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Temporal Relationship between the Transcription of Two Arabidopsis MADS Box Genes and the Floral Organ Identity Genes

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MADS box genes play important roles in specifying floral meristem and floral organ identity. We characterized the temporal and spatial expression patterns of two members of this gene family, *AGL4* and *AGL5* (for *AGAMOUS* [*AG*]-like). *AGL4* RNA initially accumulates after the onset of expression of the floral meristem identity genes but before the onset of expression of the floral organ identity genes. *AGL4* is, therefore, a putative target of the floral meristem identity genes and/or a potential regulator of the floral organ identity genes. *AGL5* is initially expressed early in carpel development, shortly after the onset of *AG* expression. The loss of *AGL5* expression in flowers of *ag* mutants, the activation of *AGL5* by ectopic expression of *AG*, and the specific binding of AG to an element in the *AGL5* promoter identify *AGL5* as a putative direct target of AG. Our study provides possible links between the establishment of floral meristem and floral organ identity as well as subsequent steps in flower development.

INTRODUCTION

Floral meristems originate as small groups of cells on the flanks of the apical and lateral inflorescence meristems. These floral meristems give rise to organ primordia, which subsequently develop into sepals, petals, stamens, and carpels. Based on morphological criteria, Arabidopsis flower development has been divided into distinct stages (Smyth et al., 1990). The floral meristem becomes discernible from the inflorescence meristem during stage 1, and sepal primordia begin to arise during stage 3. By stage 6, which initiates ~42 hr after the onset of stage 3, the sepals completely enclose the bud, and the primordia for all organ types are visible.

Considerable attention has been focused on the genes specifying floral meristem and organ identity. In Arabidopsis, the genes that play important roles in the early step of specifying floral meristem identity are *LEAFY* (*LFY*), *APETALA1* (*AP1*), *CAULIFLOWER* (*CAL*), and *APETALA2* (*AP2*) (Bowman et al., 1989, 1991a, 1993; Irish and Sussex, 1990; Schultz and Haughn, 1991; Weigel et al., 1992), all of which initiate their expression before or during stage 1 (Mandel et al., 1992a; Weigel et al., 1992; Kempin et al., 1995), except *AP2*, which is constitutively expressed (Jofuku et al., 1994). The genes required for the later step of specifying the fate of organ primordia include *AP2*, *AP1*, *APETALA3* (*AP3*), *PISTILLATA* (*PI*), and *AGAMOUS* (*AG*) (Komaki et al., 1988; Bowman et al., 1989; Irish and Sussex, 1990). Genetic studies have shown that AG and AP2 have an antagonistic relationship in specifying organ identity such that AP2 activity prevents the activity of AG in the first and second whorls, and AG activity prevents AP2 from specifying organ identity in the third and fourth whorls (Bowman et al., 1991a). *AG*, *AP3*, and *PI* are first expressed at the onset of stage 3 (Drews et al., 1991; Jack et al., 1992, 1994), and their expression depends to a large extent on the prior activity of *LFY* and *AP1*, suggesting that *LFY* and *AP1* are formally positive regulators of the later-acting floral organ identity genes (Weigel and Meyerowitz, 1993). However, because ~54 hr separates the onset of stages 1 and 3, it is likely that additional factors mediate this interaction.

Examples of genes that may be involved in this intermediate step are the five flower-specific *AGL* genes (*AG*-like), all of which encode putative DNA binding proteins with N-terminal MADS domains (Ma et al., 1991). Five other members of the MADS box–containing gene family, *AG*, *AP1*, *AP3*, *PI*, and *CAL*, have all demonstrated roles in floral morphogenesis (see review by Yanofsky, 1995). These observations establish the central role of MADS box genes in floral development and suggest that additional members of this family may function not only between stages 1 and 3 but also after the determination of floral organ identity. Characterization of these genes will provide further insight into the cascade of regulatory events that lead to the morphogenesis of a flower.

Here, we present an analysis of the expression levels of the flower-specific *AGLs* in wild-type and mutant backgrounds, and we characterize two of these genes, *AGL4* and *AGL5*, in greater detail. By using RNA gel blotting and RNA in situ hybridization

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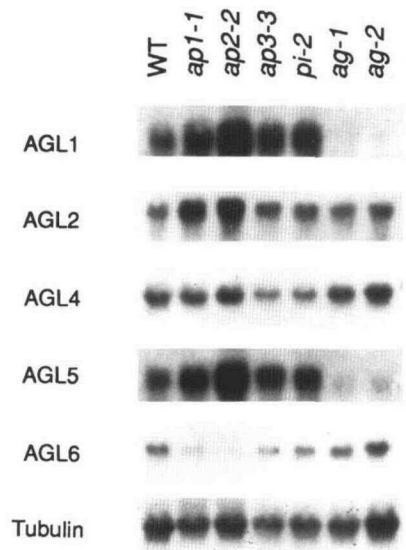

Figure 1. RNA Gel Blot Analysis of *AGL* Genes in Flowers of the Arabidopsis Wild Type and Mutants.

Total RNA (20 μg) from flowers of the wild type and indicated mutants were loaded in each lane and hybridized with probes specific for each *AGL*. The tubulin probe was used as a loading control (Marks et al., 1987). wt, wild type.

analyses with flowers from the wild type and mutants, we found that these genes are likely to be involved in two different steps of flower development. *AGL4* RNA was detected early in flower development and may mediate between the interaction of floral meristem and floral organ identity genes. *AGL5* RNA was first detected after the organ identity genes are initially expressed and is a candidate target gene of AG.

RESULTS

RNA Gel Blot Hybridizations

To characterize further the role of the *AGL* genes in flower development, RNA gel blot hybridizations were performed on total RNA isolated from wild-type flowers and the flowers of the homeotic mutants *ap1-1*, *ap2-2*, *ap3-3*, *pi-2*, *ag-1*, and *ag-2*, as shown in Figure 1. Probes specific for the 3′ end of each *AGL* gene were used to avoid cross-hybridization with other MADS box–containing genes.

The expression levels of *AGL2* and *AGL4* are relatively unaffected in the different floral homeotic mutants (Figure 1), suggesting that they are active before and/or do not require the activity of the organ identity genes. In contrast, the transcript levels of *AGL1* and *AGL5* are dramatically reduced in flowers of *ag* mutants (Figure 1). These data demonstrate that *AGL1* and *AGL5* require *AG* for wild-type levels of RNA accumulation. Transcript levels of these two genes are significantly increased in *ap2* mutant flowers, suggesting that *AP2* might negatively regulate their expression (Figure 1). *AGL6* displayed a dramatic change in RNA levels in specific mutant backgrounds. Its expression is significantly reduced in the *ap1-1* and *ap2-2* mutants and increased in the *ag* mutants (Figure 1). These data suggest that *AGL6* may be positively regulated by *AP1* and *AP2* and/or negatively regulated by *AG*. The temporal and spatial characterization of *AGL1* and *AGL2* has previously been reported by Ma et al. (1991), and *AGL6* is the subject of a separate, detailed study. Further characterization of the *AGL4* and *AGL5* expression patterns using RNA in situ hybridization is provided later in the article.

