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LEAFY COTYLEDON1 Is an Essential Regulator of Late Embryogenesis and Cotyledon Identity in Arabidopsis

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***LEAFY COTYLEDON1* (*LEC1*) is an *embryo defective* mutation that affects cotyledon identity in Arabidopsis. Mutant cotyledons possess trichomes that are normally a leaf trait in Arabidopsis, and the cellular organization of these organs is intermediate between that of cotyledons and leaves from wild-type plants. We present several lines of evidence that indicate that the control of late embryogenesis is compromised by the mutation. First, mutant embryos are desiccation intolerant, yet embryos can be rescued before they dry to yield homozygous recessive plants that produce defective embryos exclusively. Second, although many genes normally expressed during embryonic development are active in the mutant, at least one maturation phase-specific gene is not activated. Third, the shoot apical meristem is activated precociously in mutant embryos. Fourth, in mutant embryos, several genes characteristic of postgerminative development are expressed at levels typical of wild-type seedlings rather than embryos. We conclude that postgerminative development is initiated prematurely and that embryonic and postgerminative programs operate simultaneously in mutant embryos. The pleiotropic effects of the mutation indicate that the *LEC1* gene plays a fundamental role in regulating late embryogenesis. The role of *LEC1* and its relationship to other genes involved in controlling late embryonic development are discussed.**

INTRODUCTION

A unique characteristic of higher plants is that their life cycle is interrupted by a period of developmental arrest during which the mature embryo is metabolically quiescent and in a desiccated state (for review, see Crouch, 1987; Goldberg et al., 1989, 1994; Kermode, 1990; Galau et al., 1991; McCarty and Carson, 1991). This discontinuous mode of development is an integral part of the strategy used by plants to form seed. The seed habit has conferred significant selective advantages to seed plants by enhancing their ability to survive periods of adverse environmental conditions and by facilitating dispersal of the species (Steeves, 1983). A consequence of seed formation is that embryogenesis has been modified extensively from that of lower vascular plants that do not form seed (Walbot, 1978; Steeves and Sussex, 1989).

The earliest segment in the sporophytic life cycle of higher plants can be divided conceptually into four phases. The initial phase is one of morphogenesis during which the polarity and basic body plan of the plant are established and the two major embryonic organ systems are formed (Natesh and Rau, 1984; West and Harada, 1993; Goldberg et al., 1994). The axis, with its shoot–root pole, gives rise to the primary body of the vegetative plant, whereas the cotyledons are modified leaves that serve initially as embryonic storage organs but are transformed into photosynthetic organs after germination. The next two phases of embryogenesis are directly involved in processes that contribute to seed formation. During the maturation phase, storage macromolecules, including proteins, lipids, and/or carbohydrates, accumulate in the embryo to high levels, particularly in the cotyledons (Goldberg et al., 1989; Shotwell and Larkins, 1989; Huang, 1992). These reserves ultimately serve as a nutrient source for the germinating seedling until autotrophic growth commences. The third phase, designated desiccation or postabscission, is characterized by processes that permit the embryo to withstand the stresses of drying and to enter into a period of developmental arrest in a desiccated state (Crouch, 1987; Kermode, 1990; Galau et al., 1991; McCarty and Carson, 1991). The mature embryos remain metabolically quiescent until environmental conditions are conducive for the fourth phase, germination, in which growth and morphogenesis are reinitiated (Harada et al., 1988).

A variety of regulatory events must occur to coordinate the diverse cellular processes that occur during late embryogenesis. First, the maturation and desiccation phases must be

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initiated and maintained in a precisely regulated temporal sequence. Second, the finding that embryos of many plants will germinate precociously when cultured on basal media suggests that premature germination or vivipary must be actively suppressed in an embryo (Crouch, 1987; Galau et al., 1991). Third, many but not all plants enter a state of primary dormancy in which seeds will not germinate upon imbibition unless specific conditions exist (Hilhorst and Karssen, 1992). Because nondormant embryos are not viviparous, primary dormancy is distinct from the inhibition of germination that occurs during embryonic development (Hilhorst and Karssen, 1992).

Although several potential regulators of late embryogenesis and germination have been proposed, little definitive information is available. Abscisic acid (ABA) has been suggested to play regulatory roles in late embryogenesis. Although there is substantial evidence to support an involvement of ABA in suppressing the germination of maturing embryos, its role as the sole regulator of the maturation and/or desiccation phases is unlikely (Quatrano, 1986). For example, mutants unresponsive to ABA, such as *aba insensitive3* (*abi3*), are capable of synthesizing storage proteins at reduced levels and, therefore, initiating programs characteristic of the maturation phase (Koornneef et al., 1989; Finkelstein and Somerville, 1990; Nambara et al., 1992). Others have proposed that maternal factors and seed abscission may serve to regulate late embryogenesis, although the nature of these presumptive signals and the mechanisms by which they may act are not known (Galau et al., 1991).

As part of our study of embryogenesis in the representative dicotyledonous plant Arabidopsis, we identified an unusual *embryo defective* mutant from a population of plants mutagenized insertionally with T-DNA that is intolerant of desiccation; the mutant therefore dies during late embryogenesis. Prominent characteristics of this mutant are that developing embryos can be rescued before the seeds desiccate and that rescued seeds germinate to reveal the inappropriate presence of trichomes on cotyledon surfaces. We subsequently showed that the mutation is allelic with *LEAFY COTYLEDON1* (*LEC1*) and designated this allele as *lec1-2* (Meinke, 1992). To determine whether the presence of trichomes on these lateral organs represents the homeotic conversion of cotyledons to leaves, we compared the morphology and anatomy of mutant embryos with wild-type embryos and seedlings and analyzed the spatial expression of genes characteristic of embryonic and postgerminative development. We conclude that postgerminative development is activated precociously during embryogenesis and that some aspects of embryonic development are impaired in *lec1* mutants. The simultaneous expression of embryonic and postgerminative programs in mutant embryos suggests a role for the *LEC1* gene in regulating the transition from embryogenesis to postembryonic development.

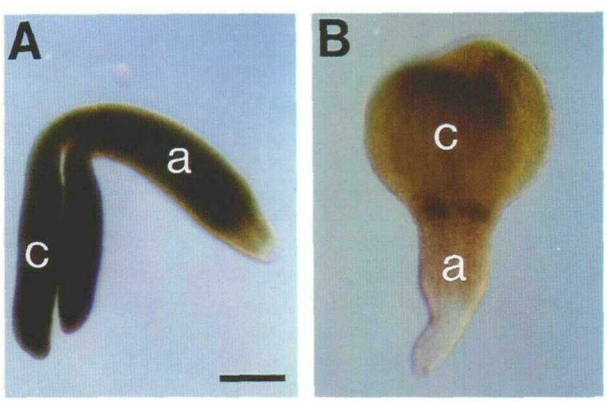

Figure 1. *lec1-2* Embryos Have an Altered Morphology.

Mutant and wild-type segregants from a single silique were dissected and photographed.

(A) Wild-type embryo. The oval-shaped cotyledons of maturing wild-type embryos fold over the elongated axis. The wild-type embryo appears uniformly green.

(B) Mutant embryo. Mutants have rounded cotyledons, shortened hypocotyls, and constricted root apices that appear clear.

a, axis; c, cotyledon. Bar = 100 μm.

RESULTS

Rescue of an *embryo defective* Mutant by Precocious Germination Reveals a Striking Phenotype

We screened 5822 T_3 families of Arabidopsis that were mutagenized insertionally with T-DNA and identified 66 *embryo defective* mutants (Yadegari et al., 1994). One exceptional mutant produced defective, pale green embryos with the morphology shown in Figure 1. Unlike wild-type embryos, mutants possess round and fleshy cotyledons that do not bend over and that often accumulate anthocyanin pigment at their tips (compare Figures 1A and 1B). The mutant's embryonic axis differs from that of the wild type in that it is shorter and constricted at the root end. In plants heterozygous for the mutation, 21% of seeds were pale green (435/2104; $\chi^2(3:1) = 21.0$; $P < 0.05$), suggesting that the phenotype results from a single-gene recessive mutation with, perhaps, slight defects in transmission.

