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# The MADS Domain Protein AGL15 Localizes to the Nucleus during Early Stages of Seed Development

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**Little is known about regulatory factors that act during the earliest stages of plant embryogenesis. The MADS domain protein AGL15 (for AGAMOUS-like) is expressed preferentially during embryogenesis and accumulates during early seed development in monocotyledonous and dicotyledonous flowering plants. AGL15-specific antibodies and immunohistochemistry were used to demonstrate that AGL15 accumulates before fertilization in the cytoplasm in the cells of the egg apparatus and moves into the nucleus during early stages of development in the suspensor, embryo, and endosperm. Relatively high levels of AGL15 are present in the nuclei during embryo morphogenesis and until the seeds start to dry in Brassica, maize, and Arabidopsis. AGL15 is associated with the chromosomes during mitosis, and gel mobility shift assays were used to demonstrate that AGL15 binds DNA in a sequence-specific manner. To assess whether AGL15 is likely to play a role in specifying the seed or embryonic phase of development, AGL15 accumulation was examined in Arabidopsis mutants that prematurely exit embryogenesis. *lec1-2* mutants show an embryo-specific loss of AGL15 at the transition stage, suggesting that AGL15 interacts with regulators in the leafy cotyledons pathway.**

## INTRODUCTION

Many of the most significant events in plant embryogenesis occur during the early stages; however, little is known about these events molecularly. In members of the Brassicaceae, such as Arabidopsis and *Brassica napus* (oilseed rape, referred to hereafter as Brassica), the main axes of the embryo are established, tissue layers are delineated, and the first signs of cytodifferentiation appear during the course of early cell divisions (reviewed in West and Harada, 1993). Because the cells involved are small, few in number, and, in zygotic embryogenesis, embedded in several layers of maternal tissue, it has been difficult to identify regulatory molecules that act specifically during early stages of the life cycle.

Several molecules that are expressed at multiple stages in the life cycle but play important roles during early embryogenesis have been identified using genetic approaches. Two Arabidopsis genes that contribute to the organization of the embryo body plan have been characterized molecularly. *emb30/gnom* and *knolle* mutants show defects in the formation and placement of new cell walls as early as the first, asymmetric cell division in the zygote. Based on the homology of EMB30/GNOM and KNOLLE to SEC7 (Shevell et al., 1994) and syntaxin (Lukowitz et al., 1996), respectively, they are thought to be involved in vesicle trafficking. *SHOOT MERISTEMLESS* (*STM*), a gene essential for the organization of the shoot apical meristem in Arabidopsis embryos, encodes a homeodomain regulatory factor that is expressed preferentially in vegetative, axillary, inflorescence, and floral meristems. *STM* mRNA can first be detected in cells at the shoot pole at approximately the 32- to 64-cell stage in Arabidopsis embryos (Long et al., 1996). The *no apical meristem* (*nam*) mutant of petunia, like *stm*, lacks a shoot apical meristem. The predicted protein product of this gene shares a conserved N-terminal domain with proteins that appear to function in transcriptional control based on their ability to activate a cauliflower mosaic virus 35S promoter in yeast. *NAM* is expressed at the late-heart stage of embryogenesis and at the boundary between the meristem and primordia during reproductive development (Souer et al., 1996).

Using polymerase chain reaction (PCR) and differential display, we previously identified *AGL15* (for AGAMOUS-like) as a sequence preferentially expressed during embryogenesis (Heck et al., 1995). *AGL15* encodes a member of the MADS domain family of regulatory proteins. The MADS domain consists of a conserved 55– to 60–amino acid residue region that includes a DNA binding domain, a dimerization domain, and a putative phosphorylation site for calmodulin-dependent protein kinases (Sommer et al., 1990). Members of the MADS domain family play pivotal roles in critical developmental events in a wide variety of organisms: in specification of mating type in yeast (Passmore et al., 1989); in responses to the environment in human cells (Norman et al., 1988); in control of

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mesoderm differentiation in Drosophila (Nguyen et al., 1994; Lilly et al., 1995) and muscle differentiation in humans (Yu et al., 1992; Breitbart et al., 1993); and in the determination of meristem and organ identity during flower development in plants (Schwarz-Sommer et al., 1990). At least 28 members of the family have been identified to date in Arabidopsis (M. Yanofsky, personal communication), including the well-known floral regulators AG, APETALA1, CAULIFLOWER, APETALA3 (AP3), and PISTILLATA and a set of closely related *AGL* genes expressed in flowers. More distantly related family members that are expressed primarily in contexts other than the flower have been identified recently (Heck et al., 1995; Rounsley et al., 1995). *AGL15* belongs to this divergent group.

AGL15 is the only MADS domain regulatory factor identified to date that is expressed in a highly preferential manner in developing plant embryos. Using AGL15-specific antibodies, we show here that the gene product accumulates in cells of the egg apparatus, is translocated into nuclei during early embryogenesis, and may play a role in regulatory networks operating before the main tissue layers are delineated in the embryo.

## RESULTS

### Production of Antibodies Specific for AGL15

To generate antibodies that would recognize AGL15 specifically, a truncated form of AGL15 lacking the conserved MADS domain was produced by overexpression in *Escherichia coli*, and purified protein was used to immunize rabbits. Anti-AGL15 antibodies were affinity purified from the immune serum by adsorption to the antigen immobilized on membrane. As Figure 1 shows, the antibody preparation recognized the truncated form of Brassica AGL15, a similarly truncated form of Arabidopsis AGL15 (Figure 1), and nontruncated forms of both Brassica AGL15 (Figure 1) and Arabidopsis AGL15 (data not shown) containing the MADS domain. To test whether there is likely to be any cross-reaction with other family members via the more weakly conserved K domain (Ma et al., 1991; Pneuli et al., 1991), which was included in the construct, AGL2 was synthesized by overexpression in *E. coli*. As Figure 1 shows, the antibodies did not recognize AGL2, which is the only other family member that has been reported to be expressed during embryogenesis in Arabidopsis (Flanagan and Ma, 1994). We showed previously that these antibodies recognize low-abundance proteins of the expected molecular mass (30 kD) in soluble extracts of Brassica embryos (Heck et al., 1995). The results reported here indicate that the antibodies are highly specific for AGL15 and can be used to determine the distribution of this particular family member in developing embryos.

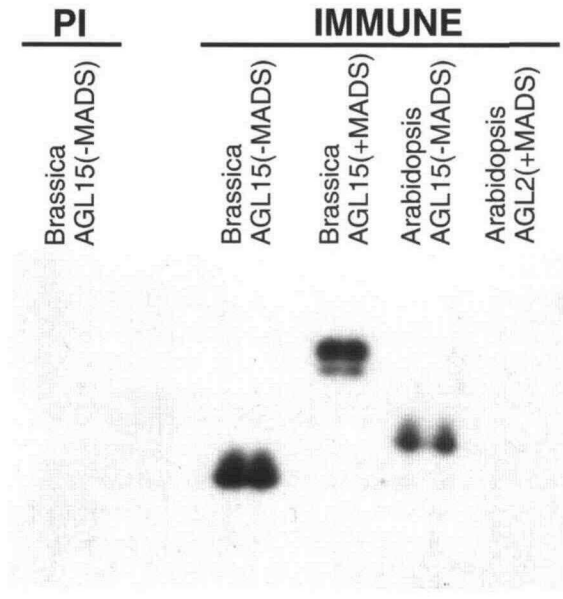


**Figure 1.** Specificity of AGL15 Antibodies.

Shown are protein gel blots of solubilized inclusion bodies (20 ng of protein per lane) probed with preimmune serum (PI) diluted 1:500 or with affinity-purified anti-AGL15 antibodies (IMMUNE) diluted 1:1500 relative to the original serum. The Brassica and Arabidopsis AGL15 or AGL2 constructs expressed in *E. coli* included the MADS domain (+MADS) or contained only the sequences C-terminal of the MADS domain (−MADS).