AGL4 RNA Accumulation in Wild-Type Flowers

RNA gel blot hybridizations showed that accumulation of *AGL4* RNA was unaffected in the different floral mutants (Figure 1). Therefore, RNA in situ hybridization studies of *AGL4* were only performed with wild-type flowers. *AGL4* RNA accumulation was first observed uniformly throughout stage 2 flower primordia, as seen in Figures 2A and 2B, and remained uniform throughout stage 8 (Figures 2C and 2D). No *AGL4* RNA was detected in stage 1 floral primordia or in the inflorescence meristem. At later stages, *AGL4* RNA is restricted to petals, the filaments of stamens, and carpels (Figures 2E to 2H). Within carpels, the level of *AGL4* RNA is particularly high in developing ovules.

AGL5 RNA Accumulates in Developing Carpels

RNA gel blot hybridizations showed that *AGL5* requires *AG* for wild-type levels of expression. To understand further the relationship between these two genes, the expression pattern of *AGL5* was compared to that previously described for *AG* (Yanofsky et al., 1990; Bowman et al., 1991b; Drews et al., 1991) and to our own probing with *AG* of wild-type tissues, as seen in Figures 3A to 3H. *AG* RNA begins to accumulate in the cells that give rise to the stamen and carpel primordia during stage 3, when the sepal primordia are first visible on the flanks of the floral meristem. *AG* RNA continues to be expressed uniformly throughout the third and fourth whorl organ primordia until approximately stage 9, when the sporogenous tissue and ovules begin to differentiate (Figures 3C and 3D). After stage 9, *AG* RNA is restricted to the filament, connective tissue, and anther walls of the stamens. In the carpels, *AG* RNA is restricted to the ovule primordia, where it accumulates to high levels, and to the ovary walls, where it accumulates to low levels. Although *AG* RNA is uniformly expressed throughout the ovule primordia until stage 12 (Figures 3E and 3F), it later becomes restricted to the endothelium, the innermost layer of cells of the inner integument that surrounds the embryo sac. *AG* RNA also accumulates in the developing stigmatic tissue at stage

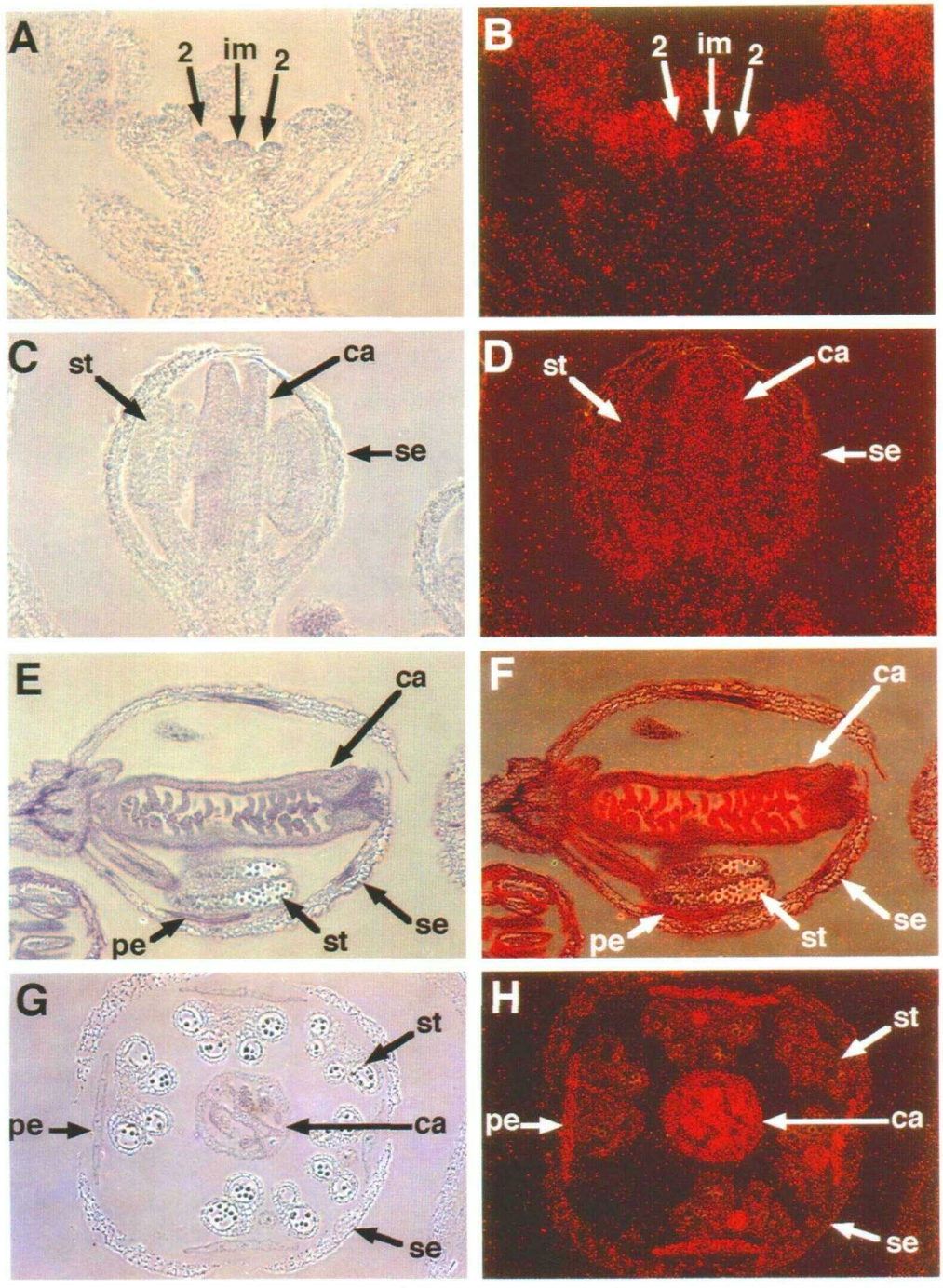

Figure 2. *AGL4* RNA Accumulation in Arabidopsis Wild-Type Floral Tissues.

Longitudinal (**[A]** to **[F]**) and horizontal (**[G]** and **[H]**) sections of wild-type floral tissues hybridized with the *AGL4*-specific antisense mRNA probe are shown. At left are bright-field images; at right are bright/dark-field (red filter) double exposures.

(A) and **(B)** Wild-type inflorescence.

(C) and **(D)** Early stage 9 flower.

(E) and **(F)** Stage 12 wild-type flower.

(G) and **(H)** Cross-section through a wild-type flower.

Numbered arrows indicate stages of floral development. ca, carpel; im, inflorescence meristem; pe, petal; se, sepal; st, stamen.