Dried defective seed of this mutant were green and unable to germinate, suggesting that the embryos are desiccation intolerant. Because viviparous seeds were occasionally observed, we attempted to rescue homozygous recessive mutants by culturing immature embryos. As represented in Figure 2, a range of 70 to 90% of mutant seed removed from siliques prior to desiccation germinated on basal media and produced seedlings; conversely, only ~10% of immature wild-type seed germinated (data not shown). Rescued mutant seedlings gave rise to homozygous plants that had no obvious abnormalities other than the presence of trichomes on their cotyledons and their production of defective progeny. With respect to its intolerance to desiccation and nondormancy, the mutation is similar to severe mutant alleles of *ABI3* (Nambara et al., 1992; Ooms et al., 1993). However, genetic tests showed that the

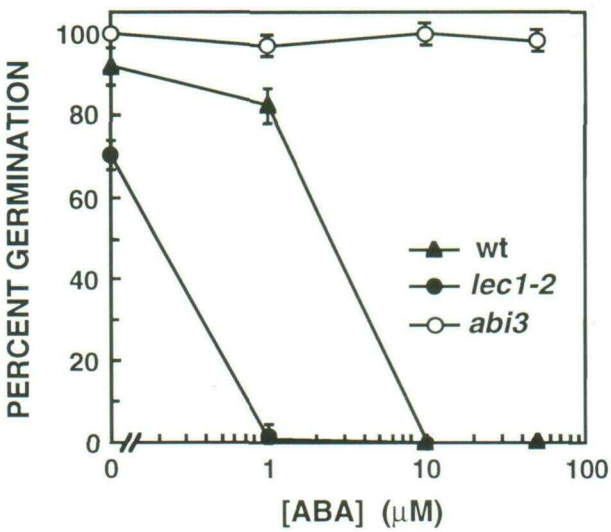

Figure 2. ABA Suppresses Germination of *lec1-2* Embryos.

Dried wild-type seed and rescued mutant seed were placed on 0.5% agar containing 0, 1, 10, and 50 μM ABA. Germination was scored after 2 weeks at 22°C. As a control, rescued *abi3-3* seeds were also tested on ABA. The data represent the average of two separate experiments, each performed in triplicate. Error bars indicate one standard deviation.

mutant complements *abi3* plants. Furthermore, we show in Figure 2 that predesiccation stage mutant seed did not germinate on 1 μM ABA, whereas immature *abi3-3* seed germinated in the presence of up to 50 μM ABA. Germination of wild-type dry seed was sensitive to 10 μM but not 1 μM ABA. Thus, this mutant is sensitive to ABA and is not an allele of *ABI3*, a conclusion that is verified by genetic mapping data.

A striking characteristic of rescued mutant seedlings is illustrated in Figure 3A. The mutant possesses trichomes on the adaxial surfaces of its cotyledons. Trichomes are normally present only on leaves, stems, and sepals, and they are never consistently observed on cotyledons of wild-type Arabidopsis seedlings germinated precociously or from dried seed, as shown in Figures 3B and 3C, respectively (Hülskamp et al., 1994). Figure 4 shows that trichomes were not induced artifactually by culturing plants on media. Unlike wild-type embryonic cotyledons (Figure 4B), which do not possess these epidermal hairs, trichomes are clearly visible on the inner surfaces of mutant cotyledons (Figures 4A and 4C). The shape of the embryonic trichomes differs from those present on wild-type leaves grown for 7 days (Figure 4D).

Two other *embryo defective* Arabidopsis mutants have been described with trichomes on their cotyledons, *lec1* and *fusca3* (*fus3*) (Meinke, 1992; Baumlein et al., 1994; Keith et al., 1994). We showed that this new mutation maps to chromosome 1, as does *lec1*, and the mutant fails to complement *lec1-1* (data not shown). Therefore, we designate this mutation *lec1-2.*

lec1 Embryos Display Postgerminative Characteristics

One interpretation of the Lec1$^-$ phenotype is that the mutation causes the homeotic conversion of cotyledons to leaves. The presence of trichomes on mutant cotyledons (Figure 3) and other work showing that protein bodies that are normally a characteristic of embryonic cotyledons are absent in *lec1-1* provide support for this hypothesis (Meinke, 1992). Because

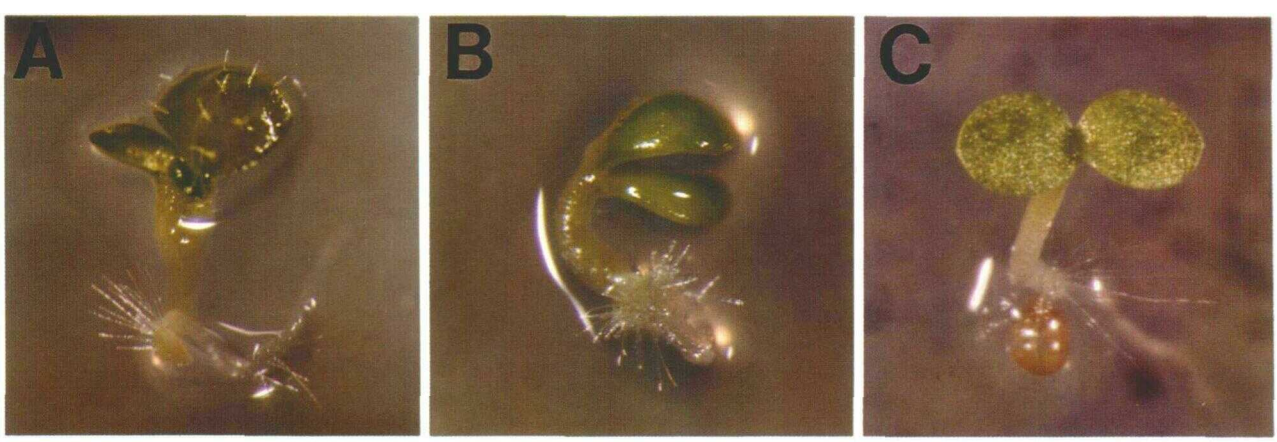

Figure 3. Precociously Germinated *lec1-2* Seedlings Have Trichomes on Their Cotyledons.

Mutant and wild-type embryos were dissected from mature green seed prior to desiccation and placed on media; wild-type dry seed were surface sterilized prior to culturing. Seedlings were photographed after 3 days.

(A) Precociously germinated mutant seedling. Trichomes appear on the adaxial surfaces of the cotyledons.

(B) Precociously germinated wild-type seedling. The fleshy cotyledons do not have trichomes.

(C) Wild-type seedling germinated from a dried seed. The flat, expanded cotyledons do not produce trichomes.

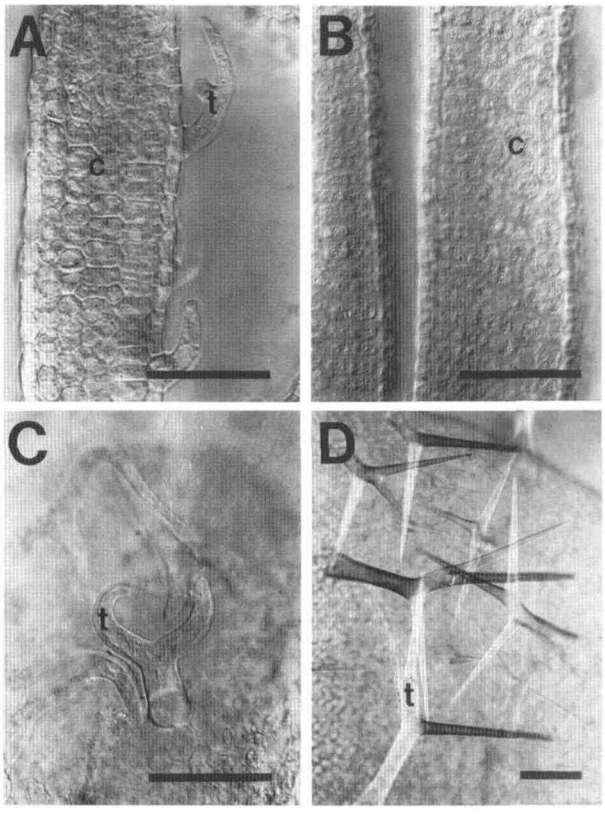

Figure 4. Trichomes Are Present on *lec1-2* Embryonic Cotyledons.

Mutant and wild-type embryos and a leaf from a seedling grown for 7 days were cleared and photographed with Nomarski optics.
(A) Mutant embryonic cotyledon. Trichomes arise from the protoderm of the inner cotyledon surface. The upper trichome is starting to branch.
(B) Wild-type embryonic cotyledon. The protoderm of wild-type cotyledons is smooth, with no indication of trichomes.
(C) Mutant embryonic cotyledon. A branched trichome is visible on the inner surface of a mutant cotyledon. Other trichomes are visible out of the plane of focus.
(D) Wild-type leaf. Mature trichomes are present on the surface of a leaf from a wild-type seedling grown for 7 days. Some of the trichomes are multiply branched.
c, cotyledon; t, trichome. Bars = 50 μm for **(A)**, **(B)**, and **(C)** and 100 μm for **(D)**.

trichomes are a postembryonic characteristic, an alternative explanation is that *lec1* is a heterochronic mutation causing postgerminative development to be initiated prematurely in *lec1* embryos. We explored these possibilities by analyzing the morphology of *lec1-2* embryos to determine whether the effects of the mutation are limited to cotyledon identity.

