The Plant Cell, Vol. 6, 1049–1064, August 1994 © 1994 American Society of Plant Physiologists

Leafy Cotyledon Mutants of Arabidopsis

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We have previously described a homeotic *leafy cotyledon* (*lec*) mutant of Arabidopsis that exhibits striking defects in embryonic maturation and produces viviparous embryos with cotyledons that are partially transformed into leaves. In this study, we present further details on the developmental anatomy of mutant embryos, characterize their response to abscisic acid (ABA) in culture, describe other mutants with related phenotypes, and summarize studies with double mutants. Our results indicate that immature embryos precociously enter a germination pathway after the torpedo stage of development and then acquire characteristics normally restricted to vegetative parts of the plant. In contrast to other viviparous mutants of maize (*vp1*) and Arabidopsis (*abi3*) that produce ABA-insensitive embryos, immature *lec* embryos are sensitive to ABA in culture. ABA is therefore necessary but not sufficient for embryonic maturation in Arabidopsis. Three other mutants that produce trichomes on cotyledons following precocious germination in culture are described. One mutant is allelic to *lec1*, another is a *fusca* mutant (*fus3*), and the third defines a new locus (*lec2*). Mutant embryos differ in morphology, desiccation tolerance, pattern of anthocyanin accumulation, presence of storage materials, size and frequency of trichomes on cotyledons, and timing of precocious germination in culture. The leafy cotyledon phenotype has therefore allowed the identification of an important network of regulatory genes with overlapping functions during embryonic maturation in Arabidopsis.

INTRODUCTION

Three critical developmental programs are elaborated during the final stages of plant embryogenesis: preparation for seed desiccation, inhibition of precocious germination, and establishment of dormancy. Although certain features of late embryogenesis have been described in detail, including changes in pigmentation, water content, desiccation tolerance, abscisic acid (ABA) concentration, mRNA complexity, and storage product accumulation, integration of these overlapping developmental events remains to be explained at the molecular level. Physiological studies alone have been insufficient to dissect the complex network of signals involved. The regulation of plant embryogenesis has been approached in part through the isolation and characterization of embryo-defective mutants (Meinke, 1991). Several types of defects in late embryogenesis have been described: (1) mutants defective primarily in the accumulation of storage products; (2) mutants with normal seed morphology but altered pigmentation; (3) mutants with altered seed morphology but normal maturation programs; and (4) mutants with fundamental defects in the regulation of late embryogenesis. The leafy cotyledon mutants described in this report represent an important addition to this final class.

The viviparous (*vp*) mutants of maize have long been used to study the genetic control of late embryogenesis in monocots because their novel phenotype, precocious germination of mutant embryos on heterozygous ears, is consistent with the loss of a regulatory factor that inhibits germination during seed development (Robertson, 1955). Emphasis has been placed on *vp1* mutant embryos, which contain normal levels of ABA but exhibit reduced sensitivity to exogenous ABA (Robichaud and Sussex, 1986). The defect in other viviparous mutants is limited to ABA or carotenoid biosynthesis (Neill et al., 1986). The *VP1* gene has been cloned by transposon tagging (McCarty et al., 1991) and shown to encode a protein that may play an important role in the regulation of transcription (Hattori et al., 1992).

The genetic control of late embryogenesis in Arabidopsis has been examined primarily through the isolation and characterization of ABA-insensitive (*abi3*) and ABA-deficient (*aba*) mutants (Koornneef et al., 1989; Rock and Zeevaart, 1991). Three *abi* loci were originally identified by screening for mutant seeds that germinated in the presence of ABA (Koornneef et al., 1984). The *abi1* and *abi2* mutations disrupt vegetative development and cause wilting in response to water stress; the defects in *abi3* are limited to seed development. Mutant *abi3* seeds contain reduced levels of storage products (Finkelstein and Somerville, 1990) but otherwise appear normal. This intermediate phenotype may be attributed to the leaky nature of the *abi3-1* mutation. Additional defects in seed development appear when double mutants (*abi3 aba*) are constructed that alter both ABA content and ABA response (Koornneef et al., 1989; Meurs et al., 1992). Similar defects were found when severe *abi3* mutant alleles were examined (Nambara et al., 1992; Ooms et al., 1993). These putative null alleles were