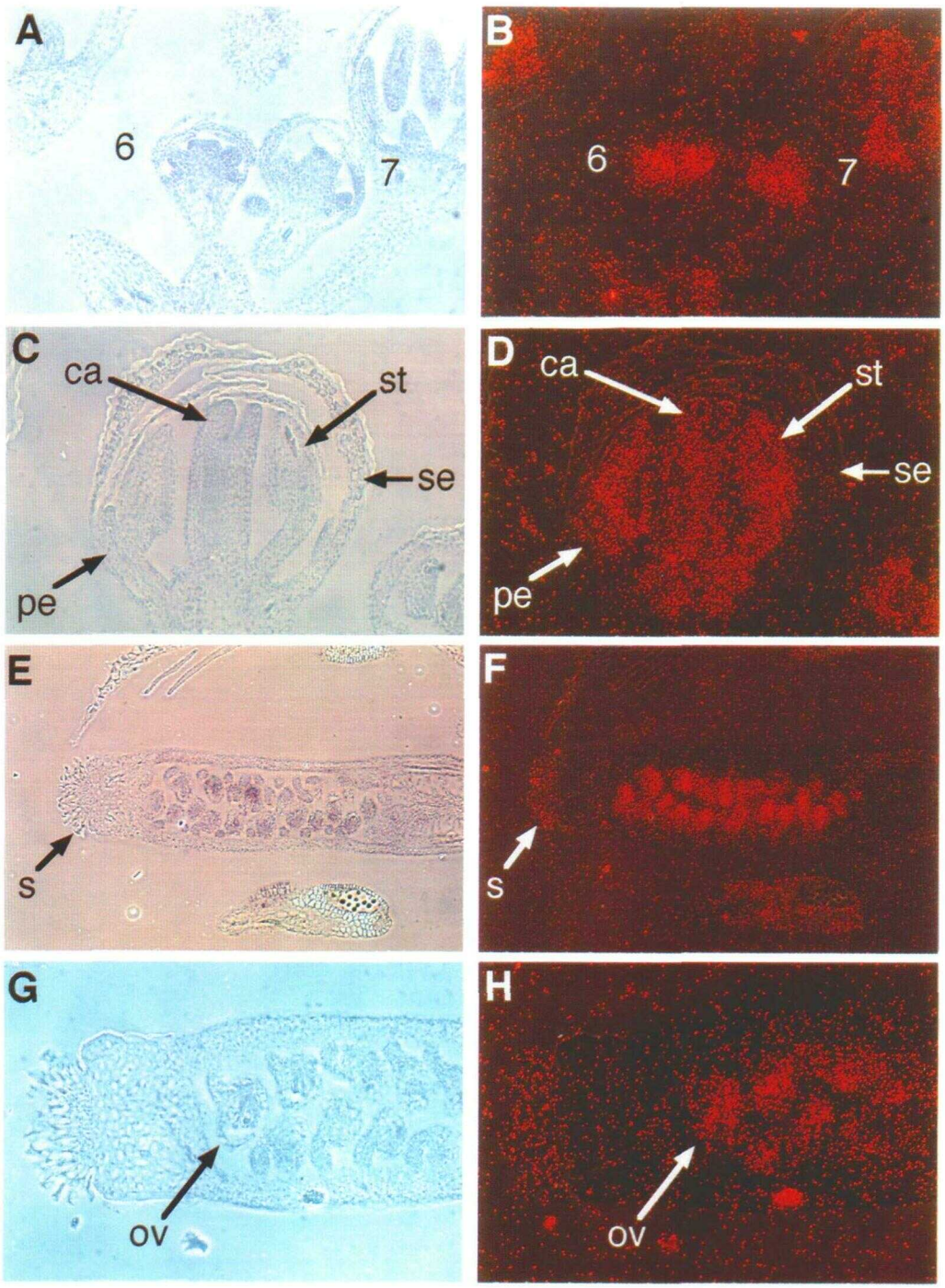

Figure 3. *AG* RNA in Arabidopsis Wild-Type Floral Tissues.

Longitudinal sections of wild-type floral tissues hybridized with the *AG*-specific antisense mRNA probe are shown. At left are bright-field images; at right are bright/dark-field (red filter) double exposures.
(A) and **(B)** Wild-type inflorescence.
(C) and **(D)** Stage 9 flower.
(E) and **(F)** Stigmatic papillae of a stage 12 flower.
(G) and **(H)** Ovules of a stage 13 flower.
Numbers indicate stages of floral development. ca, carpel; ov, ovule; pe, petal; s, stigma; se, sepal; st, stamen.

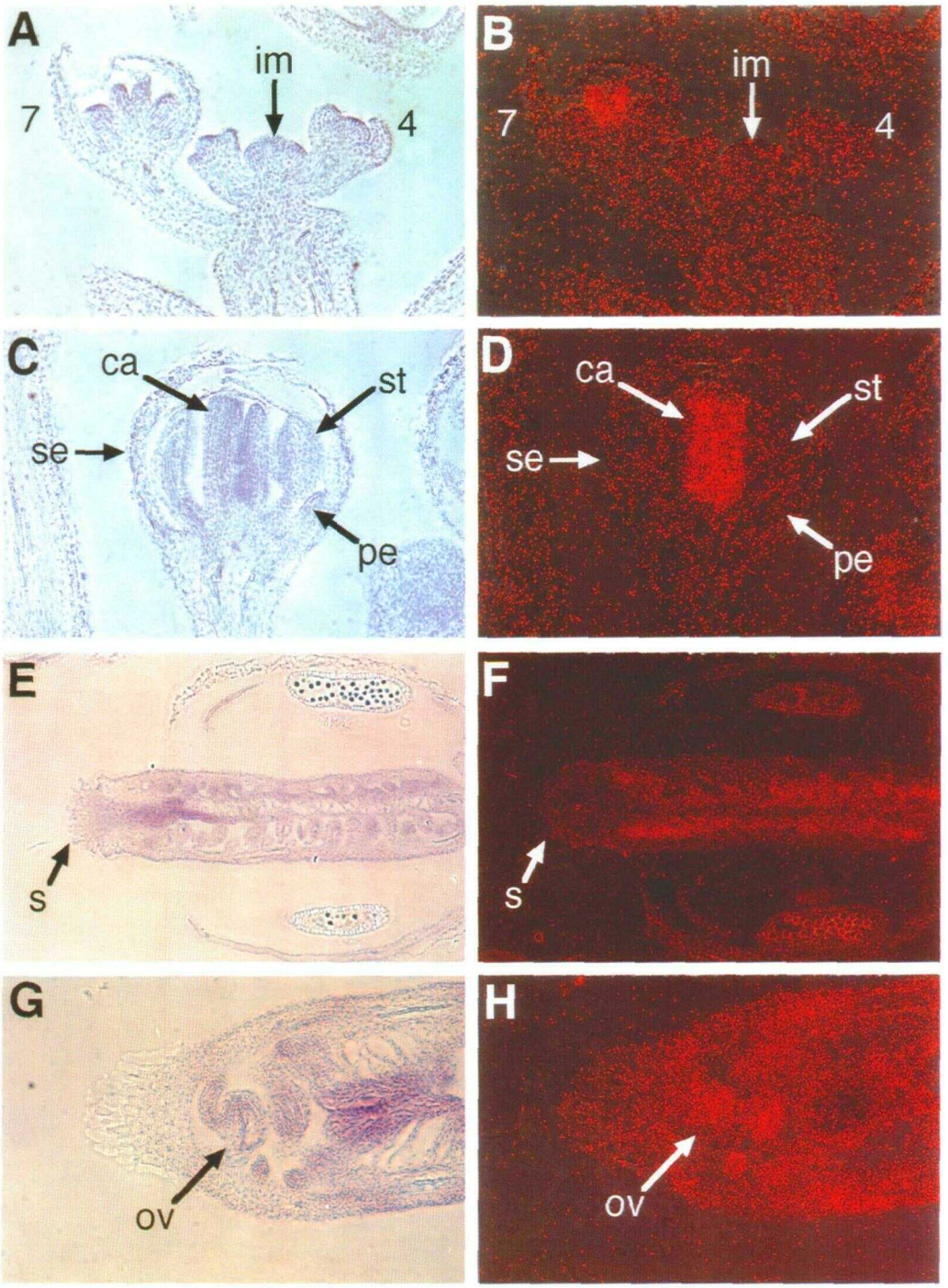

Figure 4. *AGL5* RNA in Arabidopsis Wild-Type Floral Tissues.