Figure 5 shows that there are significant differences between the shoot apices of wild-type and *lec1* embryos. Unlike the flat shoot apices characteristic of wild-type mature embryos (Figures 5D and 5E), Figures 5A to 5C show that *lec1-2* embryos possess raised apical meristems with distinct leaf

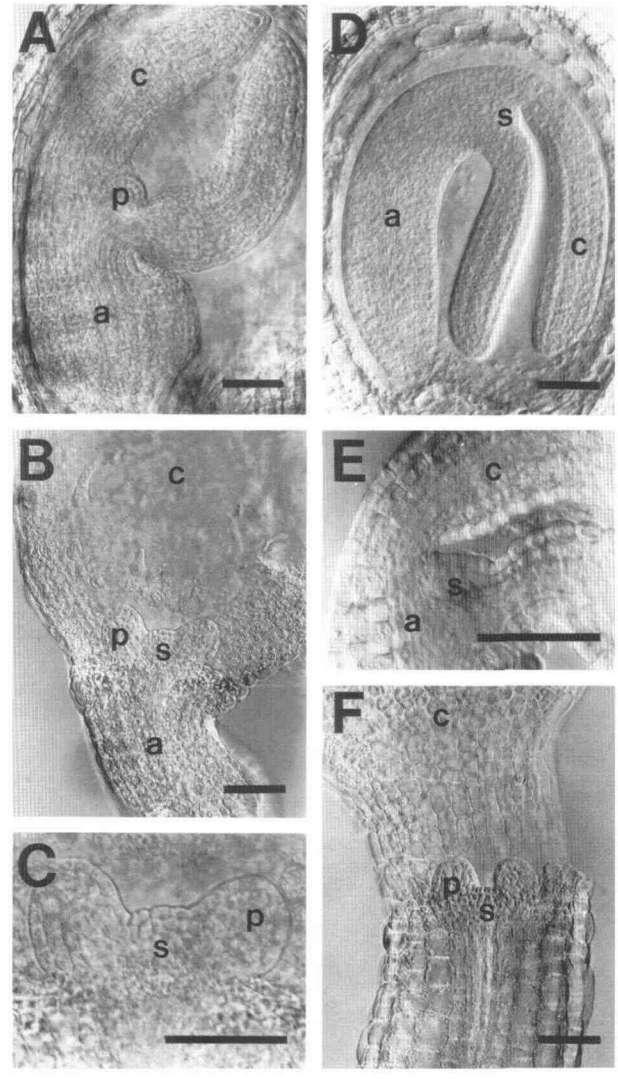

Figure 5. The Shoot Apex Is Activated Precociously in *lec1-2* Embryos.

Mutant and wild-type embryos and a 3-day-old wild-type seedling were cleared and photographed with Nomarski optics.
(A) Mature mutant embryo. A leaf primordium is visible between the two cotyledons of the mutant embryo.
(B) Mature mutant embryo. This embryo was optically sectioned to show the activated shoot apical meristem with two developing leaf primordia.
(C) Mature mutant embryo. A higher magnification photograph of the mutant embryo in **(B)**. The L_1 and L_2 cell layers of the domed shoot apical meristem and the leaf primordia emerging on the sides of the meristem are visible.
(D) Maturing wild-type embryo. The shoot apical meristem, located at the bases of the two cotyledons, is flat.
(E) Mature wild-type embryo. This higher magnification micrograph shows the L_1 and L_2 cell layers of the flat meristem. No leaf primordia are detected.
(F) Wild-type seedling. The cotyledon in the foreground was removed from a seedling grown for 3 days to facilitate viewing of the shoot apex. This active postgerminative shoot apical meristem has leaf primordia developing along its flanks.
a, axis; c, cotyledon; p, leaf primordia; s, shoot apex. Bars = 50 μm.

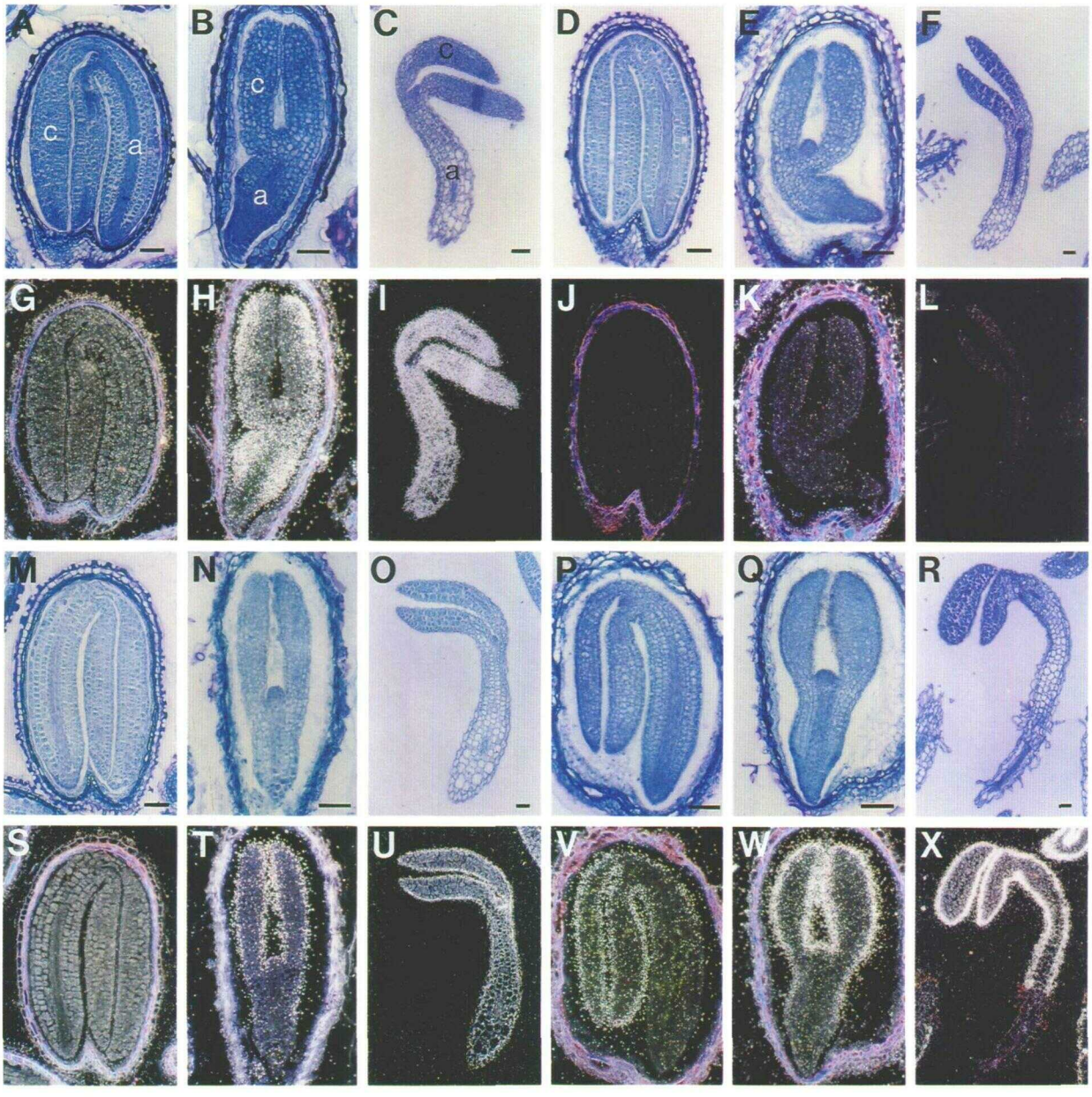

Figure 6. mRNAs Characteristic of Postgerminative Development Accumulate in *lec1-2* Embryos.

Sectioned mutant and wild-type seed and wild-type seedlings grown for 2 days were hybridized with probes for isocitrate lyase or lipid transfer protein mRNAs. Sections were photographed with dark-field optics (**[G]** to **[L]** and **[S]** to **[X]**), stained with toluidine blue, and photographed using bright-field optics (**[A]** to **[F]** and **[M]** to **[R]**).

(A) to **(C)** and **(G)** to **(I)** Isocitrate lyase antisense probe. Slides were exposed to photographic emulsion for 4 days.

(A) and **(G)** Mature, curled cotyledon–stage wild-type embryo.