### AGL15 Is Expressed throughout the Embryo and Localizes to the Nucleus

To determine the distribution and cellular localization of AGL15 in developing embryos, tissue sections of Brassica embryos were labeled with AGL15 antiserum. The system used to visualize the primary antibody generates a red-gold to brown deposit. For these experiments, AGL15 antiserum was preadsorbed against lightly fixed pieces of leaves that do not express AGL15. The patterns were identical to those seen with affinity-purified antibodies; however, the preadsorbed serum resulted in more intense color deposits. As shown in Figure 2A, the AGL15 protein, like its mRNA (Heck et al., 1995), accumulates in all tissues and cell types of a torpedo-stage (25 days after pollination [DAP]) Brassica embryo. Color deposits are associated specifically with the nuclei (Figures 2A and 2B), which were visualized by staining similar sections with 4′,6-diamidino-2-phenylindole (DAPI), a fluorescent groove-binding probe for DNA (Figure 2C). Viewing sections under high magnification revealed that AGL15 is excluded from portions of the nucleus during interphase (Figure 2D) and is associated with the chromosomes in cells undergoing mitosis (Figures 2B and 2D).

Several controls were performed to assess the specificity of the antibody reaction on tissue sections. Figures 2E and 2F show that an embryo section labeled with preimmune serum did not exhibit nuclear staining. Antiserum depleted of AGL15-specific antibodies by preadsorption with overexpressed AGL15 also did not label nuclei on embryo sections (data not shown). To confirm that the antibodies do not recognize other MADS domain family members, we incubated sections of young floral buds with antiserum. Figures 2G and 2H show that the antibodies do not label nuclei in developing floral organs, even though many different family members are expressed in this developmental context (Schwarz-Sommer et al., 1990). Figure 2I shows a similar section stained with DAPI to visualize the nuclei. On sections of shoot apices isolated just after the transition to flowering, some light staining, possibly associated with the nucleolus, was seen; however, this staining was also observed in the preimmune control and is likely to be nonspecific (data not shown).

## Developmental Regulation of AGL15 Expression and Localization

To determine how levels of AGL15 change during seed and embryo development, affinity-purified antibodies were used to probe gel blots of soluble protein extracts. As shown in Figure 3A, AGL15 could be detected in whole Brassica seeds as early as 6 DAP. The levels of AGL15 increased slightly over the next few days but began to decrease after 16 DAP, which corresponds to the transition stage of embryo development. Significant changes in AGL15 levels also occurred as the embryos exited the maturation stage. Blots of soluble protein extract prepared from maturing (30- to 60-DAP) Brassica embryos and probed with affinity-purified antibodies are shown in Figure 3B. The lane containing protein from 30-DAP embryos represents ~10 embryos; the other lanes represent approximately three embryos each. Levels of AGL15 clearly

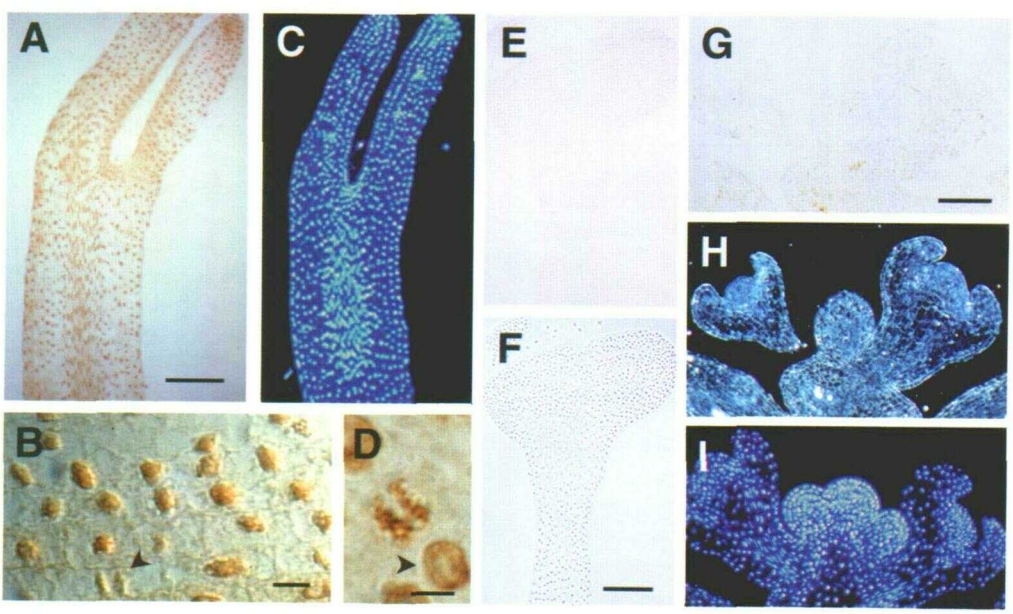


**Figure 2.** Localization of the AGL15 Protein in Brassica Tissues.

**(A)** Torpedo-stage embryo section that was treated with the AGL15 antiserum. Bar = 100 μm.
**(B)** Higher magnification of nuclear staining in the hypocotyl of the embryo shown in **(A)**, using Nomarski optics. The arrowhead indicates segregating chromosomes showing AGL15 labeling. Bar = 10 μm.
**(C)** Embryo at torpedo stage (25 DAP) with DAPI-stained nuclei.
**(D)** Higher magnification of a cell undergoing mitosis, with chromosomes showing AGL15 labeling. AGL15 is excluded from portions of the nucleus (arrowhead) during interphase. Bar = 5 μm.
**(E)** Torpedo-stage embryo section treated with preimmune serum. A bright-field image is shown.
**(F)** Tissue of the torpedo-stage embryo shown in **(E)**, viewed by using phase-contrast microscopy. Bar = 100 μm.
**(G)** Section of young floral buds and inflorescence axis treated with the AGL15 antiserum. Bar = 100 μm.
**(H)** Dark-field view of the tissues shown in **(G)**.
**(I)** Apex of the inflorescence with DAPI-stained nuclei.

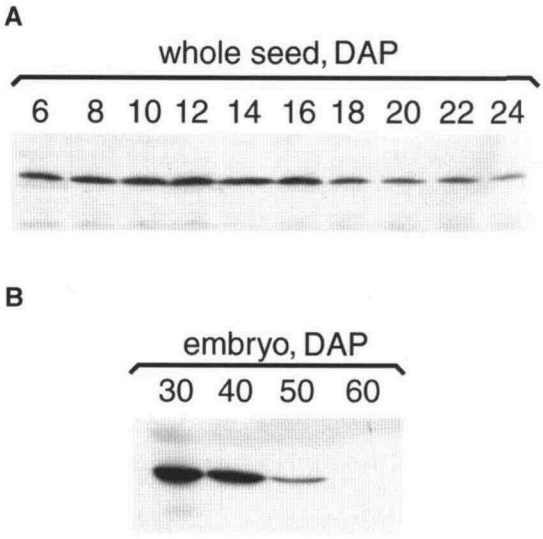


**Figure 3.** Accumulation of AGL15 in Developing Brassica Seeds and Isolated Embryos.

Protein gel blots of soluble protein extracts from Brassica whole seed or isolated embryos were probed with affinity-purified antibodies that recognize AGL15.