Longitudinal sections of wild-type floral tissues hybridized with the *AGL5*-specific antisense mRNA probe are shown. At left are bright-field images; at right are bright/dark-field (red filter) double exposures.

(A) and **(B)** Wild-type inflorescence.

(C) and **(D)** Stage 9 flower.

(E) and **(F)** Stigmatic papillae of a stage 12 flower.

(G) and **(H)** Ovules of a stage 13 flower.

Numbers indicate stages of floral development. ca carpel; im, inflorescence meristem; ov, ovule; pe, petal; s, stigma; se, sepal; st, stamen.

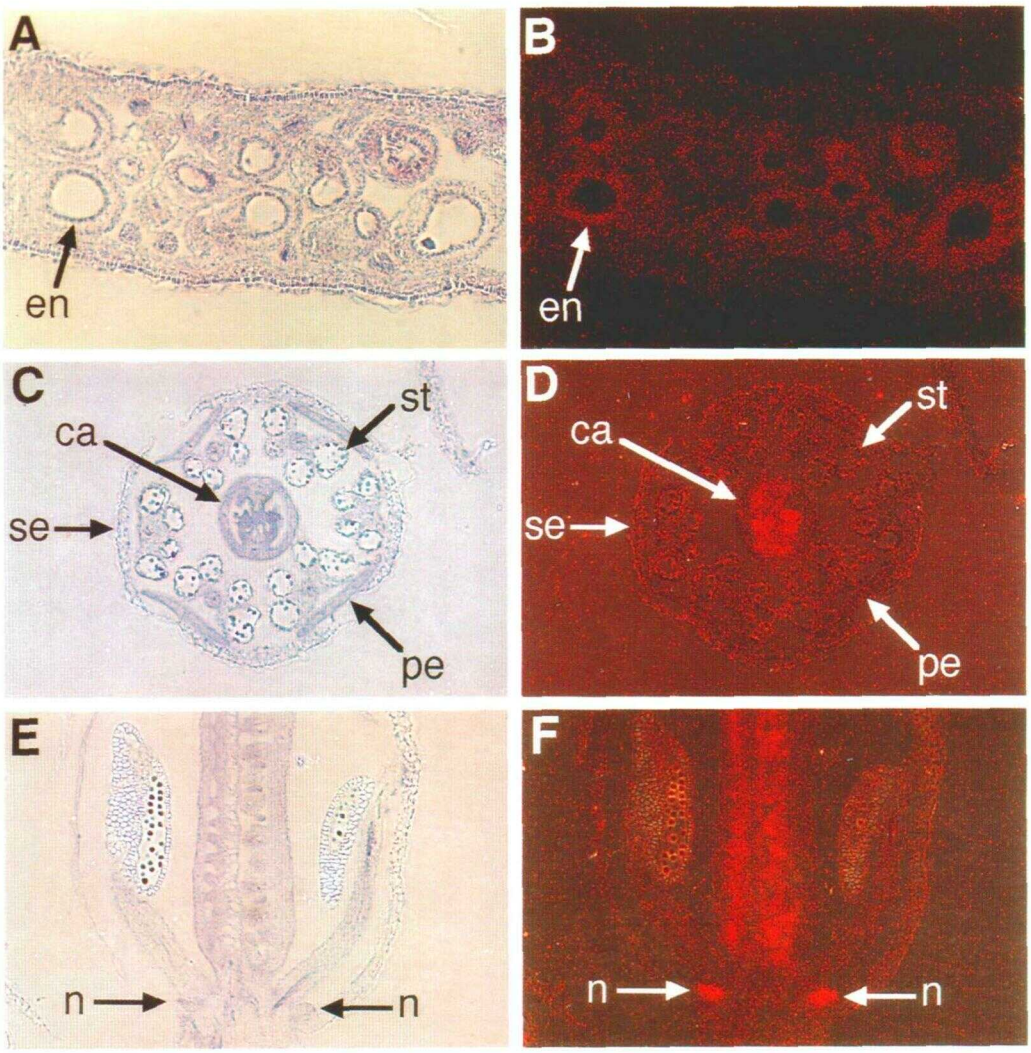

Figure 5. *AGL5* RNA in Arabidopsis Wild-Type Floral Tissues.

Longitudinal sections (**[A]**, **[B]**, **[E]**, and **[F]**) and cross-sections (**[C]** and **[D]**) of wild-type floral tissues hybridized with the *AGL5*-specific antisense mRNA probe are shown. At left are bright-field images; at right are bright/dark-field (red filter) double exposures.

(A) and **(B)** Endothelial cells of mature ovules.

(C) and **(D)** Cross-section through a later-stage flower.

(E) and **(F)** Nectaries of a stage 12 flower.

ca, carpel; en, endothelium; n, nectary; pe, petal; se, sepal; st, stamen.

11 and beyond (Figures 3E to 3H). In addition, *AG* RNA is localized to the nectaries, which are structures found at the base of the stamens (Bowman et al., 1991b).

AGL5 RNA begins to accumulate uniformly throughout the carpel primordia during stage 6 of flower development (Figures 4A and 4B). *AGL5* RNA does not accumulate in sepals, petals, or stamens at any stage of development. The uniform expression of *AGL5* in carpels is maintained through stage 8 (Figures 4C and 4D). Once ovules begin to develop, *AGL5* RNA is restricted to the ovule primordia (Figures 4E and 4F) as well as to the cells of the septum and the region of the ovary wall that contacts the septum (Figures 5C and 5D). *AGL5* RNA was not detected in the stigmatic papillae (Figures 4E and 4F), whereas *AG* RNA accumulates at low levels in this tissue type (Figures 3E and 3F). Because *AG* RNA accumulates at much higher levels than that of *AGL5*, we cannot rule out the possibility that *AGL5* is expressed in stigmatic tissue at levels not detectable by RNA in situ hybridization. As the ovules differentiate, *AGL5* RNA is limited to the inner integument and funiculus (Figures 4G and 4H). *AGL5* RNA is restricted to the endothelium

of mature ovules (Figures 5A and 5B). Interestingly, *AGL5* accumulates to high levels in the nectaries (Figures 5E and 5F), as was also reported for *AG* by Bowman et al. (1991b).

AGL5 RNA Accumulation in Floral Mutants

If *AG* is required for *AGL5* expression, as predicted by RNA gel blotting data, it would be anticipated that little or no *AGL5* RNA would be detected in *ag* mutant flowers by RNA in situ hybridization. Flowers of the severe *ag-1* mutant have normal sepals and petals; however, the stamens that normally occupy the third whorl are replaced with petals. The loss of AG activity also results in the loss of determinacy such that a new flower develops in the position of the fourth whorl carpels (Bowman et al., 1989). No *AGL5* signal was detected in the *ag-1* mutant, as seen in Figures 6A and 6B. These data support the conclusion that AG is a positive regulator of *AGL5*, although they are not sufficient to determine whether this interaction is direct or indirect. For example, the absence of *AGL5* RNA accumulation in *ag* mutant flowers could be due to the ectopic activity of a negative regulator, such as AP2 (Bowman et al., 1991a).