(B) and **(H)** Mature mutant embryo.

(C) and **(I)** Wild-type seedling grown for 2 days.

(D) to **(F)** and **(J)** to **(L)** Sense-strand control. Slides were exposed to photographic emulsion for 3.5 months.

(D) and **(J)** Mature, curled cotyledon–stage wild-type embryo.

(E) and **(K)** Mature mutant embryo.

(F) and **(L)** Wild-type seedling grown for 2 days.

(M) to **(X)** Lipid transfer protein antisense probe. **(M)** to **(O)** and **(S)** to **(U)**, slides were exposed to photographic emulsion for 6 days; **(P)** to **(R)** and **(V)** to **(X)**, slides were exposed to photographic emulsion for 3.5 months.

(M), **(S)**, **(P)**, and **(V)** Mature, curled cotyledon–stage wild-type embryos.

(N), **(T)**, **(Q)**, and **(W)** Mature mutant embryos.

(O), **(U)**, **(R)**, and **(X)** Wild-type seedling grown for 2 days.

a, axis; c, cotyledon. Bars = 50 μm.

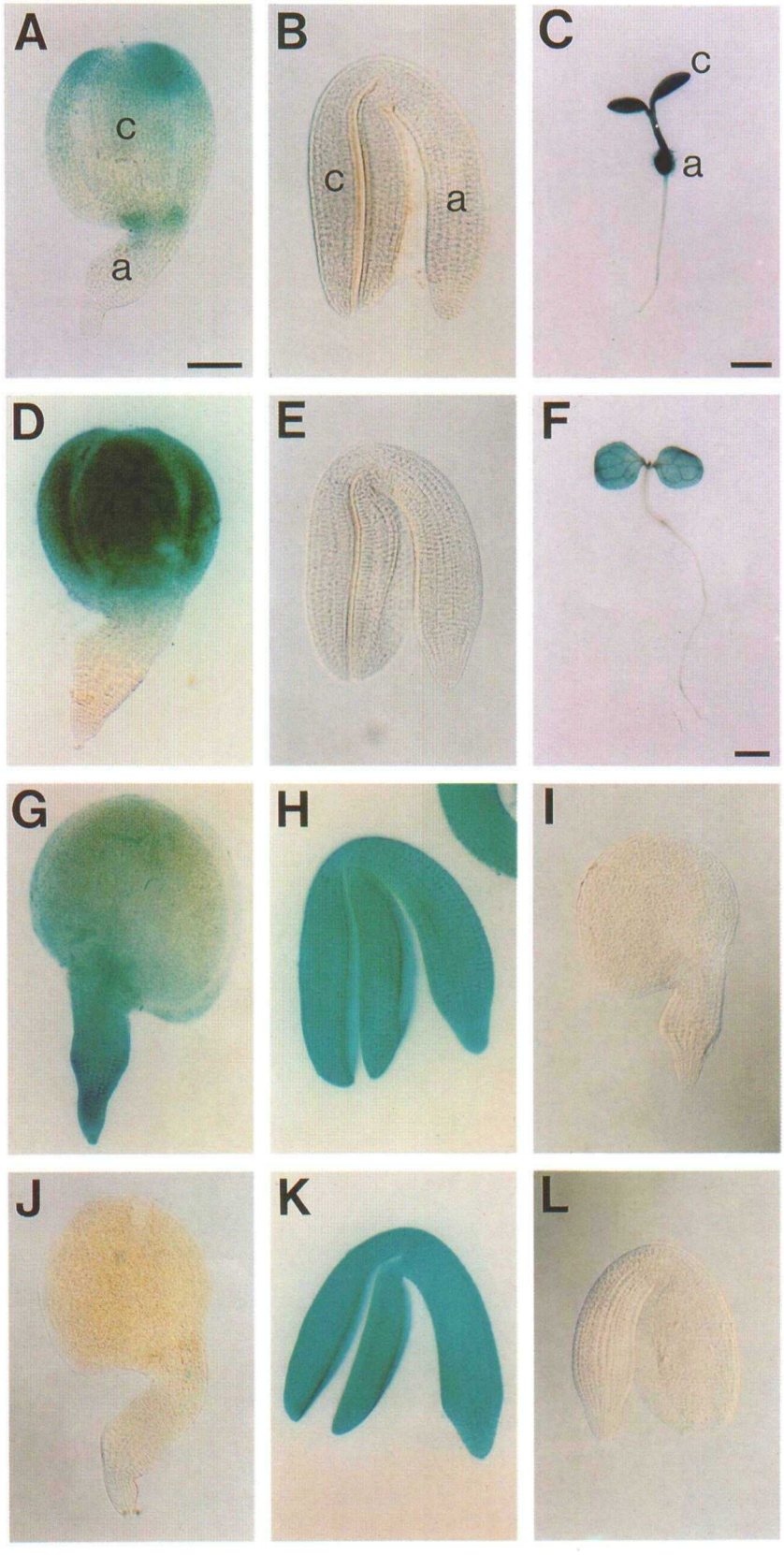

Figure 7. GUS Histochemical Staining of *lec1-2* Embryos Detects the Activity of Promoters Normally Active during Late Embryogenesis and Postgerminative Development.

primordia that resemble the postembryonic shoot apices of wild-type seedlings grown for 2 days (Figure 5F; Medford et al., 1992; Sung et al., 1992; Barton and Poethig, 1993). Thus, the normally quiescent shoot apical meristem is activated in *lec1* embryos, suggesting that postgerminative development has been initiated prematurely. We investigated this hypothesis further by determining whether genes characteristic of postgerminative development are active in the mutant.

We first asked whether isocitrate lyase genes are active in *lec1* embryos. Isocitrate lyase is a key enzyme involved in storage lipid mobilization, which is prevalent during postgerminative growth but accumulates initially at a low level late in embryogenesis and is not detected in nonsenescent leaves in a number of oilseed plants (Comai et al., 1989; Turley and Trelease, 1990). In situ hybridization experiments with embryos and seedlings shown in Figures 6A and 6G and Figures 6C and 6I, respectively, indicate that isocitrate lyase mRNA accumulated similarly in wild-type Arabidopsis. Figures 6B and 6H show that in maturing *lec1-2* embryos, however, the mRNA was prevalent in the hypocotyl region of the axes and at the outer margins and tips of the cotyledons but was either absent or present at low levels in the inner regions of the cotyledons and in the developing vascular tissue, the procambium, and root end of the axes. The specificity of the hybridization reactions is shown by the lack of appreciable binding of a sense-strand probe (Figures 6D to 6F and 6J to 6L). Thus, *lec1* embryos display characteristics of postgerminative seedlings.

A second probe was used to confirm that the timing of postembryonic development is altered in the mutant. The gene encoding a lipid transfer protein that is a homolog of the *EP2* gene from carrot is expressed specifically in the protoderm/epidermis during embryogenesis and postembryonic growth (Sterk et al., 1991). Figures 6O and 6U and Figures 6M and 6S, respectively, show that lipid transfer protein mRNA was prevalent in the epidermis of wild-type seedlings grown for 2 days but was not detected in maturing wild-type embryos when the sections were exposed for 6 days. However, this mRNA was detected in these embryos when the exposure times were increased 18-fold, as shown in Figures 6P and 6V. Unlike wild-type embryos, lipid transfer protein mRNA levels in *lec1* embryos were comparable to those seen in wild-type seedlings (Figures 6N and 6T and Figures 6Q and 6W). Furthermore, the distribution of lipid transfer protein mRNA in *lec1-2* embryos and wild-type embryos and seedlings was similar in that the mRNA was found in the cotyledons and upper axes but was not detected at the root end of the axis (Figures 6P to 6R and 6V to 6X). The lipid transfer protein gene was expressed in *lec1* embryos at a level that is characteristic of seedlings.