**(A)** Extracts prepared from seeds collected from 6 to 24 DAP. Each lane contains 300 μg of protein.

**(B)** Extracts prepared from Brassica embryos isolated from 30 (early maturation) to 60 DAP (full maturity). Each lane contains 150 μg of protein. The first lane represents ~10 embryos at 30 DAP; the second to fourth lanes represent approximately three embryos each at 40, 50, and 60 DAP.

decreased after 40 DAP, and AGL15 was undetectable in embryos by 60 DAP (Figure 3B).

The embryo represents only a small fraction of the total seed protein at early stages (6 to 10 DAP); however, relatively high levels of AGL15 were present. To investigate this apparent anomaly, AGL15 was localized on sections of ovules and young seeds, which are shown in Figure 4. Diploid maternal tissues in unfertilized Brassica ovules showed only light staining with AGL15-specific antibodies. The cells making up the egg apparatus, however, showed strong staining, as Figure 4A illustrates. Unlike the color deposits in torpedo-stage embryos (Figure 2A), the color deposits here appeared to be excluded from the nuclei and were distributed throughout the cytoplasm of the cells. This distribution changes at some point after fertilization. More diffuse and presumably cytoplasmic staining could be seen in cells of the embryo proper as late as 10 DAP (eight- to 16-cell stage), as shown in Figure 4B. However, by early globular stage (Figure 4C), and occasionally as early as the two- to four-cell stage (data not shown), staining was associated exclusively with the nuclei. Nuclear localization of AGL15 persisted through the torpedo stage in developing embryos, as shown in Figures 4D (13 DAP), 4E (20 DAP), and 2A (25 DAP). AGL15 may also be associated with the nuclei in maturing embryos; however, an increase in nonspecific staining associated with the deposition of storage proteins made localization at a cellular level difficult past the mid-maturation stage (35 DAP).

Immunolocalization revealed that expression in nonembryonic tissues also contributes to the net accumulation of AGL15 protein in young Brassica seeds. Cells in the suspensor and endosperm, as well as cells in some parts of the seed coat, also accumulated AGL15. By 11 DAP, AGL15 levels in nuclei in the suspensor and endosperm were approximately equal to levels in nuclei in the embryo proper (Figure 4C). By the time the embryo reached heart stage, the levels of AGL15 in nonembryonic tissues had declined, although levels in the embryo were still relatively high (Figure 4E). The difference in accumulation patterns may reflect the fact that the suspensor and endosperm are only present transiently during the early stages of seed development in this group of flowering plants (Meinke, 1995).

### AGL15 Localization in Monocot Embryos

AGL15 accumulation was also examined in maize embryos, which develop in a different mode (monocot with persistent endosperm) than do Brassica embryos (dicot with transient endosperm). In a previous study, we showed that maize embryo extracts contain a protein with a molecular mass of ~36 kD that is recognized by anti-AGL15 antibodies (Heck et al., 1995). As shown in Figures 4F to 4M, this protein, which presumably represents the maize homolog of AGL15, accumulates at very early stages in cells of the maize embryo, suspensor, and endosperm. As in Brassica embryos, the protein at first appeared to be more abundant in the cytoplasm than in the nucleus (4 DAP; Figure 4F). By 8 DAP, the protein was associated with the nuclei and accumulated in all tissues and cell types, including the suspensor (Figures 4G and 4H). As shown in Figures 4I to 4K, the protein associated with segregating chromosomes during mitosis. No staining was seen when preimmune serum was used (data not shown). Therefore, the pattern in maize is essentially identical to that seen in Brassica during early stages.

At later stages of development (14 DAP), maize embryos exhibited well-developed leaf primordia. The immunoreactive protein was also associated with nuclei in these organs, as shown in Figures 4L and 4M. In maize, the endosperm persists until germination. We observed high levels of the protein in the endosperm through at least 18 DAP (data not shown). As the seed desiccated, the levels of the immunoreactive protein declined. By 31 DAP, no staining was detected in the embryonic shoot, and only low levels were present in the rest of the embryo (data not shown). The fact that the protein accumulation patterns are so similar in embryos of such distantly related flowering plants as maize and Brassica supports the hypothesis that AGL15 plays an ancient and highly conserved role during seed development.

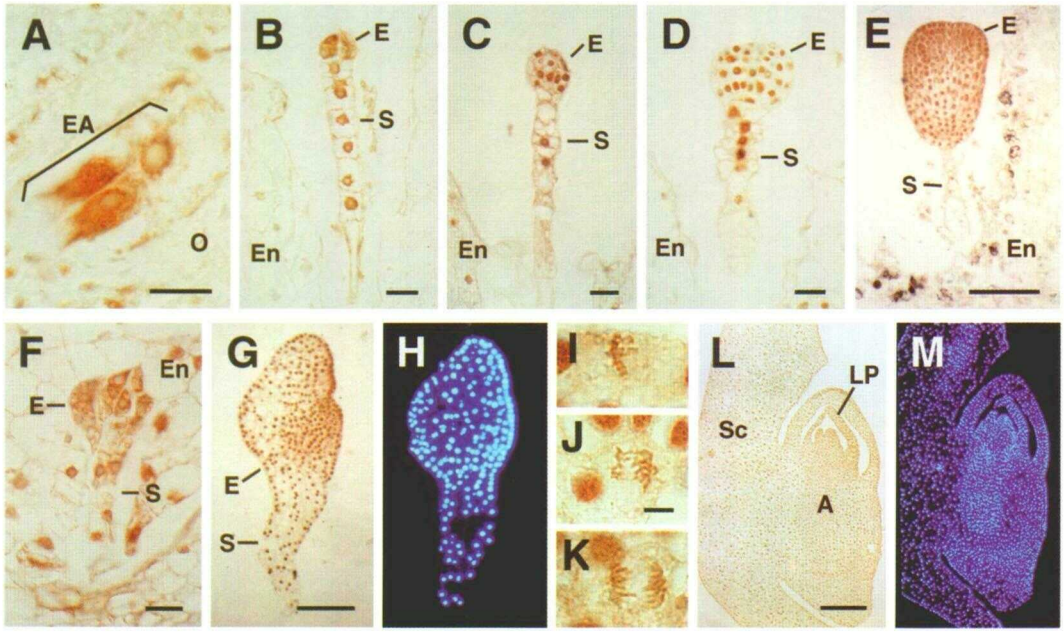


**Figure 4.** Localization of the AGL15 Protein in Developing Brassica and Maize Embryos.

Bright-field microscopy of sections treated with the AGL15 antiserum are shown. E, embryo; En, endosperm; S, suspensor.

**(A)** Unfertilized Brassica ovule. AGL15 is found at high levels in the cytoplasm of cells in the egg apparatus (EA) and at lower levels in the nuclei of cells in the surrounding ovule (O) tissues. Bar = 20 μm.

**(B)** Brassica seed at 10 DAP at the proembryo stage. AGL15 is present in the cytoplasm of cells in the embryo proper. Endosperm cells contain lower levels of AGL15 than embryo and suspensor cells. Bar = 20 μm.

**(C)** Brassica seed at 11 DAP at the globular stage. AGL15 staining is associated with nuclei in the embryo proper, suspensor, and endosperm. Bar = 20 μm.