To determine whether AP2 plays a role in regulating *AGL5*, RNA in situ hybridizations were performed with *ap2* mutant flowers. In the severe *ap2-2* mutant, carpels develop in the first and fourth whorls, and second and third whorl organs are usually absent (Komaki et al., 1988; Bowman et al., 1991a). *AGL5* RNA accumulates in the carpels of *ap2-2* flowers by at least stage 5 of development (Figures 6C and 6D). Later *AGL5* RNA accumulation in all carpels is similar to that in the wild type (data not shown). The presence of additional carpels in the *ap2-2* mutant most likely accounts for the elevated levels of *AGL5* RNA observed in the RNA gel blots. These results support the hypothesis that AG is a positive regulator of *AGL5* because, as has been demonstrated, *AG* expression expands into the first whorl of *ap2* mutants (Drews et al., 1991). However, these results do not rule out the possibility that AP2 plays a negative regulatory role.

If AG is a positive regulator of *AGL5*, then it would be anticipated that little or no *AGL5* RNA would accumulate in the flowers of *ag ap2* double mutants, as observed in the *ag* single mutant. If AP2 is a negative regulator and AG is not required for activation of *AGL5*, then high levels of *AGL5* RNA accumulation would be expected in the flowers of *ag ap2* double mutants. To investigate this possibility, RNA in situ hybridizations were performed with flowers of *ag-1 ap2-2* double mutants. The first whorl organs of the double mutant are leaflike, and the second and third whorl organs are intermediate between petals and stamens. Similar to the *ag-1* single mutant, a new flower develops in the position of the fourth whorl, and this pattern can be repeated many times (Bowman et al., 1991a). No *AGL5* RNA was detected in the *ag-1 ap2-2* double mutant (Figures 6E and 6F), verifying that AG is formally a positive regulator of *AGL5*. These data do not allow us to conclude whether AP2 also has a role as a negative regulator.

If AG activates *AGL5*, it would be expected that *AGL5* RNA would be detected in a pattern similar to that of *AG*, namely, in both stamen and carpel primordia. However, *AGL5* RNA was only detected in carpel primordia, indicating that *AGL5* may be negatively regulated in the third whorl of wild-type flowers. Two possible candidates for this negative regulation are *AP3* and *PI*; these genes are expressed by stage 3 in second and third whorl primordia (Jack et al., 1992, 1994). To address whether *AP3* and *PI* have roles in negatively regulating *AGL5*, RNA in situ hybridizations were performed with flowers from *ap3-3* and *pi-1* mutants. The mutations of the *ap3-3* allele or *pi-1* allele result in the conversion of the second whorl petals into sepals and the third whorl stamens into carpels that often fuse to the fourth whorl carpels (Bowman et al., 1989, 1991a; Hill and Lord, 1989; Jack et al., 1992). *AGL5* RNA was observed in both the third and fourth whorl developing carpels of *ap3-3* (Figures 6G and 6H) and *pi-1* (data not shown) mutants, beginning at approximately stage 5. Expression of *AGL5* in late *ap3-3* (data not shown) and *pi-1* carpels (Figures 6I and 6J) was similar to the pattern observed in wild-type carpels.

AG Binds to the *AGL5* Promoter

RNA gel blot and RNA in situ hybridization studies identified *AGL5* as a candidate target gene of AG. Therefore, we sequenced the 5′ flanking region of *AGL5* to determine whether it contains an AG consensus binding site. The DNA binding consensus sequence for AG has been identified using a strategy combining polymerase chain reaction and gel mobility shift assays. Shiraishi et al. (1993) identified the consensus as 5′-TT(A/T/G)CC(A/T)$_6$GG(A/T/C)AA-3′, and Huang et al. (1993) identified it as 5′-TT(A/T)CC(A/T)(A/t)$_2$(T/A)NNGG(A/T/C)(A/T)$_2$. These consensus binding sites share the CC(A/T)$_6$GG core to which the mammalian and yeast MADS box gene products, serum response factor (SRF) and MCM1, respectively, bind (Norman et al., 1988; Passmore et al., 1988; Mueller and Nordheim, 1991). In the promoter of *AGL5*, 537 bp 5′ of the beginning of the longest cDNA, a stretch of 16 bp conforms to the AG consensus binding site, as shown in Figure 7A. The site in the promoter of *AGL5* is 5′-TTACCAAAA-AAGGAAA-3′, which exactly matches the consensus sequences determined by Shiraishi et al. (1993; Figure 7B) and Huang et al. (1993).

It has previously been demonstrated that in vitro, the maize ortholog of AG, ZAG1, can specifically bind the consensus site found in the *AGL5* promoter (Schmidt et al.,1993). To determine whether AG also binds this consensus site, a gel shift assay was performed after incubating in vitro–transcribed and in vitro–translated AG with a probe containing either a wild-type or a mutated consensus sequence. The mutated consensus sequence was generated by replacing the CC(A)$_6$GG core with GG(A)$_6$CC. This exchange has been demonstrated to prevent binding of the human serum response element by the SRF (Hayes et al., 1988) as well as the AG consensus site of the *AGL5* promoter by ZAG1 (Schmidt et al., 1993). A shifted