Together, our results suggest that some facets of postembryonic development are initiated prematurely in *lec1* embryos. To determine when postgerminative traits can first be observed during *lec1* embryogenesis, we analyzed the temporal expression of reporter genes encoding β-glucuronidase (GUS) that were fused with promoters from an isocitrate lyase or a lipid transfer protein gene and transferred into *lec1-2* plants by genetic crosses (Thoma et al., 1993; Zhang et al., 1994). Figures 7A to 7C and Figures 7D to 7F, respectively, show that the isocitrate lyase and lipid transfer protein fusion genes were expressed similarly with the endogenous genes in wild-type embryos and seedlings and mutant embryos (compare with Figure 6). GUS activity was high in specific regions of mutant embryos and wild-type seedlings but was low or undetectable in maturing wild-type embryos. Wild-type and *lec1* embryos without the fusion genes did not possess detectable GUS activity (Figures 7I and 7L). The regulated activities of these fusion genes are consistent with other results (Thoma et al., 1993; Zhang et al., 1994; J.Z. Zhang, C. Santes, M. Engel, C.S. Gasser, and J.J. Harada, unpublished data). We monitored GUS activities in *lec1-2* embryos at successive embryonic stages and determined that the activities of both promoters could first be detected at the linear cotyledon stage (data not shown). By this criterion, the earliest indication of *LEC1* gene activity during embryogenesis was observed after the cotyledons had formed and elongated.

lec1 Embryos Simultaneously Express Embryonic and Postgerminative Programs of Gene Expression

Our experiments suggest that *LEC1* affects both cotyledon and axis functions because many aspects of postembryonic development are initiated prematurely in both organ systems of mutant embryos. These findings alone do not invalidate the

Figure 7. (continued).

Embryos and seedlings containing GUS fusion genes were stained and photographed, as described in Methods.
(A) to **(C)** Isocitrate lyase promoter.
(D) to **(F)** Lipid transfer protein promoter.
(G) and **(H)** Late embryogenesis–abundant promoter.
(J) and **(K)** β-Conglycinin α′ subunit promoter.
(I) and **(L)** Control embryos not containing a GUS construct.
(A), **(D)**, **(G)**, **(J)**, and **(I)** Mature mutant embryos.
(B), **(E)**, **(H)**, **(K)**, and **(L)** Mature, curled cotyledon–stage wild-type embryos.
(C) and **(F)** Wild-type seedlings grown for 2 days.
a, axis; c, cotyledon. Bars = 100 μm; bar in **(A)** applies to all panels except **(C)** and **(F)**.

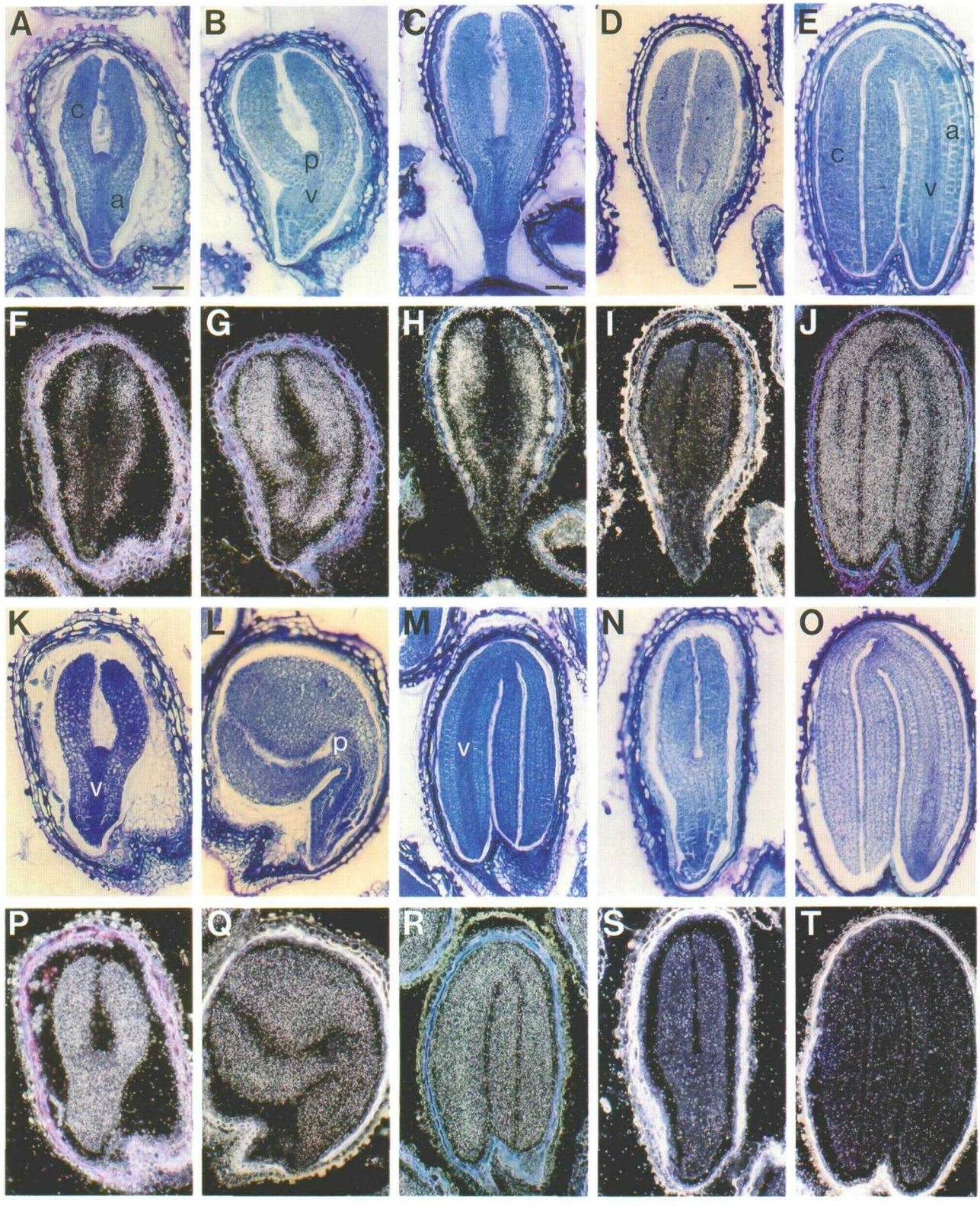

Figure 8. mRNAs Characteristic of Embryonic Development Accumulate in *lec1-2* Embryos.

hypothesis that *LEC1* specifies cotyledon identity because *lec1* embryos may not make a factor, normally produced in cotyledons, that suppresses postembryonic development in the embryonic axis. To determine whether the mutation causes the complete homeotic conversion of *lec1* embryonic cotyledons to leaves and therefore abolishes cotyledon-specific processes, we asked whether genes specifically expressed during late embryogenesis are active in *lec1* cotyledons.

Analyses of two mRNAs that normally accumulate primarily during the maturation phase of embryogenesis, those encoding the A isoform of the 12S storage protein cruciferin and the oil body membrane protein oleosin provided several clues about the effect of the *lec1* mutation on late embryogenesis (Pang et al., 1988; Huang, 1992). First, as shown in Figures 8A to 8C and 8F to 8H and Figures 8K, 8L, 8P, and 8Q, respectively, cruciferin and oleosin mRNAs were present in both the axes and cotyledons of *lec1-2* embryos, showing that both organ systems are competent to express these maturation stage–specific genes. Surprisingly, cells in the cotyledons and the hypocotyl region of the axes that accumulate these embryogenesis-specific mRNAs also express isocitrate lyase genes at a level characteristic of seedlings. The concurrent expression of embryonic and postgerminative programs in a single cell has not been previously described (Bisgrove et al., 1994). Second, the temporal and spatial modulations in cruciferin mRNA levels observed in the sequentially staged mutant embryos in Figures 8A to 8D and 8F to 8I are similar to those observed in wild-type embryos. As with other storage protein mRNAs in wild-type embryos, cruciferin mRNA levels increase then decrease during *lec1* embryogenesis, and the mRNA is present initially in the outer face of cotyledons before becoming prevalent in the inner region (Pang et al., 1988; Perez-Grau and Goldberg, 1989; Fernandez et al., 1991). Third, the tissue specificity of mRNA accumulation was maintained in mutant embryos. In both *lec1-2* and wild-type embryos, cruciferin mRNA was not detected in the procambium (Figures 8A to 8C and 8F to 8H and Figures 8E and 8J, respectively), whereas oleosin mRNA was present in this tissue system (Figures 8K, 8L, 8P, and 8Q and Figures 8M and 8R, respectively). The only obvious difference between mutant and wild-type embryos is that both maturation stage mRNAs did not accumulate in the extreme root end of *lec1-2* embryos and in leaf primordia. The vivipary observed in some *lec1-2* embryos and the absence of the mRNAs in their embryonic roots may indicate that, similar to shoot apices, root apical meristems are active prematurely. Together, the results show that gene expression programs that are normally temporally distinct occur simultaneously in *lec1* embryos.

