificity. What mechanisms, then, allow these proteins to direct the development of different organs?

Some answers to the problem of how highly similar transcription factors can achieve their distinct *in vivo* functional specificities are emerging from studies on the *Drosophila* homeodomain regulatory proteins, which include the homeotic (HOM) proteins (reviewed in Mann, 1995). HOM proteins, *in vitro*, can recognize the same binding sites with similar, although not identical, affinities. Subtle differences in DNA binding may account for part of the unique regulatory specificities of the HOM proteins (Dessain *et al.*, 1992; Ekker *et al.*, 1992), but selective protein-protein interactions with additional cofactors, like the extradenticle (EXD) homeodomain protein, also seem to make an important contribution to that specificity (Chan *et al.*, 1994; Mann and Chan, 1996). In addition, it appears that the target site itself plays an important role, because EXD prefers to bind cooperatively with different HOM proteins depending on subtle differences in the heterodimer binding site (Chan and Mann, 1996). On the basis of the lack of correlation between DNA-binding properties and functional specificity, it has been suggested previously that interactions with additional (unknown) cofactors are probably crucial for the activity of the MADS-domain organ identity proteins (Krizek and Meyerowitz, 1996a; Riechmann *et al.*, 1996b), and the results described in this article further support this suggestion. It is interesting to note that, despite their importance, protein-protein interactions (or whichever other mechanism is responsible for the homeodomain protein functional specificity) do not appear to make it possible for the *Drosophila* homeodomain regulatory proteins to withstand a drastic change in DNA-binding specificity (comparable to that generated by the MEF2A substitution) without losing a significant portion of their biological activity. All HOM proteins, as well as other homeodomain regulatory proteins such as fushi tarazu (FTZ), have a Gln at amino acid 50 in the third helix of the homeodomain. This residue can

be an important sequence-specificity determinant for the homeodomains, and changing it from Gln to Lys (the residue found at that position of the bicoid protein; BCD) in the FTZ homeodomain enables the mutant FTZ protein to bind *in vitro* with high affinity to BCD-binding sites (GGATTA), in contrast to the wild-type FTZ protein, which binds with high affinity to a CCATTA motif but only weakly to a GGATTA site (Percival-Smith *et al.*, 1990). This mutant FTZ-Q50K protein only retained weak wild-type FTZ activity in gene activation and in phenotypic rescue assays: it could not rescue *ftz*⁻ mutant animals to adulthood, although it conferred partial embryonic rescue (Schier and Gehring, 1993). Conversely, a BCD mutant protein with a Lys to Gln change at position 50 recognizes Antennapedia (ANTP)-class target sites instead of BCD sites, and this altered-specificity BCD mutant protein was shown to lack wild-type BCD activity because it could not rescue anterior pattern defects in *bcd*⁻ embryos (Hanes *et al.*, 1994). A chimeric FTZ protein that contained part of the muscle segment homeobox (MSH) homeodomain was not able to complement an *ftz*⁻ mutant, although the embryos could show some cuticular rescue phenotype that was dependent on gene dosage (Furukubo-Tokunaga *et al.*, 1992). This chimeric FTZ protein was defective in binding to an ANTP-class target site that is recognized by wild-type FTZ but not by MSH, and the analysis of additional FTZ mutant proteins indicated that as little as a sixfold reduction of the *in vitro* DNA-binding activity from the wild-type level may lead to a severe defect of *ftz* function in flies (Furukubo-Tokunaga *et al.*, 1992). Because we did not detect any clear difference between the organ identity activity of the MEF2 chimeric proteins and the corresponding wild-type proteins in both the gain-of-function phenotypes and the phenotypic rescue assays, our results indicate that the activities of the floral MADS domain homeotic proteins are independent of specificity in DNA binding to a higher degree than are those of the homeodomain regulatory proteins of *Drosophila*.

If we postulate that specific cofactors exist for the plant MADS domain proteins, it is conceivable that the cofactors' own binding to DNA may contribute a large portion of the energy for formation of the multiprotein-DNA complex. By this view, binding of the plant MADS domain proteins to their target sites would not require optimal DNA contacts to be made for sufficient binding affinity and might, therefore, effectively function in a largely specificity-independent manner. Precedents for this view exist in the MADS domain protein family. SRF mediates a cellular response to growth factor stimulation, the rapid activation of many immediate-early genes. SRF forms a ternary complex at the *c-fos* (a prototypic immediate-early gene) serum response element by binding to the DNA and recruiting accessory proteins, the ternary