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identified either by germination on media containing Uniconazol, an inhibitor of gibberellin biosynthesis (Nambara et al., 1992), or by scoring for mutant seeds that remained green late in development and germinated immediately after harvest (Ooms et al., 1993). None of these *abi3* mutants produce leafy cotyledons or viviparous embryos that germinate within the silique. The *ABI3* gene has recently been isolated through map-based cloning and shown to encode a protein with regions of sequence similarity to the *VP1* gene of maize (Giraudat et al., 1992). *ABI3* therefore appears to play a central role in modulating ABA response and regulating gene expression during embryo development in Arabidopsis.

Several *fusca* (*FUS*) genes of Arabidopsis also appear to serve important regulatory functions at this stage of the life cycle (Castle and Meinke, 1994). Recessive *fusca* mutants exhibit inappropriate accumulation of anthocyanin in developing cotyledons of mutant seeds (Müller, 1963). Most of these seeds germinate but fail to develop beyond the seedling stage (Castle and Meinke, 1994). At least 12 complementation groups of *fusca* mutations have been defined by Miséra et al. (1994). Several mutants described previously as defective in light response pathways (e.g., the *constitutive photomorphogenic* mutant *cop1* and the *deetiolated* mutant *det1*) have a *fusca* phenotype as well (Castle and Meinke, 1994). Several tagged *fusca* alleles have been identified among transgenic populations produced by Agrobacterium-mediated seed transformation (Feldmann, 1991; Castle et al., 1993). Three *FUSCA* genes have now been cloned and sequenced: *FUS1/COP1* (Deng et al., 1992), *FUS2/DET1* (Pepper et al., 1994), and *FUS6* (Castle and Meinke, 1994). These genes appear to perform distinct but overlapping functions in transduction of developmental and environmental signals during embryogenesis (Castle and Meinke, 1994).

Two *fusca* mutations (*fus3* and *fus10*) alter seed morphology in addition to promoting anthocyanin accumulation (Miséra et al., 1994). These two mutations do not interfere with vegetative development if immature embryos are germinated before desiccation. Two *fus3* alleles isolated in different laboratories have recently been examined in detail (Bäumlein et al., 1994; Keith et al., 1994). Homozygous mutant seeds are desiccation intolerant, occasionally viviparous, lack storage materials, and germinate precociously in culture. These features support the conclusion that *FUS3* plays an important role in regulating several maturation programs during plant embryogenesis.

The *leafy cotyledon* (*lec1*) mutant of Arabidopsis exhibits the most dramatic alteration in late embryogenesis (Meinke, 1992). Mutant embryos have a reduced hypocotyl and rounded cotyledons that remain green unusually late in development. Mutant embryos also lack the protein and lipid bodies characteristic of wild-type embryos. Mutant seeds are deformed, occasionally viviparous with a protruding root inside the silique, and desiccation intolerant at maturity. Immature embryos rescued in culture produce viable plants that appear normal except for the presence of leafy cotyledons with trichomes on their adaxial surface. Based on the severity of this mutant phenotype, we proposed that *LEC1* is required for activation of a wide range of embryo-specific programs and plays a more global function than either *VP1* or *ABI3*. According to this model, mutant embryos germinate precociously because they fail to activate programs that normally inhibit germination during seed development. Mutant cotyledons then revert to a basal (leaflike) developmental state. This view is consistent with the origin of cotyledons as modified leaves during plant evolution. According to our initial model, mutants of Arabidopsis with trichomes on cotyledons were not identified previously by screening at the seedling stage because inhibition of trichome formation and acquisition of desiccation tolerance were viewed as coupled processes. Any mutation that allowed trichome formation on cotyledons was therefore thought to result in desiccation intolerance at maturity.