**(D)** Brassica seed at 13 DAP at the late globular stage. Bar = 20 μm.

**(E)** Brassica seed at 20 DAP at the heart stage. AGL15 levels are lower in nuclei in the endosperm than in the embryo. Bar = 100 μm.

**(F)** Maize seed at 4 DAP at the early transition stage. AGL15 labeling is associated with the cytoplasm in the embryo and nuclei in the endosperm. Bar = 20 μm.

**(G)** Maize embryo at 8 DAP at the late transition stage. AGL15 labeling is associated with nuclei in the embryo and suspensor. Bar = 100 μm.

**(H)** Maize embryo at 8 DAP at the late transition stage. Nuclei are stained with DAPI.

**(I)** to **(K)** High-magnification views of segregating chromosomes at metaphase in **(I)** and at anaphase in **(J)** and in **(K)** in 8-DAP maize embryo cells. Bar in **(J)** = 5 μm for **(I)** to **(K)**.

**(L)** Maturing maize embryo at 14 DAP. Nuclei in the scutellum (Sc), axis (A), and leaf primordia (LP) contain AGL15. Bar = 200 μm.

**(M)** Maturing maize embryo at 14 DAP. Nuclei are stained with DAPI.

### Expression of AGL15 in Arabidopsis Mutants

We extended our analysis to Arabidopsis, reasoning that investigation of mutations that alter embryo development might lead us to possible modifiers of AGL15 expression. As shown in Figure 5, the pattern of AGL15 accumulation in wild-type Arabidopsis embryos (Wassilewskija ecotype) is very similar to that seen in Brassica. AGL15 accumulates in nuclei in the proembryo (Figure 5A). Relatively high levels are present through globular and transition stages (Figures 5B and 5C) and into later stages of development, such as the torpedo stage (Figure 5D). AGL15 also accumulates in nuclei of the endosperm throughout its development (Figures 5C and 5D). Interestingly, AGL15 expression in suspensor cells appears to be confined to the period before the transition to bilateral symmetry. Nuclei in the suspensor were labeled at the eight-cell stage (Figure 5E) and at a later globular stage (data not shown), but no immunostaining could be detected by the transition stage (Figure 5C). Maternal seed coat tissues may also accumulate low levels of AGL15, but staining in these tissues was variable. The brown color in the endothecium appears to be due to compounds it accumulates and not to AGL15 staining (Figure 5C). Cells in this tissue were equally dark on sections treated with preimmune serum.

Because AGL15 accumulates at high levels only during the embryonic or seed phase of the life cycle, we focused our attention on Arabidopsis mutants that appear to exit this phase prematurely and enter the postgerminative phase before the

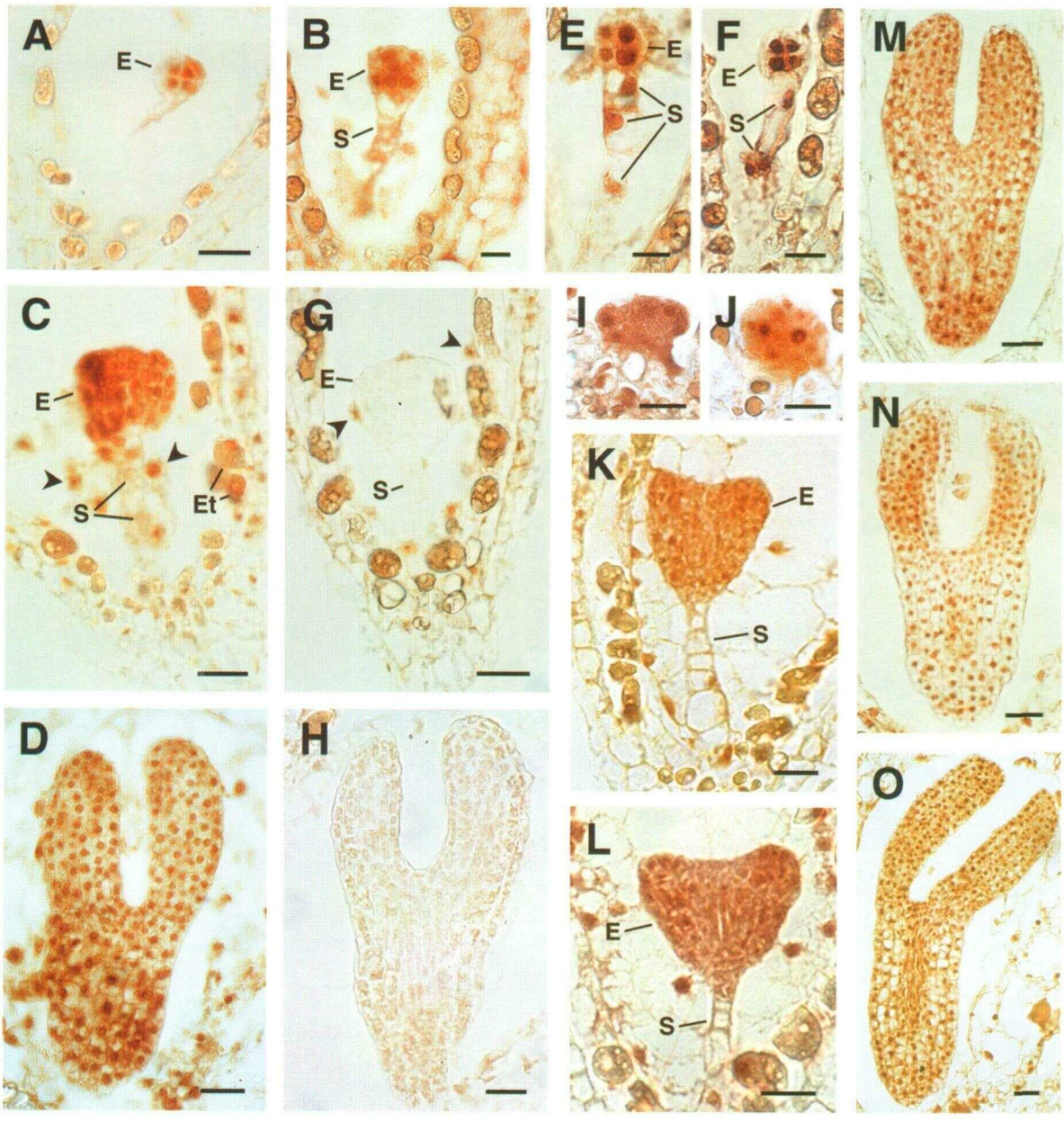


**Figure 5.** Localization of the AGL15 Protein in Developing Arabidopsis Embryos.

Bright-field microscopy of sections treated with the AGL15 antiserum. E, embryo; S, suspensor. Unless otherwise indicated, bars = 20 μm.

**(A)** Wild type at the proembryo stage.

**(B)** Wild-type embryo at the globular stage. Bar = 10 μm.

**(C)** Wild-type embryo at the transition stage. Nuclei in the suspensor do not label with anti-AGL15 antibodies. Arrowheads indicate labeled endosperm nuclei. The brown color in the endothecium (Et) is not due to AGL15 staining.

**(D)** Wild-type embryo at the torpedo stage.

**(E)** Wild-type eight-cell embryo. Labeled nuclei in the suspensor are visible. Bar = 10 μm.

**(F)** Eight-cell embryo of a *lec1-2* homozygote. Labeled nuclei in the suspensor are visible. Bar = 10 μm.

**(G)** *lec1-2* homozygote at the transition stage. Nuclei in the embryo are not labeled. Arrowheads indicate labeled endosperm nuclei.