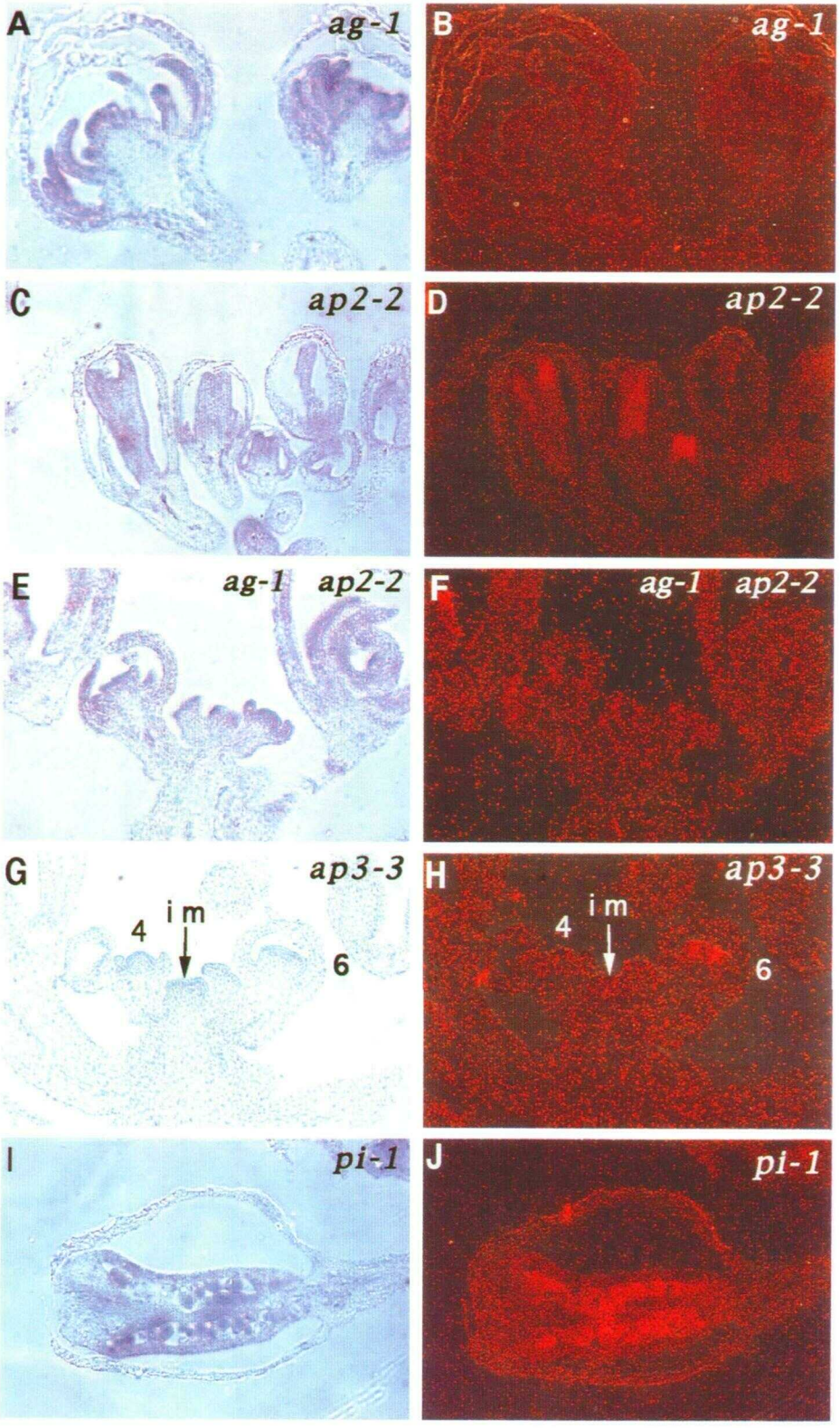

Figure 6. *AGL5* RNA Accumulation in the Flowers of Mutants.

A

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-1067  GAAAATGATGAGGAATGGGCAAAACACAAAAGAGTTTCCTTTCGTAACT
-1017  ACAATTAATTAATGCAAATCTGAGAAAGGGTTCATGGATAATGACTACA
-967   CACATGATTAGTCATTCCCCGTGGGCTCTCTGCTTTCATTTACTTTATT
-917   AGTTTCATCTTCTCTAATTATATTGTCGCATATATGATGCAGTTCTTTT
-867   GTCTAAATTACGTAATATGATGTAATTAATTATCAAAATAATATTAACG
-817   ACATGCAATGTATATAGGAGTAGGGCAATAAAAAGAAAAGGAGAATAAA
-767   AAGGGATTACCAAAAAAGGAAAGTTTCCAAAAGGTGATTCTGATGAGAA
-717   ACAGAGCCCATACCTCTCTTTTTTCCTCTAAACATGAAAGAAAAATTGG
-667   ATGGTCCTCCTTCAATGCTCTCTCCCCACCCAATCCAAACCCAACTGTC
-617   TTCTTTCTTTCTTTTTTCTTCTTTCTATTTGATATTTTCTACCACTTAA
-567   TTCCAATCAATTTCAAATTTCAATCTAAATGTATGCATATAGAATTTAA
-517   TTAAAAGAATTAGGTGTGTGATATTTGAGAAAATGTTAGAAGTAATGGT
-467   CCATGTTCTTTCTTTCTTTTTCCTTCTATAACACTTCAGTTTGAAAAAA
-417   AACTACCAAACCTTCTGTTTTCTGCAAATGGGTTTTTAAATACTTCCAA
-367   AGAAATATTCCTCTAAAAGAAATTATAAACCAAAACAGAAACCAAAAAC
-317   AAAAAATAAAGTTGAAGCAGCAGTTAAGTGGTACTGAGATAATAAGAAT
-267   AGTATCTTTAGGCCAATGAACAAATTAACTCTCTCATaattcatcttcc
-217   catcctcacttctctttctttctgatataattaatcttgctaagccagG
-167   TATGGTTATTGATGATTTACACTTTTTTTTAAAAGTTTCTTCCTTTTCT
-117   CCAATCAAATTCTTCAGTTAATCCTTATAAACCATTTCTTTAATCCAAG
-67    GTGTTTGAGTGCAAAAGGATTTGATCTATTTCTCTTGTGTTTATACTTC
-17    AGctagggcttatagaaatg...
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B

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AGL5                5'-TT   A   CC (A)6 GG   A   AA-3'
AG CONSENSUS        5'-TT(A/T/G)CC(A/T)6GG(A/T/C)AA-3'
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Figure 7. AG Consensus Binding Site within the *AGL5* Promoter.

(A) The 5′ flanking region of *AGL5*. The position of the AG consensus binding site is underlined. Introns are given in uppercase letters; exons are in lowercase letters.

(B) Comparison of the sequence located −746 from the *AGL5* ATG to the AG consensus binding site as determined by Shiraishi et al. (1993).

complex was observed when the AG protein was incubated with the wild-type probe, and this probe was competed away with excess, unlabeled wild-type probe, as shown in Figure 8. AG did not bind the mutant probe.

Ectopic Activation of AGL5 in Cauline Leaves

RNA in situ hybridization experiments demonstrated that *AG* is required for activation of *AGL5* in flowers, and the ability of AG to bind the *AGL5* promoter suggests that this interaction may be direct. To gain further insight into the role of AG as an activator of *AGL5*, we asked whether ectopic expression of *AG* in leaves could activate an *AGL5* promoter–β-glucuronidase (*GUS*) fusion (Jefferson et al., 1987). The 5′ flanking region (2.1 kb) of *AGL5* was fused to the *GUS* gene and introduced into Arabidopsis. The resulting transgenic plants were crossed to another transgenic line that constitutively expresses the *Brassica* ortholog of *AG* (*BAG1*) under the control of the cauliflower mosaic virus 35S promoter (Mandel et al., 1992b). Cauline leaves from wild-type (data not shown) and the 35S–*BAG1* plants had no GUS activity, as illustrated in Figure 9A. A low level of GUS activity was observed in the primary vein in cauline leaves from the 2.1-kb *AGL5–GUS* plants (Figure 9B). In contrast, cauline leaves from plants that contained

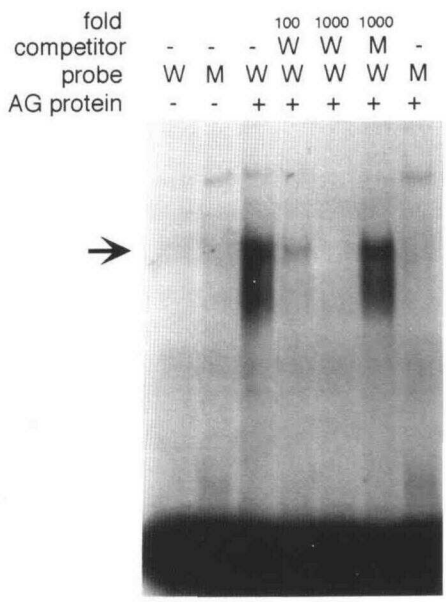

Figure 8. DNA Mobility Shift Assay with AG.