To study the effects of the mutation on the desiccation phase of embryogenesis, we used a fusion gene consisting of the promoter from the late embryogenesis–abundant gene *DC-8* and the GUS reporter gene (Goupil et al., 1992). As shown in Figures 7G and 7H, respectively, GUS activity was detected in both *lec1-2* and wild-type maturing embryos. The staining of mutant cotyledons appeared less intense than wild type, although this difference may reflect the decreased thickness of the cotyledon when viewed in this orientation. To compare the timing of fusion gene activation in mutant and wild-type embryos, we constructed plants homozygous for the GUS fusion gene and heterozygous for *lec1-2*. Segregating wild-type and *lec1* embryos were obtained from consecutive siliques along an inflorescence to monitor GUS activities at successive embryonic stages. We showed, in all inflorescences tested, that the late embryogenesis–abundant promoter becomes active at identical embryonic stages in both wild-type and mutant embryos, suggesting that this aspect of the desiccation program is regulated correctly in *lec1* embryos (data not shown).

Whereas our results showed that several genes characteristically expressed during the maturation and desiccation phases are active in *lec1* embryos, we also showed that there are defects in some aspects of late embryogenesis. A GUS fusion gene driven by the promoter from the gene encoding the α′

Figure 8. (continued).

Sectioned mutant and wild-type seed were hybridized with probes for cruciferin and oleosin mRNAs, as described in Methods. Sections were photographed using dark-field optics (**[F]** to **[J]** and **[P]** to **[T]**), stained with toluidine blue, and photographed using bright-field optics (**[A]** to **[E]** and **[K]** to **[O]**).

(A) to **(J)** Cruciferin antisense probe. Slides were exposed to photographic emulsion for 2 days. Mutant embryos in **(A)** to **(D)** and **(F)** to **(I)** are arranged in a developmental progression, from youngest to oldest.

(A) and **(F)** Immature mutant embryo.

(B) and **(G)** Mature, curled cotyledon–stage mutant embryo.

(C) and **(H)** Viviparous mutant embryo containing anthocyanin.

(D) and **(I)** Viviparous mutant embryo with anthocyanin and beginning to desiccate.

(E) and **(J)** Mature, curled cotyledon–stage wild-type embryo equivalent in age to the mutant embryo in **(B)** and **(G)**.

(K) to **(M)** and **(P)** to **(R)** Oleosin antisense probe. Slides were exposed to photographic emulsion for 40 days.

(K), **(L)**, **(P)**, and **(Q)** Mature mutant embryos.

(M) and **(R)** Mature, curled cotyledon–stage wild-type embryo.

(N), **(O)**, **(S)**, and **(T)** Sense-strand control. Slides were exposed to photographic emulsion for 40 days.

(N) and **(S)** Mature mutant embryo.

(O) and **(T)** Mature, curled cotyledon–stage wild-type embryo.

a, axis; c, cotyledon; p, leaf primordia; v, developing vascular tissue. Bars = 50 μm; Bar in **(A)** applies to all panels except **(C)**, **(D)**, **(H)**, and **(I)**.

subunit of the 7S storage protein β-conglycinin was transferred into a *lec1-2* plant by crossing, and progeny from this plant were stained for GUS activity (Hirai et al., 1994). As shown in Figure 7J, mutant segregants did not stain in spite of extensive incubation with the substrate even though GUS activity was high in wild-type segregants (Figure 7K). Other experiments established that the fusion gene was present in the *lec1-2* embryos (data not shown). We conclude that the mutation adversely affects the expression of at least some genes normally expressed during the maturation stage.

Our findings that *lec1* cotyledons possess trichomes yet express genes characteristic of late embryogenesis suggest that these cotyledons have been partially converted rather than completely transformed into leaves. Comparison of the anatomy of postgerminative *lec1-2* cotyledons with the cotyledons and primary leaves of wild-type plants is consistent with this conclusion. Mesophyll cell shape in mutant postgerminative cotyledons, shown in Figure 9C, more closely resemble those of the primary leaves (Figure 9B) than the cotyledons (Figure 9A) of wild-type plants. However, mutant cotyledons lack the organized cellular arrangement that is characteristic of leaves, particularly in the palisade layer underlying the adaxial epidermis. The results suggest that the cellular organization of *lec1* cotyledons is intermediate between that of wild-type cotyledons and leaves.

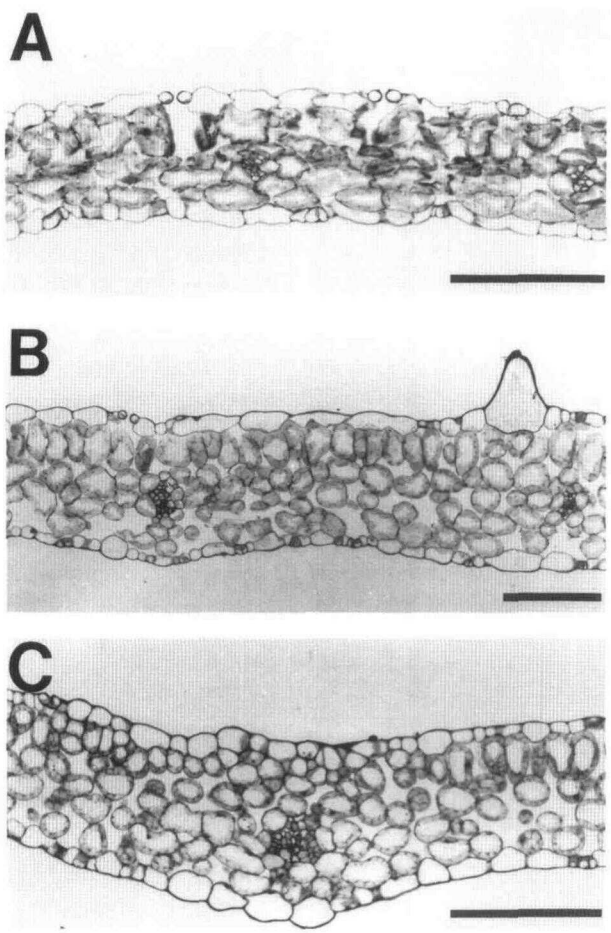

Figure 9. Cellular Organization of *lec1-2* Cotyledons Is Intermediate between That of Wild-Type Leaves and Cotyledons.

Light micrographs of transverse sections through wild-type and mutant cotyledons from seedlings grown for 4 days and from primary leaves of wild-type seedlings grown for 10 days.

(A) Wild-type cotyledon. There is no indication of organization of mesophyll cells into a palisade cell layer.

(B) Wild-type leaf. Elongated palisade cells are organized into a characteristic layer beneath the epidermis. A trichome precursor is visible developing from an epidermal cell.

(C) Mutant cotyledon. The mesophyll cells appear to be organized into a palisade cell layer in parts of but not throughout the cotyledon.

Bars = 100 μm.

Genetic Interactions between *LEC1* and *FUS3*

fus3 is another *embryo defective* mutation with effects similar to those of *lec1*. Both mutants are desiccation intolerant and sometimes viviparous, often accumulate anthocyanin throughout the cotyledons (particularly at low temperature), possess trichomes on embryonic cotyledons and an activated shoot apical meristem, and are sensitive to ABA (Baumlein et al., 1994; Keith et al., 1994). As shown in Figures 10A and 10C, the primary morphological differences between the two mutants are that *lec1* embryonic axes are significantly shorter than those of *fus3*, and *fus3* cotyledons are generally bent over the axis unlike *lec1* embryos. To investigate the relationship between the two genes, we constructed plants homozygous for both mutations by crossing homozygous *lec1-2* and *fus3-3* plants and by identifying double mutants among rescued F_2 progeny through genetic crosses with each parental line. As represented in Figure 10B, *lec1-2 fus3-3* embryos from three double mutant plants exhibit a consistent phenotype. Although they resemble *lec1* embryos at a gross morphological level, closer examination shows that the double mutants are smaller than either single mutant at the same embryonic stage, their axes are intermediate in size between the two parents, and they accumulate much less anthocyanin under low-temperature conditions (Figure 10B). Because the two mutations do not display epistasis, the simplest interpretation is that the *LEC1* and *FUS3* genes participate in distinct regulatory pathways.