**(H)** *lec1-2* homozygote at the early maturation stage.

**(I)** Wild-type chalazal endosperm at the torpedo stage.

**(J)** *lec1-2* homozygote chalazal endosperm at the transition stage.

**(K)** *lec1-1* homozygote at the heart stage.

**(L)** *lec2* homozygote at the heart stage.

**(M)** *lec2* homozygote at the early maturation stage.

**(N)** *fus3-3* homozygote at the early maturation stage.

**(O)** *abi3-6* homozygote at the early maturation stage.

seed is shed. These include the leafy cotyledon mutants *lec1*, *lec2*, and *fusca3* (*fus3*) and the abscisic acid (ABA)–insensitive mutant *abi3* (Meinke, 1992; Nambara et al., 1992, 1994, 1995; Keith et al., 1994; Meinke et al., 1994; West et al., 1994). All of the leafy cotyledon mutants show a partial transformation of the cotyledons into leaves. *abi3* mutants do not show cotyledon transformations but, like *lec1* and *fus3* mutants, are desiccation intolerant and viviparous, suggesting that the embryos fail to complete late embryogenesis programs. LEC1 is thought to act upstream of LEC2 in the leafy cotyledons pathway (Meinke et al., 1994), whereas FUS3 and ABI3 act in separate pathways (Keith et al., 1994; Meinke et al., 1994; West et al., 1994).

The levels and pattern of AGL15 accumulation were indistinguishable from those of the wild type in most cases; however, one mutation had a specific and dramatic effect. AGL15 accumulated normally during early stages in embryos homozygous for the *lec1-2* mutation. As shown in Figure 5F, AGL15 was present in both the embryo and the suspensor nuclei at the eight-cell stage; it was also present at high levels in nuclei at the globular stage (data not shown). By the transition stage, however, when the embryo first exhibits signs of bilateral symmetry, AGL15 was undetectable (Figure 5G). No immunostaining could be seen in the embryo at later stages (Figure 5H). Accumulation in the endosperm was not affected by this mutation. Endosperm nuclei near the embryo (Figure 5G) or at the chalazal pole (Figure 5J) showed no change relative to the wild type (Figures 5C and 5I). As shown in Figure 5K, the other mutant allele of *LEC1* did not produce the same effect. AGL15 was present at relatively high levels even at the heart stage in *lec1-1* homozygous mutant embryos. Similarly, as shown in Figures 5L to 5O, no change in levels or overall pattern was seen in embryos homozygous for the *lec2*, *fus3*, and *abi3* mutations, even when the strongest mutant alleles available were tested.

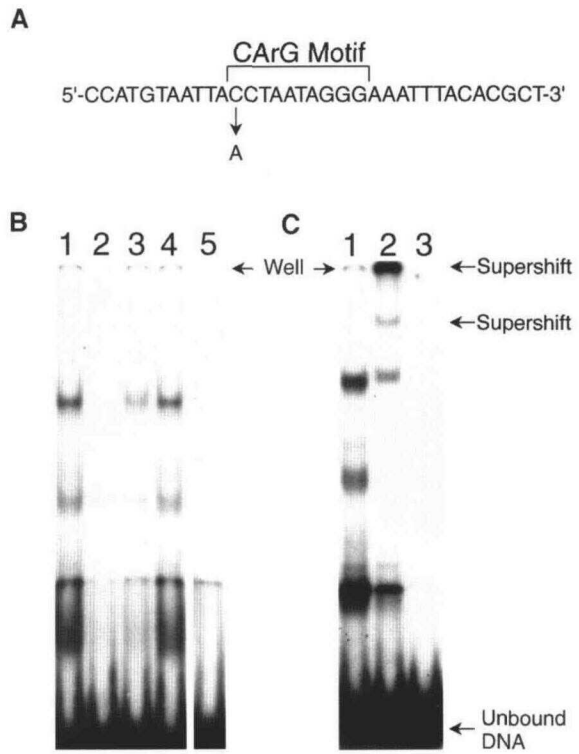


**Figure 6.** Gel Mobility Shift Assays of AGL15–DNA Interactions.

**(A)** Sequence of the portion of the yeast *STE6* promoter used for gel mobility shift assays. The canonical MADS domain binding site (CArG motif) is indicated. In the DNA fragment containing the modified CArG motif, a highly conserved C residue was changed to an A residue as indicated.

**(B)** Autoradiography of gel mobility shift experiments. Lane 1 contains overexpressed AGL15 incubated with radioactive DNA fragments containing the CArG motif; lane 2, overexpressed AGL15 incubated with radioactive DNA fragments containing the modified CArG motif; lane 3, the same reaction shown in lane 1 plus a 100-fold excess of nonradioactive DNA fragments containing the CArG motif; lane 4, the reaction shown in lane 1 plus a 100-fold excess of nonradioactive DNA fragments containing the modified CArG motif; lane 5, overexpressed truncated AGL15 lacking the MADS domain incubated with radioactive DNA fragments containing the CArG motif.

**(C)** Autoradiography of gel mobility supershift experiments. Lane 1 contains overexpressed AGL15 incubated with radioactive DNA fragments containing the CArG motif in the presence of the preimmune serum; lane 2, AGL15 incubated with DNA in the presence of the immune serum; lane 3, radioactive DNA fragments, including the CArG motif incubated with the immune serum.

## AGL15 Specifically Binds DNA in Vitro

Gel retardation assays were used to demonstrate that AGL15 binds DNA in a sequence-specific manner. Because the in vivo targets for AGL15 are not known, we used a region of the yeast *STE6* promoter that contains a canonical MADS domain protein binding site (known as a CArG motif). This sequence, which is shown in Figure 6A and corresponds to the binding site of the MADS domain protein MCM1, was previously used to demonstrate specific binding of the DEFICIENS–GLOBOSA (DEF A–GLO) heterodimer (Schwarz-Sommer et al., 1992).

As shown in Figure 6B, overexpressed AGL15 binds to $^{32}$P-labeled DNA fragments containing the CArG motif and slows their migration in gels (lane 1). A second set of modified $^{32}$P-labeled DNA fragments, which had a single base pair change in the CArG motif, was also prepared (Figure 6A). No AGL15–DNA complexes formed when these fragments were used as the target (Figure 6B, lane 2), demonstrating that AGL15 binding is sequence specific. When nonradioactive DNA fragments containing the CArG motif were provided in excess, they competed with radioactive DNA fragments containing the CArG motif for binding of AGL15 (Figure 6B, lane 3). When nonradioactive fragments containing the modified CArG motif were provided in excess, they had no effect on AGL15 binding at the CArG motif (Figure 6B, lane 4).

The MADS domain was essential for binding to the CArG motif; overexpressed AGL15 lacking the MADS domain did not

bind DNA (Figure 6B, lane 5). The addition of the AGL15 antiserum to the binding reaction decreased the intensity of the shifted bands and led to the appearance of the radiolabel in bands of lower mobility (supershifted bands), as shown in Figure 6C (lane 2), confirming that AGL15 was present in the DNA–protein complex. In the presence of preimmune serum, no supershift was observed (Figure 6C, lane 1). AGL15 antiserum added to the radiolabeled DNA fragments in the absence of AGL15 did not cause any shift or supershift of the DNA (Figure 6C, lane 3).