In this report, we demonstrate that the leafy cotyledon phenotype is not limited to a single mutation. Two additional mutants with related phenotypes (*lec2* and *fus3*) and a second allele of *lec1* are described. One mutant (*lec2*) produces desiccation-tolerant seeds that germinate at maturity. This mutation demonstrates that our initial model concerning trichome formation was incorrect; desiccation-tolerant seeds with a leafy cotyledon phenotype can be recovered. The surprising feature of *lec2* is that trichomes on cotyledons are found only when immature embryos are germinated before desiccation. Seedlings grown from dry seeds no longer display trichomes on their cotyledons, even though these trichomes were initiated during embryogenesis. Apparently, these cells are not desiccation tolerant and therefore are lost following germination of dry seeds. We also describe the developmental anatomy of mutant embryos, the ABA sensitivity of immature embryos in culture, and the phenotypes of double mutants, all of which support our initial model that the leafy cotyledon phenotype results from a fundamental defect in plant embryogenesis that is not limited to ABA signal transduction pathways. We have therefore identified a network of three related genes (*LEC1*, *LEC2*, and *FUS3*) that together with *ABI3* play critical roles in the regulation of late embryogenesis in Arabidopsis.

RESULTS

Developmental Anatomy of *lec1* Embryos

The developmental anatomy of *lec1* embryos is illustrated in Figure 1. Analysis of early embryos was facilitated by the availability of homozygous mutant plants produced from seedlings rescued in culture. Mutant seeds were indistinguishable from wild-type controls early in development. No defects were seen in suspensor formation, cotyledon initiation, or endosperm development through the heart stage (Figure 1A). Abnormalities were first detected at the torpedo stage (Figure 1B). Vacuolation of hypocotyl cells became pronounced at this stage and the shape of mutant embryos was distorted. Additional defects became apparent as development proceeded. The hypocotyl