complex factors (reviewed in Treisman, 1994). Ternary complex factors, which are members of the Ets-domain family of proteins, such as SAP-1 and Elk-1, do not bind alone to the *c-fos* Ets-like site but require the prior assembly of the SRF-DNA binary complex. However, it has been shown that Elk-1 (which can bind independently to its own high-affinity binding sites) can recruit SRF into a ternary complex on Ets-CArG elements that do not support formation of the SRF-DNA binary complex because the respective SRF target sequence is suboptimal (Latinkic and Lau, 1994; Latinkic *et al.*, 1996). Moreover, the analyses of the immediate-early genes *nur77* and *pip92* revealed that their activation is mediated through "mutated" CArG sequences, significantly altered from the consensus such that they are not expected to bind strongly to SRF (Williams and Lau, 1993; Latinkic and Lau, 1994). These results showed that, similar to the way in which a low-affinity Elk1-DNA interaction can be compensated by the high-affinity SRF-serum response element interaction (Treisman *et al.*, 1992), a poor CArG box (low-affinity SRF-DNA interaction) can function in conjunction with a high-affinity Ets site (Latinkic and Lau, 1994; Latinkic *et al.*, 1996). A mechanism similar to this latter one may account for the capability of the plant MADS domain proteins to work, with respect to organ identity determination, in a manner that seems to be largely independent of specificity in DNA binding.

Dimerization of the Plant MADS Domain Proteins

The analysis of the DNA-binding activity of the SRF and MEF2 chimeric proteins revealed that, in general, either one of the two MADS domains of a dimer is sufficient to confer to the complex the capability to bind to a particular site. Examples are the binding of SRF-AP3/PI and of AP3/SRF-PI (but not of AP3/PI) to probe S1; the binding of the MEF2 chimeric proteins to the "hybrid" CArG box A, which contains a half site that matches the MEF2 specificity (CCATTTT~~TT~~AG), and their failure to bind to CArG box B, which differs from site A in one base that changes that half site (CCATTTT~~TT~~TGG); the binding of the AGNML/MEF2-AGML heterodimer to probes B and D (which are not bound by a MEF2-AGML homodimer) and to probe ME (which is not bound by an AGNML homodimer); and the binding of MEF2-AP3/PI and of AP3/MEF2-PI (but not of AP3/PI) to probe ME and of AP3/MEF2-PI (but not of MEF2-AP3/MEF2-PI) to probes B and D. The absence of binding of the MEF2-AP3/PI complex to probes B and D was the only exception to this behavior of the MADS domains. Three different mutant SRF proteins, which had single amino acid changes in the MADS domain that abolished DNA binding but did not compromise dimerization, could form DNA-binding heterodimers with

wild-type SRF (Sharrocks *et al.*, 1993a). Therefore, it may be a general feature that (either) one of the MADS domains of a dimer is sufficient to allow the complex to bind DNA (as long as the integrity of the other MADS domain is preserved, because deletion of the amino-terminal half of the MADS domain of AG and AP3 prevents the heterodimers formed with wild-type AG and PI from binding DNA; Riechmann *et al.*, 1996b). It is likely that each MADS domain contributes to the overall specificity (i.e., optimal high-affinity binding site) of a particular dimer. But it is also apparent from the results reported herein that such particular optimal site can be recognized (perhaps with a different affinity) when it is optimum for only one of the two MADS domains of the dimer.

This observation raises the question of whether the chimeric proteins retained the organ identity activity of the corresponding wild-type proteins because they heterodimerize with some other endogenous MADS-domain proteins. AG and AP1 bind to DNA as homodimers (Riechmann *et al.*, 1996a), and genetic analyses have not yet uncovered any essential, or functional, heterodimerization partner for them, which may argue against that possibility considering that SRF-AP1, MEF2-AP1, SRF-AG, and MEF2-AG exhibit the appropriate organ identity activity in the absence of endogenous AP1 or AG, as was shown in the complementation experiments. However, AP1 and AG are not forced to homodimerize, and they can form heterodimeric DNA-binding complexes (Huang *et al.*, 1996; Riechmann *et al.*, 1996a). For example, AG can bind DNA as a heterodimer with AGL1, AGL2, or AGL3 (Huang *et al.*, 1996; similar results have been obtained with the homologous *Antirrhinum* proteins, Davies *et al.*, 1996). Although the plant MADS domain family of proteins is large, MADS-box genes usually show restricted domains of expression, the floral realm being the most frequent one (Ma *et al.*, 1991; Rounsley *et al.*, 1995). From this point of view, ectopic induction of expression from the *AGL5* promoter in the cauline leaves of 35S::SRF-AG and 35S::MEF2-AG plants may be considered an indication that these two proteins, as homodimers, function like AG; but, on the other hand, *AGL3* (but not *AGL1* or *AGL2*) is expressed in vegetative tissues and floral tissues (Ma *et al.*, 1991; Huang *et al.*, 1995). Definitive elucidation of this issue will require a detailed understanding of the network of possible interactions among all the MADS domain proteins that may be coexpressed in the same cells and will also require the availability of mutant alleles of all of the different MADS box genes.