DISCUSSION

The *lec1* Mutation Affects Cotyledon Identity and the Timing of Postgerminative Development

The *lec1* mutation profoundly affects many aspects of late embryogenesis and postgerminative development. The pleiotropic

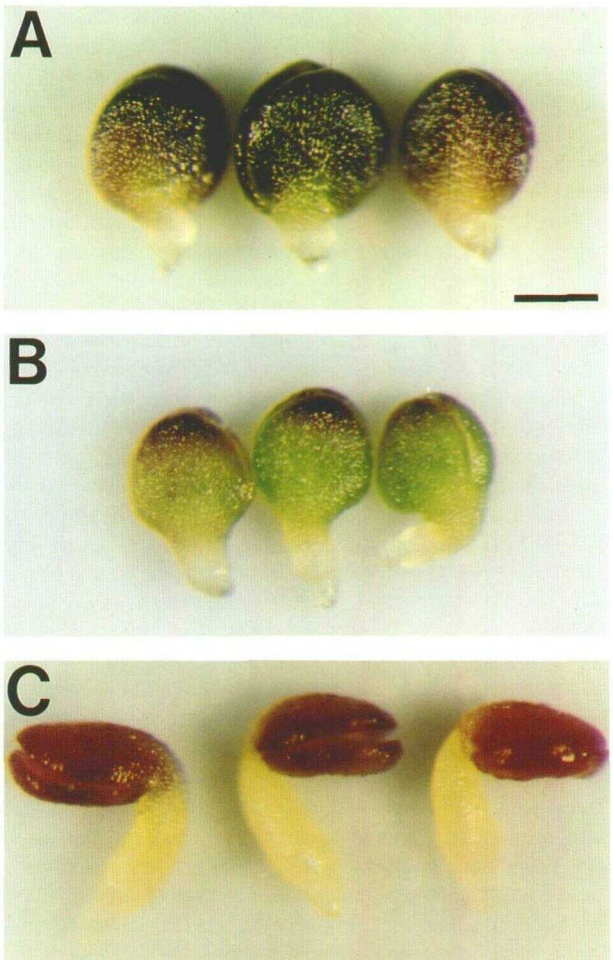

Figure 10. Lack of Epistasis between *LEC1* and *FUS3*.

Embryos at identical developmental stages were dissected from plants grown at 18°C to accentuate anthocyanin accumulation.
(A) *lec1-2* embryos. The embryonic axis is very short and clear. Anthocyanin accumulates throughout the cotyledons.
(B) *lec1-2 fus3-3* double mutant embryos. Although these embryos are shaped similarly to *lec1-2* embryos, their axes are intermediate in length to *lec1-2* and *fus3-3*. Anthocyanin accumulates only in the tips of the cotyledons.
(C) *fus3-3* embryos. The axis is similar in length to a wild-type axis. Anthocyanin accumulates throughout the cotyledons.
Bar = 200 μm.

effects of the mutation strongly suggest a regulatory role for *LEC1* in late embryogenesis and implicate two possible interpretations of the mutation.

One explanation is that *lec1* is a homeotic mutation that converts cotyledons to leaves. Homeosis, defined broadly as the assumption by one part of an organism of the characteristics from another, occurs frequently in plants and other organisms, and the analysis of homeotic mutations has provided significant insights into the mechanisms that underlie many developmental processes (Sattler, 1988; Coen and Meyerowitz, 1991; St Johnston and Nüsslein-Volhard, 1992). Although the presence of trichomes on cotyledons (Figures 3 and 4) and the absence of protein bodies in embryonic cotyledons provide support for the hypothesis that mutant cotyledons are completely replaced by leaves, several aspects of the Lec1$^-$ phenotype are inconsistent with this premise (Meinke, 1992; Meinke et al., 1994). First, *lec1* embryonic cotyledons do not possess stipules that are normally associated with leaves in Arabidopsis (Figure 5). Second, isocitrate lyase genes that are not expressed at significant levels in nonsenescent leaves of wild-type plants are active in mutant cotyledons (Figures 6 and 7). Third, genes expressed exclusively in embryos and not in leaves of wild-type plants, specifically those encoding cruciferin, oleosin, and a late embryogenesis–abundant protein, are active in *lec1* embryonic cotyledons (Figures 7 and 8). Although *lec1* cotyledons have acquired some leaf traits, they maintain many facets of cotyledon identity. We note that the ectopic induction of trichomes on cotyledons has been observed in transgenic plants that constitutively express *GLABROUS1*, a gene required for trichome initiation (Larkin et al., 1994).

These aspects of the phenotype and others described in the following discussion also suggest that postembryonic development has been initiated prematurely in *lec1* embryos. Specifically, the shoot apical meristems and, perhaps, the root apical meristems of mutant embryos resemble these structures in postembryonic plants, indicating that they have been activated precociously (Figures 5 and 8). Additionally, the activities of isocitrate lyase and lipid transfer protein genes in both axes and cotyledons of *lec1* embryos are more characteristic of postgerminative seedlings than embryos of wild-type plants (Figures 6 and 7). Thus, an alternative interpretation is that *lec1* is a heterochronic mutation. By contrast to homeosis, which implies a change in the location of a developmental process, heterochrony implicates variations in the rate of development (Lord and Hill, 1987; Poethig, 1990; Conway and Poethig, 1993). The simultaneous expression of embryonic and postgerminative development in mutant embryos is consistent with the behavior of other heterochronic mutations (Poethig, 1990). The *fus3* mutation has also been described as heterochronic (Keith et al., 1994).

On the other hand, two lines of reasoning argue against a heterochronic interpretation of *lec1*. First, trichomes do not normally form at any stage of cotyledon development, and therefore, an acceleration of postembryonic development would not be predicted to induce trichome formation (Meinke et al., 1994). This argument is based on the assumption that cotyledons and leaves, which are heteroblastic forms, cannot be interconverted. However, others have shown that the fate of leaf primordia at the shoot apex of *Brassica napus* embryos could be altered by precociously germinating embryos at different developmental stages (Finkelstein and Crouch, 1984). For example, the leaf primordia develop into leaves with characteristic trichomes when mature seeds are germinated in culture. By contrast, precocious germination of immature embryos

converts these leaf primordia into cotyledon-like organs that lack trichomes and that synthesize storage proteins. Although it is unclear whether cotyledon fate can be altered similarly by the genetic induction of precocious germination, the results suggest that leaf identity may be determined after primordium formation and may be influenced by physiological factors. In this regard, we note that the first detectable indication of postembryonic development in *lec1* embryos is observed after cotyledons have been initiated.

The phenotype of another *LEC* mutation, *lec2*, has been used as a second argument against the involvement of heterochrony in the mutant phenotype (Meinke et al., 1994). Precociously germinated *lec2* embryos produce trichomes on their cotyledons, although mutant embryos are desiccation tolerant, and they accumulate some storage proteins in cotyledons (Meinke et al., 1994). The desiccation tolerance of *lec2* embryos has been interpreted to indicate that defects in late embryogenesis are not a prerequisite for trichome formation on cotyledons. However, additional characterization of the embryonic phenotype of *lec2* mutants is necessary before this conclusion can be validated. For example, the *lec2-1* mutation appears to have subtle effects on late embryonic development (Meinke et al., 1994). Furthermore, it is not clear that the *lec2-1* mutant represents a severe allele of the gene. Leaky mutations of *ABI3* are desiccation tolerant, whereas severe alleles are intolerant (Koornneef et al., 1989; Nambara et al., 1992; Ooms et al., 1993).

The Lec1$^-$ phenotype is probably best represented as a partial conversion of cotyledons to leaves. The simultaneous expression of leaf characters and cotyledon-specific traits in *lec1* embryonic cotyledons and the intermediate cellular organization of *lec1* cotyledons provide support for this conclusion (Figures 3, 4, 7, 8, and 9). The chimeric nature of the phenotype makes it difficult to assign *lec1* definitively to either class of mutation. Homeosis and heterochrony are often difficult to distinguish, particularly in plants, because of their continuous and polar mode of development (Sattler, 1988; Poethig, 1990). Moreover, these two types of phenomena are not mutually exclusive, and some phenotypes may be classified as examples of both (Sattler, 1988). Although the available data do not distinguish between these two possibilities, as described in the following discussion, they do provide insight into the mechanistic role of the *LEC1* gene in controlling late embryogenesis.

Control of Late Embryogenesis

Our data indicate that many aspects of the maturation and desiccation phases are not affected by the *lec1* mutation. The temporal and spatial expression of the cruciferin, oleosin, and late embryogenesis–abundant genes are similar in both mutant and wild-type embryos (Figures 7 and 8). However, the intolerance of the mutant to desiccation, the lack of detectable activity of the β-conglycinin α′ promoter, and the absence of protein bodies in *lec1* cotyledons clearly indicate that there are defects in key facets of these phases (Figure 7; Meinke, 1992). In this regard, the altered ratios of specific storage proteins could account for protein body defects, as has been observed in a maize mutant (Shotwell and Larkins, 1989). Together, the results show that the *lec1* mutation affects the initiation and/or maintenance of some late embryonic programs.