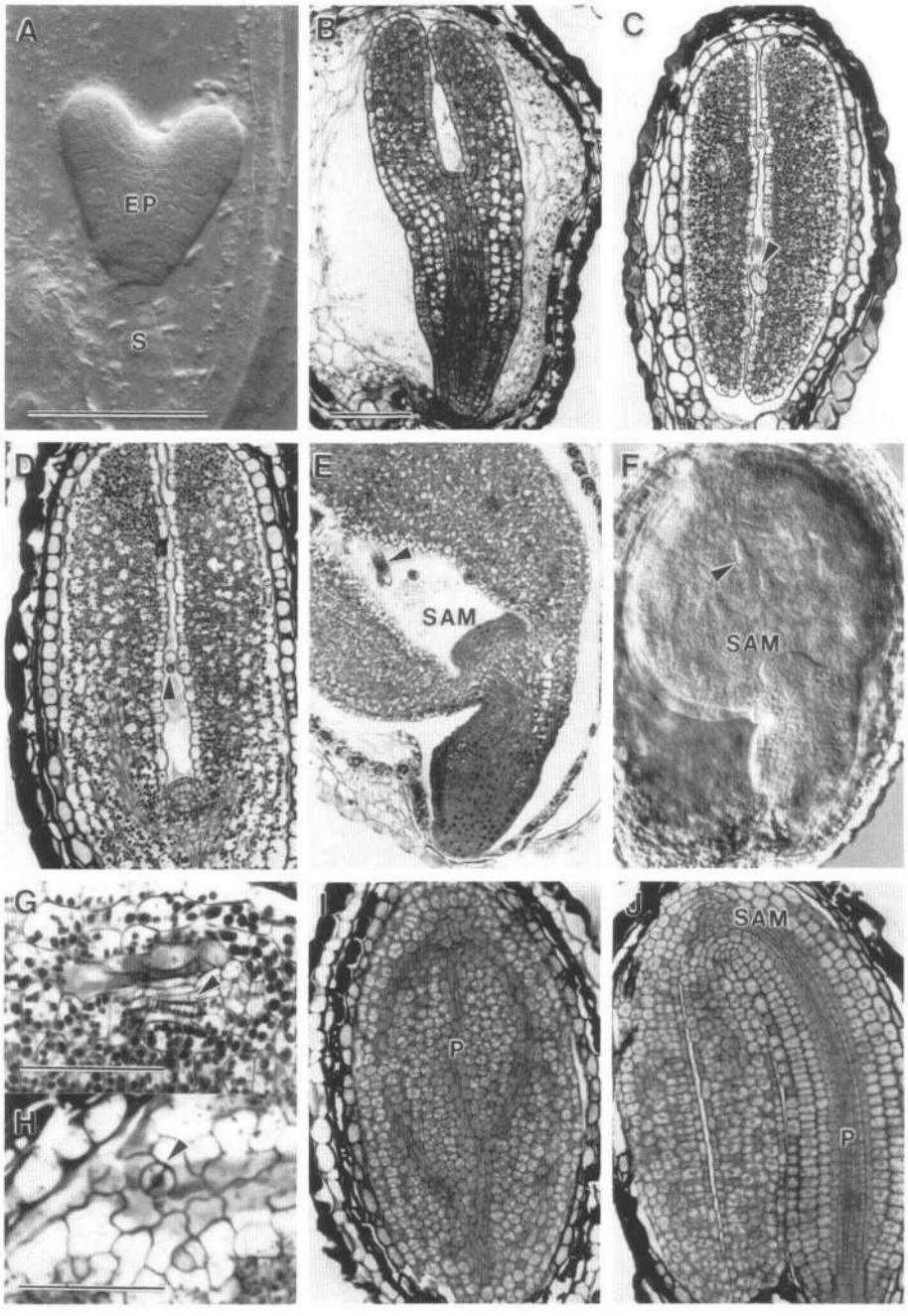

Figure 1. Developmental Anatomy of *lec1-1* Mutant Embryos.

(A) Nomarski image of a cleared mutant seed at the heart stage of development. S, suspensor; EP, embryo proper.

(B) to **(E)** Light micrographs of mutant seeds at the torpedo stage in **(B)** and cotyledon stages in **(C)** to **(E).** SAM, shoot apical meristem; arrowhead, trichome initial.

(F) Nomarski image of a cleared mutant seed at the cotyledon stage. SAM, shoot apical meristem; arrowhead, trichome initial.

(G) Internal region of a mutant cotyledon showing vascular tissue with mature xylem elements (arrowhead).

(H) Paradermal section through the cotyledon surface showing precocious stomate (arrowhead) in a mutant embryo.

(I) Longitudinal section of a wild-type cotyledon. P, procambium lacking mature vascular elements.

(J) Longitudinal section of a wild-type embryo showing the hypocotyl (right) and two cotyledons (left). SAM, shoot apical meristem; P, procambium.

Bars = 100 μm.

failed to elongate further during the cotyledon stage and was reduced in length at maturity. Several protoderm cells on the adaxial surface of mutant cotyledons enlarged and differentiated into trichome initials (Figures 1C to 1F). Internal cells of mutant cotyledons began to enlarge, vacuolate, and resemble leaf mesophyll cells. Intercellular spaces were prominent in mutant cotyledons. The dense packing of cells observed in wild-type cotyledons (Figures 1I and 1J) was disrupted. The shoot apical meristem was greatly enlarged and often included leaf primordia (Figures 1D to 1F). The root apex was normal in appearance but became active prematurely in viviparous seeds.

Mature xylem elements were often found in vascular tissues of mutant cotyledons (Figure 1G). In contrast, the vascular system of wild-type cotyledons (Figure 1I) was composed of procambium without differentiated elements. Stomata were found on the surface of mutant cotyledons (Figure 1H), whereas stomata in wild-type cotyledons did not appear until after germination. Plastids with large starch grains were prominent at the tips of mutant cotyledons. Mutant embryos lacked the mature protein bodies characteristic of wild-type embryos. Lipid bodies were rare in mutant cotyledons and present in reduced numbers in the hypocotyl. These results indicate that mutant embryos develop normally at first but fail to activate a wide range of embryo-specific programs after the heart stage. Embryos then enter a germination pathway and differentiate leafy cotyledons with features normally restricted to vegetative leaves.