Oligonucleotides containing the AG consensus binding site found in the *AGL5* promoter (W) or mutated site (M) were end labeled and incubated with 8 μL of an in vitro translation reaction programmed with (+) or without (−) *AG* RNA. Some of the binding reactions were incubated in the presence of excess (100- or 1000-fold) unlabeled competitor oligonucleotides (W or M). The position of the shifted complex is indicated by the arrow. The free probe is shown at the bottom of the gel.

Figure 6. (continued).

Longitudinal sections of mutant floral tissues hybridized with the *AGL5*-specific antisense mRNA probe are shown. At left are bright-field images; at right are bright/dark-field (red filter) double exposures.

(A) and **(B)** *ag-1*.

(C) and **(D)** *ap2-2*.

(E) and **(F)** *ag-1 ap2-2*.

(G) and **(H)** *ap3-3*.

(I) and **(J)** *pi-1*.

Numbers indicate stages of floral development. im, inflorescence meristem.

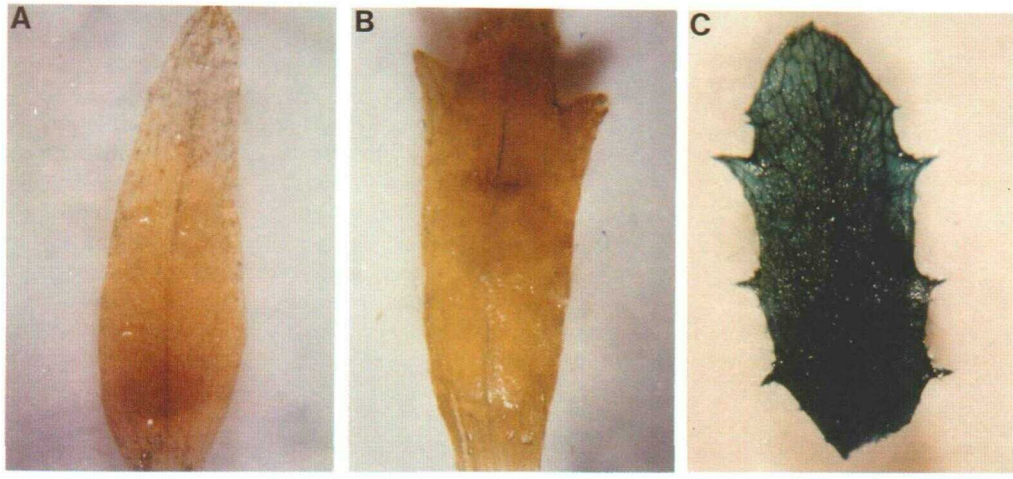

Figure 9. Histochemical Staining for GUS Activity in Cauline Leaves.

(A) A leaf from a transgenic plant containing the 35S–*BAG1* construct.
(B) A leaf from a transgenic plant containing the 2.1-kb *AGL5–GUS* construct.
(C) A leaf from a transgenic plant containing both the 35S–*BAG1* and *AGL5–GUS* constructs.

both the 2.1-kb *AGL5–GUS* and 35S–*BAG1* constructs stained an intense blue throughout the leaf (Figure 9C), demonstrating that ectopic AG activity in cauline leaves activates *AGL5*.

Restriction Fragment Length Polymorphism Mapping

AGL4 was placed on the restriction fragment length polymorphism (RFLP) map using an XbaI polymorphism between Landsberg *erecta* (L*er*) and Columbia ecotypes. These data place *AGL4* on the top of chromosome 3, which is 12 centimorgans above marker 302. *AGL5* was placed on the RFLP map using an XbaI polymorphism between Wassilewskija and L*er* ecotypes. These data placed *AGL5* on the lower region of chromosome 2, between markers λAt429 and λAt336. Neither position corresponds to a previously described floral mutant on the genetic map.

DISCUSSION

Genetic and molecular studies have shown that the meristem identity genes *AP1* and *LFY* are positive regulators of the floral organ identity genes *AG*, *AP3*, and *PI* (Weigel and Meyerowitz, 1993). However, the onset of *LFY* and *AP1* expression (Mandel et al., 1992a; Weigel et al., 1992) occurs at least 2 days prior to that of the organ identity genes, suggesting that this interaction may not be direct. In contrast to the rapid progress made in understanding floral meristem and organ specification, little is known about the genes that might act as intermediates between these two sets of genes or about the downstream targets of the organ identity genes. Here, we identify *AGL4* as a candidate intermediate gene between these two early steps in flower development and *AGL5* as a downstream gene regulated by AG.

AGL4 RNA Accumulates Before the Organ Identity Genes

AGL4 is initially expressed during stage 2 of flower development (Figures 2A and 2B), which is before the onset of expression of the floral organ identity genes but after the onset of expression of the floral meristem identity genes. These data suggest that *AGL4* acts as an intermediate between these two steps of development. This early accumulation of *AGL4* RNA is uniform throughout the floral primordium. *AGL4* RNA continues to accumulate uniformly in petals and in specific cell types of stamens and carpels throughout development (Figures 2E to 2H), suggesting that *AGL4* may also be important in later steps.

Several other genes are expressed in a temporal pattern similar to *AGL4* and therefore may have related roles. The *AGL2* gene of Arabidopsis is closely related to *AGL4*, sharing 98% identical residues in the DNA binding MADS domain (Ma et al., 1991). The onset of *AGL2* expression also occurs during stage 2 of flower development (Flanagan and Ma, 1994). The parallel expression patterns and sequence similarity suggest that *AGL2* and *AGL4* encode redundant functions that regulate the same target genes and may explain why mutations in these genes have not yet been identified.

The expression patterns of *AGL4* and *AGL2* are also similar to that of the petunia MADS box gene *fbp2* (for floral binding protein 2 gene) in that they are expressed after the floral meristem has been established but before the organ identity

genes are active (Angenent et al., 1992). Angenent et al. (1992) have shown that in transgenic petunia plants cosuppressed for *fbp2*, *fbp6* (the *AG* putative ortholog) is not expressed in late flowers. These data support the idea that *fbp2* may activate the floral organ identity gene *fbp6*. Given the early expression patterns of *AGL4* and *AGL2* and the fact that these two genes and *fbp2* are very similar but not orthologs, it is possible they have similar roles. Recent isolation and characterization of the Antirrhinum *fimbriata* gene have shown that it acts as a mediator between the floral meristem and floral organ identity genes (Simon et al., 1994). Although *fimbriata* is not a MADS box gene, the onset of its expression coincides with that of *AGL2* and *AGL4*, suggesting that the Arabidopsis ortholog of *fimbriata* may interact with these two MADS box genes to regulate the floral organ identity genes.

AGL5 Is Positively Regulated by AG

Although there has been progress in identifying intermediates between the floral meristem and floral organ identity genes, little is known about the regulatory genes that control the steps following the onset of the activation of the floral organ identity genes. The lack of *AGL5* RNA accumulation in the flowers of *ag* or *ag ap2* mutants, as determined by RNA gel blotting and in situ hybridization studies (Figures 1 and 6), allowed us to identify *AGL5* as a gene that requires AG for its activation. The ability of ectopic *AG* to activate *AGL5* in cauline leaves provides additional support for this conclusion (Figure 9) and also demonstrates that AG is sufficient to activate *AGL5* in the absence of other carpel-specific factors. It has been observed that ectopic *AG* activity results in altered leaf morphology with carpelloid characteristics, both in Arabidopsis and tobacco (Mizukami and Ma, 1992; Kempin et al., 1993). This transformed leaf morphology may be due to the activation of carpel-specific genes such as *AGL5*.