## DISCUSSION

### Distribution of AGL15 Protein in the Seed

To make predictions about the role of factors like AGL15 in development, it is important to determine when and where the protein accumulates. Three plant MADS domain proteins, FLORAL BINDING PROTEIN1 (FBP1), AP3, and AGL15, have now been localized in nuclei (Cañas et al., 1994; Jack et al., 1994; this study), which is consistent with their anticipated function as transcription factors (Schwarz-Sommer et al., 1990). Comparisons of the distribution of the mRNA and protein indicated that both *FBP1* and *AP3* are subject to post-transcriptional regulation (Cañas et al., 1994; Jack et al., 1994), as are several other members of the MADS domain family of genes, including *myocyte-specific enhancer-binding factor 2* (*MEF2*) (Yu et al., 1992; Breitbart et al., 1993) and *Zea endosperm MADS box a* (*ZEMa*) (Montag et al., 1995). In the case of FBP1, which is involved in the regulation of floral organ identity in petunia, mRNA and protein accumulate at early stages of stamen development. However, at later stages, only mRNA can be detected in these organs. The absence of detectable protein indicates that the translation of *FBP1* mRNA may be blocked at later stages or alternately that the protein may be degraded rapidly after synthesis (Cañas et al., 1994). In any case, FBP1 is likely to exert most of its effects in stamens at early stages and to play less of a role in regulation during later stages.

Unlike FBP1, the distributions of AGL15 protein and mRNA are essentially coincident at all stages in embryos. We previously demonstrated that *AGL15* mRNA is present in all cells in Brassica embryos at the torpedo stage (Heck et al., 1995), and Rounsley et al. (1995) showed that *AGL15* mRNA is present in all cells of Arabidopsis embryos at the eight-cell stage. Immunolocalization of AGL15 in developing Brassica and Arabidopsis embryos indicated that the protein also accumulates in all cells throughout embryo development. The levels of both mRNA and protein decline as the embryo matures, but the accumulation pattern does not change qualitatively during later stages. The widespread distribution of AGL15 protein in the embryo suggests that AGL15 is unlikely to act as a regulator of organ or cell-type specific programs, except in combination with factors that might be more restricted in distribution.

Immunolocalization experiments gave us a different view of AGL15 expression than we had had previously (Heck et al., 1995; Rounsley et al., 1995). It is clear that AGL15 is expressed in more seed tissues than the mRNA localization studies had originally indicated. The ability to detect the accumulation of specific mRNAs using in situ hybridization techniques is limited in situations in which the signal is low and difficult to distinguish from background, that is, when transient expression, small numbers of cells, and/or low levels of expression are involved, as in early stages of embryogenesis. Using a highly sensitive detection system, we found that AGL15 protein accumulates at relatively high levels in cells in the suspensor and the endosperm, as well as in the embryo, in all three species that we examined. Reporter gene studies have subsequently confirmed that the *AGL15* promoter is active in these cells in Arabidopsis (G.R. Heck and D.E. Fernandez, unpublished observations). Immunolocalization also indicated that cells in seed tissues that are strictly maternal in origin transiently express AGL15 in some species (Brassica and maize). Because of high levels of nonspecific binding in seed coat tissues (i.e., signal with sense probes), the results of in situ hybridizations performed previously with sections of developing Brassica seeds were inconclusive (D.E. Fernandez, unpublished observations). Finally, at least some elements of AGL15 protein structure appear to have been conserved evolutionarily (Heck et al., 1995), which made it possible to use immunohistochemistry to compare the pattern of expression in distantly related species. Because of the differences in codon bias between monocots and dicots (Campbell and Gowri, 1990), comparable experiments localizing mRNAs would be difficult if not impossible without first isolating the maize homolog of AGL15.

### AGL15-DNA Associations

Localization of AGL15 in sections of fertilized and unfertilized ovules revealed that a dramatic change in the cellular localization of the protein occurs during early embryogenesis. Although the conserved MADS domain contains a bipartite nuclear localization signal (Raikhel, 1992), the protein appears to be confined to the cytoplasm of the cells constituting the egg apparatus before fertilization. In the early stages of embryogenesis, generally before the embryo reaches the 16-cell stage, AGL15 is translocated into the nucleus. In subsequent cell divisions, AGL15 segregates with the chromosomes. A similar association has been reported in the case of Xenopus nuclear factor 7, a maternally expressed putative transcription factor (Li et al., 1994), and may represent a mechanism to ensure equal distribution of regulatory factors to daughter cells.

Our DNA binding studies established that AGL15 has the ability to bind in a sequence-specific manner to DNA in vitro. AGL15 recovered from bacterial inclusion bodies is functional in binding assays; therefore, unlike DEF A and GLO (Schwarz-Sommer et al., 1992; Tröbner et al., 1992), but like AG (Shiraishi et al., 1993), AGL15 is capable of binding as a homodimer and

does not require a binding partner to form a stable complex with DNA. The target site used in these studies, from the yeast *STE6* promoter, does not represent the optimal in vivo binding site, which will have to be determined experimentally. Previous studies demonstrating sequence-specific binding "preferences" in vitro (Huang et al., 1996) suggest that each member of the MADS domain family may bind to a slightly different spectrum of sites in vivo. Because plant MADS domain proteins lack any known motifs that function as transcriptional activators (Pneuli et al., 1991), transcription of target genes may involve interactions with other regulatory factors.

The timing of the translocation of AGL15 into the nucleus suggests that AGL15 could regulate genes that are activated before the main tissue layers are delineated in the embryo. A well-studied example of a regulatory factor that is similarly translocated during early embryogenesis is the morphogen DORSAL in Drosophila. DORSAL is synthesized from maternal RNA and is anchored in the cytoplasm by another protein, CACTUS. At an early stage in development, a signal transduction cascade leads to the phosphorylation of DORSAL and/or CACTUS. The subsequent dissociation of the complex and the translocation of DORSAL into nuclei on the ventral side of the embryo establish the dorsoventral axis (St. Johnston and Nüsslein-Volhard, 1992; Ingham, 1994). Although the uniform distribution of AGL15 appears to preclude a relationship between AGL15 action and the delineation of axes in plant embryos, the observation that the cellular localization of AGL15 is developmentally regulated establishes the potential for this kind of control of morphogens and other regulatory factors during the early stages of plant embryogenesis.

### Developmental Role of AGL15

What kinds of developmental processes are complexes containing AGL15 likely to regulate? One possible developmental role for AGL15 would be to specify the embryo or seed phase of the life cycle. *AGL15* promoter activity (G.R. Heck and D.E. Fernandez, unpublished observations) and mRNA levels are significantly higher (up to five- to 10-fold) during the embryonic phase than after germination (Heck et al., 1995; Rounsley et al., 1995). Accumulation of protein at relatively high levels is restricted to the products of double fertilization (embryo, suspensor, and endosperm) and associated maternal tissues. All of these are capable of producing embryos and/or expressing cellular differentiation programs unique to the seed phase of the life cycle. In genetic backgrounds in which embryo development or signaling systems are disrupted in some way (*suspensor*, *raspberry*, and *embryo-defective* mutants in Arabidopsis), cells in the suspensor can undergo extra divisions (Schwartz et al., 1994; Yadegari et al., 1994), organize into tissues (Yadegari et al., 1994), synthesize storage products (Schwartz et al., 1994), and in some cases, make additional embryos (*twin* mutant; Vernon and Meinke, 1994).

Despite the obvious morphological and physiological differences that exist, there are also a number of parallels between embryo and endosperm cells in terms of activating programs of gene expression. Storage proteins accumulate transiently in the endosperm as well as in the embryo and suspensor in faba bean (Panitz et al., 1995). In maize, the endosperm accumulates high levels of seed-specific storage products, and many of the mutations resulting in defective kernels (*dek* mutants) affect both embryo and endosperm development (Neuffer and Sheridan, 1980).