Regardless of whether or not it is the heterodimerization with other endogenous MADS domain proteins that allows each of the individual organ identity proteins to tolerate drastic changes in DNA-binding specificity without losing its activity *in vivo*, the fact that one MADS domain of the dimer can be sufficient

to confer a certain DNA-binding specificity to the complex should be regarded in the context of the plant MADS box multigene family. A recurrent theme when families of transcription factors that function as dimers are considered (including the MADS domain proteins) is the suggestion that heterodimerization allows the formation of different complexes with novel DNA-binding specificities or affinities for different sites. This conception emanated mostly from studies of leucine zipper proteins that showed that cross-family dimerization of Fos/Jun and ATF/CREB factors altered DNA binding specificity (Hai and Curran, 1991). On the contrary, the results that we have described argue against this idea, in its broadest terms, for the MADS domain proteins. No drastically novel DNA-binding specificities seem to be generated through heterodimerization, because an asymmetric or "hybrid" CARG box is recognized by a heterodimer as well as by the two corresponding homodimers. It is interesting to note that, for the leucine zipper proteins, a single normal binding domain in the dimer is insufficient to mediate DNA binding (Gentz *et al.*, 1989; Landschulz *et al.*, 1989) but that the opposite result has been obtained with the MADS domain proteins (see above). If heterodimerization among MADS domain proteins does not create completely novel DNA-binding specificities, it can certainly change the DNA-binding affinities of the resulting complexes (see, for example, Figure 7B). However, as it was previously discussed (Riechmann *et al.*, 1996b) and is exemplified by the conservation of the organ identity activities in the different SRF and MEF2 chimeric proteins, those slight changes or differences in DNA-binding affinities do not seem to contribute to the functional specificity of the plant MADS domain proteins. It nonetheless remains a possibility that heterotypic dimerization may contribute to the generation of regulatory diversity within the MADS domain family of proteins by a mechanism other than changing DNA-binding specificities/affinities.

Conservation of the MADS Domain Sequence

Another question that emerges, given that a part of heterologous and divergent MADS domains can functionally substitute for the corresponding region of AP1, AP3, PI, and AG, is why the MADS domains of these four proteins are so highly conserved (Figure 1A; Theißen *et al.*, 1996). Although we have characterized the organ identity activity of the chimeric proteins and found no significant differences with the corresponding wild-type proteins, it is possible that there are functional constraints not detectable in our assays (an example could be a slight decrease in fertility as a consequence of the mutations that would, in nature, transform AG into MEF2-AG). In addition, we could consider a quali-

tative and a quantitative side in the function of the homeotic proteins, the qualitative being the determination of organ identity and the quantitative the complete development of floral organs. Although at present it is not understood what qualitative and quantitative may mean in molecular terms, there is evidence that both aspects exist. For example, although both 35S::SRF-AP3 35S::SRF-PI and 35S::MEF2-AP3 35S::MEF2-PI flowers consist of two outer whorls of petals and stamens interior to those (the organ identity activity is preserved in the chimeric proteins), the shape of the petals can be slightly different (compare Figure 4, K and L). Also, MEF2-AP1 seems to be more dependent on its expression levels to achieve a correct AP1 activity; and AG may be capable, at least in some cases, of rescuing the determinacy defect of *ag-3* mutant flowers to a higher degree than SRF-AG or MEF2-AG. It is possible that these quantitative aspects are somehow translated into a selection pressure that would determine the conservation of the MADS domain sequences of AP1, AP3, PI, and AG. Further elucidation of this issue will require the expression of the chimeric genes under the control of the regulatory regions of the corresponding wild-type genes (at present poorly characterized), to assure a level and timing of expression as similar and accurate as possible, in transgenic lines of the pertinent mutant backgrounds.

We have found that the organ identity activity of the MADS domain proteins AP1, AP3, PI, and AG does not depend on their DNA-binding specificity. The elucidation of the molecular mechanisms that permit this independence is a challenge for the immediate future, as it is to discover the reasons for the MADS domain sequence conservation shown by these proteins. In addition, we have found that the concept of heterodimerization as a way to increase the regulatory potential of dimeric transcription factors by means of creating new DNA-binding specificities does not seem to apply for the plant MADS domain proteins.

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