To investigate further the relationship between *AG* and *AGL5*, the temporal and spatial patterns of RNA accumulation for the two genes were compared (Figures 3 and 4). During stage 6 and beyond, the RNA accumulation pattern of *AGL5* mirrors that of *AG* in carpels, except that *AGL5* RNA is not detected in stigmatic tissue (Figures 4E and 4F). The only *AGL5* signal detected outside of carpels is in the nectaries, which are at the base of the stamens (Figures 5E and 5F). Interestingly, *AG* is also expressed in nectaries. The overlapping expression patterns of *AG* and *AGL5* suggest that *AGL5* plays a role in carpel and ovule development under the positive influence of AG. However, it is unclear whether activation of *AGL5* by AG is direct or indirect, because *AGL5* RNA is first detected ~42 hr after the onset of *AG* expression. The presence of a perfect match to an AG consensus binding site in the promoter of *AGL5* (Figure 7) and the fact that AG specifically binds to this sequence (Figure 8) support the possibility of a direct interaction between AG and *AGL5*.

Although we demonstrated that AG positively regulates *AGL5* in carpels, it is apparent that AG is unable to activate *AGL5* in stamens, suggesting that *AGL5* is negatively regulated in the third whorl. RNA in situ hybridization experiments showed that *AP3* and *PI* can be considered formal negative regulators of *AGL5*, because *AGL5* RNA accumulates in the third whorl organs of both *ap3* and *pi* mutant flowers (Figures 6G to 6J). However, because carpels form in the third whorl of *ap3* and *pi* mutant flowers and because *AGL5* is expressed in a carpel-specific manner, the negative regulation by *AP3* and *PI* may be indirect.

The fact that *AGL5* is expressed in an organ-specific manner makes it a useful tool for identifying developing carpel tissue. For example, *AGL5* is expressed in fourth, third, and first whorl carpels of flowers of the wild type, *ap3* and *pi* mutants, and *ap2* mutants, respectively. In contrast, *AG* RNA expression occurs in a region-specific pattern in that it accumulates in third and fourth whorl organ primordia, regardless of organ type. For example, in flowers of *ag* mutants, *ag* RNA accumulates in the third and internal whorls, which will develop as petals and sepals rather than as stamens and carpels (Gustafson-Brown et al., 1994). Therefore, *AGL5* can be used as an early marker for identifying cells destined to form carpels, whereas *AG* can be considered an early marker for the third and fourth whorls.

Floral morphogenesis is a complex process involving a cascade of regulatory events that culminate in the formation of a flower. Most of the major regulators of the early step of specifying floral meristem identity as well as of the later step of specifying floral organ identity are MADS box genes. Our results here indicate that members of this family of genes may have a broader role in flower development, acting as intermediates between these steps as well as acting in the subsequent steps of organ differentiation.

METHODS

RNA Isolation and Gel Blot Analysis

Total RNAs were isolated using the method of Crawford et al. (1986), size fractionated by gel electrophoresis on formaldehyde-agarose gels, transferred to Hybond-N nylon membranes (Amersham), and hybridized according to standard procedures with ^{32}P-radiolabeled probes. The *AGL1* (for *AGAMOUS* [*AG*]-like) probe was synthesized from a 420-bp EcoRI fragment of pCIT4217, *AGL2* from a 500-bp EcoRI-XbaI fragment of pCIT4221, *AGL4* from a 410-bp EcoRI-BglII fragment of pCIT4213, *AGL5* from a 400-bp HincII fragment of pCIT2243, *AGL6* from a 725-bp HindIII-EcoRI fragment of pCIT3209, and tubulin from an EcoRI-BamHI fragment excised from a clone isolated by Marks et al. (1987).

In Situ Hybridization

Fixation of tissue and hybridization conditions were the same as previously described (Drews et al., 1991), with minor modifications. The *AGL5* ^{35}S-labeled antisense mRNA was synthesized from a HindIII-digested pCIT2242 template. The *AGL4* ^{35}S-labeled antisense mRNA

was synthesized from an EcoRI-digested pSR12 template. Probes were used at a final concentration of 2.5 × 10^7 cpm/mL.

Image Processing

Photographic slides were scanned and digitized using a Nikon Coolscan (Nikon Inc., Melville, NY). Brightness, contrast, and color balance were adjusted using Adobe Photoshop 2.5 (Adobe Systems Inc., Mountain View, CA). Composite figures were printed using a Tektronix Phaser IIsdx dye sublimation printer (Tektronix Inc., Wilsonville, OR).

Gel Shift Analysis

A plasmid containing the *AG* cDNA with an artificial initiation codon preceding the MADS box was in vitro transcribed (T7 RNA polymerase) and translated using a wheat germ extract–TNT-coupled system (Promega) according to the manufacturer's specifications. Part of each translation reaction product was labeled with ^{35}S-methionine and analyzed on an 8% SDS–polyacrylamide gel. The unlabeled AG protein was assayed for DNA binding by equilibrating 8 μL of programmed lysate for 5 min at room temperature in a mobility shift buffer (10 mM Hepes, pH 7.8, 50 mM KCl, 1 mM EDTA, 10 mM DTT, 2 mg/mL BSA, 100 μg/mL salmon sperm DNA, 10% glycerol). Labeled dimerized oligonucleotides containing the consensus binding site (0.5 ng) were added, and the reactions (25 μL final volume) were incubated at room temperature for 20 min. Samples were then loaded onto a 4% polyacrylamide gel and electrophoresed at 30 mA. Electrophoresis was followed by drying of the gel and autoradiography. The wild-type (5′-AATTGGATTACCAAAAAAGGAAAGTT-3′) and mutant (5′-AATTGGATTAGGAAAAAACCAAAGTT-3′) binding site probes used in this experiment were generated by the fill-in reaction using the Klenow fragment of DNA polymerase I and ^{32}P-α-dATP.

Agrobacterium tumefaciens-Mediated Transformation and Histochemical Staining

The 2.1-kb *AGL5* promoter–β-glucuronidase (*GUS*) fusion construct was introduced into Agrobacterium strain ASE and used to transform the Nossen ecotype of Arabidopsis using a modified root explant procedure based on the previously described method of Valvekens et al. (1988). GUS histochemical staining was performed essentially as described by Jefferson et al. (1987). Cauline leaves were cleared of chlorophyll by washing in 70% ethanol. The cauliflower mosaic virus 35S–*BAG1* construct (provided by M.A. Mandel, University of California at San Diego, La Jolla, CA) was in the Landsberg *erecta* (*Ler*) ecotype, and the 2.1-kb *AGL5–GUS* construct was in the Nossen ecotype.

Restriction Fragment Length Polymorphism Analysis

Restriction fragment length polymorphism (RFLP) mapping filters were scored, and the results were analyzed with the Macintosh version of the Mapmaker program as described by Rieter et al. (1992).

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