The hypothesis recently advanced by Friedman (1994) concerning the evolutionary origin of the endosperm predicts that embryo and endosperm cells share elements specifying "identity" at some level. Friedman suggests that the endosperm actually originated as a separate "sister" embryo that later assumed a nutritive role. In apomictic species, in which the embryos are not products of fertilization, the cells that assume embryonic identity are generally in the unreduced megagametophyte or in maternal tissues, such as the nucellus and the inner integument (Koltunow, 1993). Although the levels that accumulate in maternal tissues vary considerably from species to species, these cells clearly have the capacity to express AGL15.

To test the hypothesis that AGL15 contributes to the establishment and/or maintenance of embryo/seed identity, we examined the effect of mutations in *LEC*, *FUS3*, and *ABI3* genes on AGL15 protein accumulation during embryogenesis. Defects in these genes lead to a partial loss of embryo identity and/or premature exit from embryo-specific programs (Meinke, 1992; Nambara et al., 1992, 1994, 1995; Keith et al., 1994; Meinke et al., 1994; West et al., 1994). AGL15 accumulated in the normal spatial and temporal pattern in embryos homozygous for the *lec2*, *fus3-3*, or *abi3-6* mutations. In homozygous *lec1-2* embryos, AGL15 accumulated at normal levels during early stages. Once the embryos made the transition to bilateral symmetry, however, the protein could no longer be detected. The decline in AGL15 levels preceded the appearance of morphological abnormalities in the embryo during late heart stage. Levels did not decline in the endosperm, which is not altered phenotypically by the *lec1-2* mutation. Therefore, the effect of *lec1-2* on AGL15 accumulation appears to be both stage specific and embryo specific.

Interestingly, embryos that are homozygous for the *lec1-1* mutation do not show this effect even though the two alleles are indistinguishable otherwise (Meinke et al., 1994). A trivial explanation of this result would be that the *lec1-2* embryos carry another defect that is responsible for the loss of AGL15. We believe this is unlikely, given the coincidence between the embryo-specific loss of AGL15 and the onset of the embryo-specific mutant phenotype. Another explanation is based on the possibility that AGL15 and LEC1 may interact directly in some way. If AGL15 and LEC1 interact as parts of the same complex, the results can be explained in terms of differential stability. For instance, a complex containing *lec1-1* may be stable but nonfunctional, whereas a complex containing *lec1-2* may be unstable and targeted for rapid degradation. Once the *LEC1* locus has been characterized molecularly, we should be able to test for these kinds of effects.

If AGL15 is interacting with the leafy cotyledons regulatory pathway at the level of LEC1, it is perhaps not surprising that mutations in the *LEC2*, *FUS3*, and *ABI3* genes have no effect on the accumulation of AGL15 in the embryo. Based on the number of late-embryogenesis programs disrupted in the mutants (Meinke et al., 1994), the current models place LEC1 at or near the top of the leafy cotyledons pathway. LEC2 is thought to act downstream of LEC1, and recent studies suggest that FUS3 may function in a different pathway (West et al., 1994). *abi3* mutants show a loss of some late embryogenesis programs but no loss of embryo identity (Nambara et al., 1992, 1994, 1995). Based on double mutants and ABA sensitivity, ABI3 is also thought to act in a separate pathway (Meinke et al., 1994). *lec1* mutations have no effect on accumulation of AGL15 in young embryos; therefore, LEC1 is not required for the activation of *AGL15* expression. Our results indicate that LEC1 plays a role in maintaining high levels of AGL15 during later stages of embryogenesis. LEC1 may have a direct role, which would be the case if it interacts with the *AGL15* gene product, or a more indirect role, possibly influencing other gene products that affect *AGL15* transcription and/or turnover rates.

In summary, the data presented in this study support the premise that AGL15 plays an important role in the regulatory networks operating during embryogenesis. AGL15 is expressed before fertilization but remains in the cytoplasm until after the first few rounds of cell division. Assuming that AGL15 acts as a transcription factor, it must move into the nucleus, where it can associate with target sequences. The fact that this process is developmentally regulated suggests that AGL15 function is likely to have a special, as yet undefined developmental significance for the establishment of the new generation. AGL15, or molecules related to it, may perform equivalent functions in all flowering plants. We found that the pattern of accumulation is essentially identical in monocots and dicots, despite their divergence in modes of embryogenesis. Finally, we have shown that AGL15 interacts, at least genetically, with another putative regulator, LEC1, that is involved in maintenance of the embryonic phase. Further study of components that interact at molecular and genetic levels with AGL15 should lead to a better understanding of the roles regulatory molecules play in the elaboration of early developmental programs in the seed.

## METHODS

### Plant Material

Oilseed rape (*Brassica napus* cv Tower) plants were grown in an environmental chamber (Conviron; Controlled Environments Inc., Pembina, ND), using a 16-hr-light (370 $\mu$E $m^{-2}$ $sec^{-1}$ at flower level at 15°C)/8-hr-dark (10°C) regime. Flowers on the primary inflorescence were hand pollinated on the day of anthesis.

*Arabidopsis thaliana* (Wassilewskija and Columbia ecotypes) plants were grown at 22°C in constant light (88 $\mu$E $m^{-2}$ $sec^{-1}$). Flowers were tagged the day before they fully opened. The *lec1-1*, *lec1-2*, and *lec2* mutations (seed provided by D. Meinke, Oklahoma State University, Stillwater) were in a Wassilewskija background, whereas the *abi3-6* and *fus3-3* mutations (seed provided by P. McCourt, University of Toronto, Ontario, Canada) were in a Columbia background. Homozygous mutants were obtained by rescuing seeds containing embryos that accumulate high levels of anthocyanin from a heterozygous plant, as described previously (Meinke et al., 1994).

Hybrid maize (*Zea mays*) plants (W64A × W438) were field grown during the summer of 1995 and hand pollinated.

Developmental stage was estimated in all cases based on chronological age (days after pollination [DAP]) and verified by examination of embryo morphology.

### Constructs for Protein Overexpression

Overexpression of a truncated form of Brassica AGL15-1 lacking the MADS domain (residues 62 to 258) was described previously (Heck et al., 1995). A sequence encoding all of Brassica AGL15-1 except for the last six amino acids (residues 1 to 258) was amplified from a Brassica transition-stage embryo cDNA library (Heck et al., 1995), using polymerase chain reaction (PCR) and *AGL15-1* specific oligonucleotides flanked by NcoI and BamHI restriction sites. Constructs to overexpress Arabidopsis AGL15 were produced by amplifying sequences corresponding to different portions of the coding sequence from a previously characterized Arabidopsis *AGL15* cDNA clone (Heck et al., 1995). The PCR products encoded amino acid residues 1 to 268 (for the construct including the MADS domain) and residues 62 to 268 (for the construct lacking the MADS domain) flanked by NcoI and XhoI restriction sites. A cDNA sequence encoding full-length Arabidopsis AGL2 and flanked by NcoI and XhoI restriction sites was previously amplified from a Wassilewskija silique library (Heck et al., 1995). All of the PCR products were cloned into pBluescript SK− (Stratagene, La Jolla, CA) that had been digested with EcoRV and tailed with a thymidine residue using Taq polymerase. The inserts were subsequently excised with the appropriate restriction enzymes and cloned into the expression vector pET15b (Novagen, Madison, WI). Expression was induced in *Escherichia coli* host cells BL21(DE3), and the overexpressed protein was purified by isolation of inclusion bodies as described previously (Heck et al., 1995).