The simultaneous expression of genes characteristic of both embryonic and postgerminative development in a single cell is an exceptional characteristic and emphasizes the regulatory nature of the mutation (Bisgrove et al., 1994). Although embryonic and postgerminative development occur concurrently in some precociously germinating embryos, both programs have not been shown to occur in the same cell (Finkelstein and Crouch, 1984; Bisgrove et al., 1994; Jakobsen et al., 1994). Thus, an obvious consequence of the mutation is that postgerminative development is initiated prematurely in *lec1* embryos. Germination is normally actively suppressed during embryogenesis, and ABA has been implicated as an endogenous inhibitor of this process. For example, ABA inhibits the precocious germination of excised embryos, viviparous mutants have lower levels of or lower sensitivity to ABA, and application of ABA synthesis inhibitors to seed can cause vivipary (Quatrano, 1986; Crouch, 1987; Galau et al., 1991). However, *lec1* mutants do not appear to be defective in ABA synthesis or perception. Homozygous recessive vegetative plants are not susceptible to wilting (as are mutants defective in ABA synthesis), an ABA-inducible late embryogenesis–abundant promoter is correctly regulated in *lec1* embryos, and mutant embryos remain sensitive to ABA (Figure 2; data not shown). Furthermore, *abi3* and *lec1* mutants differ in that *abi3* embryos do not accumulate anthocyanin and rescued seedlings do not possess trichomes on their cotyledons (Koornneef et al., 1989; Nambara et al., 1992; Ooms et al., 1993). These findings, coupled with the recent report that *lec1 abi3* double mutants do not display epistasis, suggest that *LEC1* and *ABI3* participate in distinct regulatory pathways (Meinke et al., 1994). Thus, multiple pathways are likely to be required to inhibit the premature germination of embryos.

Why do mutations in the *LEC1*, *FUS3*, and *ABI3* genes cause similar defects in late embryogenesis? A working hypothesis is that these genes act primarily to initiate or maintain distinct aspects of the maturation phase, as suggested by the finding that the three mutations each inactivate different maturation phase–specific genes. Specifically, the β-conglycinin α′ promoter, which is inoperative in *lec1* embryos, is active in *fus3* and *abi3* mutants, whereas mRNA encoding the cruciferin A isoform accumulates in *lec1* and *fus3* mutants but is not detected in *abi3* embryos (Figure 7; Nambara et al., 1995; S. Naito, personal communication). These results suggest that the maturation phase is regulated in a combinatorial fashion. Several independent regulatory pathways, minimally defined as those regulated by *LEC1*, *FUS3*, and *ABI3*, may operate concurrently to regulate different sets of genes required for embryo maturation. This thesis is consistent with the findings that genes expressed during the maturation phase are regulated by different *cis*-acting DNA sequences and *trans*-acting factors (Thomas, 1993). Furthermore, the *ABI3* gene product has been implicated as a regulatory protein because it is homologous

to the maize transcription factor VP1 (McCarty et al., 1991; Giraudat et al., 1992). The failure to maintain all aspects of the maturation and desiccation phases may be sufficient to induce premature germination. Additionally, *LEC1* and *FUS3* may be required to suppress trichome formation and anthocyanin production, whereas *ABI3* is required to establish responsiveness to ABA. Understanding the functions of these genes and identifying other genes in these regulatory pathways are essential to understanding the control of late embryogenesis.

METHODS

Plant Material and Propagation

The *LEAFY COTYLEDON1-2* (*lec1-2*) mutant was identified from a population of *Arabidopsis thaliana* ecotype Wassilewskija (Ws-O) lines mutagenized with T-DNA insertions that were generated by Ken Feldmann, University of Arizona (Feldmann and Marks, 1987; Feldmann, 1991). Plants were grown in soil under constant light at 22°C. *aba insensitive 3-3* (*abi3-3*) and *fusca 3-3* (*fus3-3*) lines and *lec1-1* lines were generously provided by Peter McCourt, University of Toronto, and David Meinke, Oklahoma State University (Meinke, 1992; Nambara et al., 1992; Keith et al., 1994). Arabidopsis lines containing GUS fusion genes regulated by promoters from the genes encoding the α′ subunit of β-conglycinin (Hirai et al., 1994), the late embryogenesis–abundant protein DC-8 (Goupil et al., 1992), a lipid transfer protein (Thoma et al., 1993), or isocitrate lyase (Zhang et al., 1994) were kindly provided by Satoshi Naito (Hokkaido University), Renee Sung (University of California, Berkeley), Chris Somerville (Carnegie Institution of Washington, Stanford, CA), and the Harada laboratory.

Homozygous *lec1-2* plants were generated by germinating on basal media either intact seed or dissected embryos obtained from mature green siliques before they became desiccated (Olsen et al., 1993). To enhance anthocyanin production, plants were grown at 18°C under constant lights. Growth under cycling lights or at 18°C had no effect on the mutant phenotype, other than the enhancement of anthocyanin accumulation at low temperature.

Genetic Mapping

A homozygous *lec1-2* plant was crossed with the multiply marked line W-100 obtained from the Arabidopsis Biological Resource Center. Individual F_2 plants were scored for the segregation of *lec1* progeny and visible morphological markers. Only 9% of the plants homozygous for *angustifolia* were also heterozygous for *lec1-2* (14/153), in contrast to the 66% frequency expected for the independent assortment. We conclude that *LEC1-2* is linked with *angustifolia* on chromosome 1.

Germination Assay

Rescued immature seeds (*lec1-2* and *abi3-3*) were placed on 0.5% agar plates containing 0, 1, 10, or 50 μM (±)-*cis*,*trans*-ABA (Sigma) and incubated at 22°C in a growth chamber with 16-hr light/8-hr dark cycles. Dried wild-type (Ws-O) seed were surface sterilized, plated as described in a previous section, and vernalized for 2 days at 4°C prior to incubation in the growth chamber. After 2 weeks, seedlings were scored for germination using the emergence of seedlings from the seed coats, radical elongation, and cotyledon expansion as the criteria. Cotyledon expansion was scored to differentiate between germinating seedlings and viviparous seed.

GUS Histochemical Assays

To construct mutant plants containing GUS fusion genes, flowers from homozygous *lec1-2* plants were emasculated, dusted with pollen from plants homozygous for the GUS fusion gene, and covered with plastic wrap. The next day, the stigmas were pollinated again using the same donor lines. F_3 progeny from F_2 plants heterozygous for *lec1-2* were stained for GUS activity to identify plants homozygous for the GUS fusion genes.

GUS histochemical assays were performed as described previously by Dietrich et al. (1992). Embryos and seedlings containing GUS fusion genes with the following promoters were stained for GUS activity at 37°C for the indicated times: the isocitrate lyase and the lipid transfer protein promoters, overnight; the late embryogenesis–abundant promoter, 1.5 hr; the β-conglycinin α′ promoter, wild-type embryos, 5 hr; *lec1-2* embryos, 72 hr. Embryos were destained overnight in 70% ethanol and processed for microscopy using Nomarski optics as described in the following section. Stained seedlings were photographed directly after destaining using a Zeiss SV11 stereomicroscope (Carl Zeiss, Inc., Thornwood, NY).

Light Microscopy

For whole mount analyses, embryos, seed, and seedlings were dehydrated in ethanol, cleared in methyl salicylate, and photographed with a Nikon Optiphot 2 microscope (Nikon, Inc., Melville, NY) using Nomarski optics.

To investigate leaf and cotyledon anatomy, wild-type first leaves were collected from seedlings 10 days after germination, and mutant and wild-type cotyledons were collected from seedlings 4 days after germination. Cut sections of the organs were fixed in 2% paraformaldehyde, 1.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, for 3 hr at room temperature, then rinsed in 0.1 M sodium cacodylate buffer, pH 7.2, four times for 15 min each (Karnovsky, 1965). The tissue was then dehydrated in a series of increasing concentrations of ethanol and embedded in Historesin (Reichert-Jung, Leica Instruments GmbH, Heidelberg, Germany) per the manufacturer's directions. Two-micrometer sections were taken with glass knives, laid on microscope slides, and stained in 0.1% toluidine blue. Photographs were taken with an Olympus Vanox microscope (Olympus America, Inc., Lake Success, NY).

In Situ Hybridization

Experiments were performed as described previously by Dietrich et al. (1989). Sections hybridized with antisense probes for the A isoform of cruciferin (Pang et al., 1988), isocitrate lyase (Comai et al., 1989), oleosin (M. Murase and J.J. Harada, unpublished data), and lipid transfer protein (kindly provided by Sacco de Vries, Agricultural University, Wageningen, The Netherlands) were exposed to photographic emulsion for 1 to 2 days, 3 to 4 days, 40 days, and 6 days or 3.5 months, respectively. As a negative control, a sense-strand probe was prepared,

reacted with tissue sections, and exposed to photographic emulsion for the same length of time as each of the experimental probes. In all cases, no signal above background was detected using the sense-strand probe.

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