### Antibody Preparation

Production of polyclonal antibodies that recognize AGL15 was described previously (Heck et al., 1995). For protein gel blot analysis, antibodies that recognized the overexpressed truncated Brassica AGL15-1 were blot affinity purified from the antiserum as described by Tang (1993). For immunohistochemistry, immune and preimmune sera were preadsorbed to remove serum components that bind nonspecifically to fixed plant tissues, using a protocol established by Jack et al. (1994). Pieces (~4 $mm^2$) of fully expanded Brassica leaves that did not express AGL15 were fixed for 1 hr under vacuum with 4% (w/v) freshly prepared paraformaldehyde and 0.02% (v/v) Triton X-100 in 50 mM potassium phosphate buffer, pH 7. The leaf pieces were washed for several hours with a large volume and multiple changes of PBST buffer (PBST is 237 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4$, 1.4 mM $KH_2PO_4$, 0.1% Tween 20, pH 7.3). A solution consisting of 10% (v/v) preimmune or immune serum, 0.05% (w/v) BSA fraction V in 0.9 ×

PBST was added to the fixed leaf pieces (~5 mL of diluted serum per 1 g of leaf tissue) and incubated overnight with gentle mixing at 4°C. The preadsorbed serum was removed by aspiration and stored at 4°C with 0.05% (w/v) sodium azide. Sera prepared in this manner could be used for up to several months.

### Immunodetection of AGL15 on Protein Gel Blots

Developing Brassica embryos or whole seeds were flash frozen in liquid nitrogen, and soluble protein extracts were prepared as described previously (Heck et al., 1995). Extracts from seeds (300 μg per lane), embryos (150 μg per lane), or protein from solubilized bacterial inclusion bodies (20 ng per lane) were separated on denaturing 15% polyacrylamide gels before blotting onto Immobilon polyvinylidene fluoride membranes (Millipore Corp., Bedford, MA). Blots were incubated with affinity-purified antibodies (final dilution of ~1:1500 [v/v] relative to the original immune serum) or with preimmune serum (final dilution of 1:500 [v/v]). The immunoreactive protein was visualized using the Lumi-GLO system (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD), with the secondary antibody diluted 1:5000 (v/v). The blot was exposed to x-ray film (Biomax MR; Eastman Kodak Co., Rochester, NY) for 1 min.

### Immunohistochemistry

Plant tissues were fixed overnight at 4°C with 4% (w/v) freshly prepared paraformaldehyde and 0.02% Triton X-100 in 50 mM potassium phosphate buffer, pH 7. Samples >3 mm (maize embryos and young seeds, Arabidopsis siliques) were cut into smaller pieces in fixative, using a double-edged razor blade. Fixed tissues were embedded in Paraplast Plus (Sherwood Medical, St. Louis, MO) and sectioned (7 μm thick) with a steel knife. Sections were mounted on ProbeOn Plus microscope slides (Fisher Scientific, Pittsburgh, PA).

For immunohistochemistry, dewaxed and rehydrated sections were treated for 10 min at room temperature with proteinase K (100 or 500 μg/mL in PBS, depending on the tissue). The slides were washed for 10 min in TBST (20 mM Tris, pH 7.4, 150 mM NaCl, 0.1% Tween 20) and blocked with 5% (v/v) normal goat serum in TBST for 30 min at room temperature. After an overnight incubation at 4°C with immune or preimmune serum (preadsorbed against leaf pieces and diluted 1:1000 relative to the original serum volume with 5% goat serum in TBST), the slides were washed thoroughly with TBST, and the primary antibody was visualized by using the Vectastain Elite ABC kit (Vector Labs, Inc., Burlingame, CA). This system uses a biotinylated secondary antibody and a preformed avidin and biotinylated horseradish peroxidase macromolecular complex, which is visualized by incubation with diaminobenzidine (Sigma) to generate a red-gold to brown deposit. Detection of the primary antibody was performed according to the directions of the manufacturer, except that more extensive washes (10 min in TBST) and a longer incubation with the secondary antibody (2 hr at room temperature) were used. Detection with diaminobenzidine was performed as described in a protocol provided by Jack et al. (1994). Slides were examined on a Zeiss Axioskop (Carl Zeiss, Ltd., Oberkochen, Germany) or Zeiss Ultraphot II, using bright-field or Nomarski optics and photographed using Ektachrome 160T color slide film (Eastman Kodak).

To visualize nuclei, proteinase-treated sections were incubated for 10 min at room temperature with 1 μg/mL of 4′,6-diamidino-2-phenylindole (DAPI) and 10 mM EDTA in PBS with gentle agitation in the dark. Slides were washed briefly in PBS, and sections were sealed under a coverslip in a small pool of PBS with 10 mM EDTA. After an overnight incubation at 4°C in the dark, DAPI staining was examined using a UV filter and epifluorescent illumination.

To prepare the figures, slides were scanned using a Kodak professional RFS 2035 film scanner, and the images were assembled into plates, using Adobe Photoshop 3.0 (Adobe, Inc., Mountain View, CA).

### Gel Mobility Shift Assays

The region containing the CArG motif of the yeast *STE6* promoter was reconstituted using partially complementary primer pairs that were extended using PCR. Oligonucleotide 1, 5′-CCATGTAATTACCTAATAGGGAA-3′, and oligonucleotide 2, 5′-AGCGTGTAAATTTCCCTATTAGG-3′, were used to generate the wild-type sequence. A sequence containing a modified CArG motif was created using oligonucleotide 2 and oligonucleotide 3, 5′-CCATGTAATTAACTAATAGGGAA-3′. These fragments were cloned into pBluescript SK− that had been digested with EcoRV and tailed with a single thymidine residue, using Taq polymerase. The plasmids were subsequently digested with EcoRI and HindIII, and the inserts were gel purified. The overhanging ends of each insert were filled in with $^{32}$P-dATP, using the Klenow fragment of DNA polymerase I, generating 52-bp radioactive DNA fragments.

AGL15 and a truncated form lacking the MADS domain were overexpressed in *E. coli* BL21(DE3) cells and solubilized as described by Heck et al (1995). Proteins were renatured by dialyzing for 8 hr at 4°C through progressively decreasing concentrations of urea (6, 4, 2, and 0 M) in 1× binding buffer (60 mM KCl, 12 mM Hepes, pH 7.5, 4 mM Tris-Cl, pH 7.5, 1 mM EDTA, 1 mM DTT, and 10% glycerol). To inhibit proteolysis, 1 mM phenylmethylsulfonyl fluoride was added to the dialysis buffer. The renatured protein preparations were cleared by centrifuging, and aliquots of the supernatant were frozen at −80°C.

DNA binding reactions were performed in 20-μL volumes (1 × binding buffer, 10 μg of BSA, 1 μg of poly(dI-dC), and 10% immune or preimmune rabbit serum) in the presence of up to 7 μg of renatured overexpressed protein and 1 to 8 × $10^4$ cpm of probe. Reaction components were incubated for 10 min at room temperature before adding the probe and for an additional 10 min at room temperature after adding the probe. Samples were centrifuged (16,000*g*) at room temperature for 10 min, and 15 μL of the reaction was loaded onto a 5% polyacrylamide gel containing 0.5 × TBE (1 × TBE is 8.9 mM Tris base, 8.9 mM boric acid, and 0.2 mM EDTA) and electrophoresed at room temperature. Gels were dried and exposed to x-ray film (Biomax MR; Kodak).

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