Mutants with Related Phenotypes

Interpretation of any developmental mutant is difficult when only a single mutant allele is available. We therefore decided to screen for other mutants with leafy cotyledons to expand our understanding of this phenotype and the genetic control of late embryogenesis in Arabidopsis. Three additional mutants were found that produced trichomes on cotyledons. One of these (*fus3-1*) was originally isolated and characterized as a *fusca* mutant with a defective seed phenotype (Müller and Heidecker, 1968). Seeds from this line were provided by H. Bäumlein (Gatersleben, Germany) to construct double mutants with *lec1*. Additional features of this mutant have recently been described (Bäumlein et al., 1994). We discovered the leafy cotyledon phenotype of *fus3-1* seedlings when immature embryos were rescued in culture. This feature was not evident in Gatersleben, where homozygous lines were maintained by planting green seeds in soil. Under these conditions, fragile trichome initials on mutant cotyledons became desiccated, reduced in size, and difficult to identify. We report here that *fus3-1* is more similar in phenotype to *leafy cotyledon* mutants than to *fusca* mutants. Another *fus3* allele with the same pattern of development (*fus3-3*) has recently been isolated and characterized by Keith et al. (1994).

Two additional mutants with a leafy cotyledon phenotype were found among transgenic lines produced by Agrobacterium-mediated seed transformation (Feldmann, 1991). These families were included in a collection of putative embryo-defective mutants identified at the University of Arizona by R. Goldberg, R. Fischer, and J. Harada (University of California) and colleagues. We screened dry seeds from 85 of these lines deposited at the Arabidopsis stock center (Ohio State University) for the presence of defective seeds with a *lec1* phenotype. Two families identified in this screen were shown in subsequent generations to be segregating for a recessive mutation that resulted in trichome initiation on mutant cotyledons. One of these lines (CS2922) was shown through complementation tests to be allelic to *lec1-1*. The phenotype of this new allele (*lec1-2*) is indistinguishable from the original allele. No differences were found in general morphology, internal anatomy, or response in culture. This allele has also been studied in detail elsewhere (M.A.L. West, K.L. Matsudaira, R.L. Fischer, R.B. Goldberg, J.L. Zimmerman, and J.J. Harada, manuscript in preparation). The second line (CS2728) exhibited a different phenotype and defined a separate genetic locus (*lec2*). Mapping with visible markers indicated that *lec2* was on chromosome 1, but the precise location remains to be determined because data with linked visible markers gave contradictory estimates of map position.

Morphology of *fus3*

The phenotypes of *lec1-2*, *lec2*, and *fus3-1* embryos and rescued plants are shown in Figure 2. The *fus3* phenotype was similar to that described above for *lec1*. Trichomes were initiated on mutant cotyledons during embryogenesis and became prominent when immature embryos were germinated in culture (Figures 2-II-H and 2-II-I). Mutant seeds were desiccation intolerant, occasionally viviparous, lacked protein bodies, and accumulated anthocyanin at maturity. The vascular pattern of mutant cotyledons was intermediate between that of wild-type leaves and cotyledons, as noted previously for *lec1* (Meinke, 1992). Stomata and differentiated vascular elements were occasionally observed in mutant cotyledons.

Several differences in mutant phenotypes were noted that appeared to be independent of genetic background. Seeds and embryos of *fus3* were paler than *lec1* controls grown under similar conditions (Figures 2A and 2C to 2F). The pattern of anthocyanin accumulation also differed. Pigmentation in *fus3* was most pronounced at the cotyledon/hypocotyl junction (Figures 2-II-C to 2-II-F), whereas anthocyanin in *lec1* embryos was usually limited to cotyledon margins (Figures 2-I-C and 2-I-D). The *fus3* hypocotyl was not altered in shape and as a result the embryo was usually curled at maturity (Figures 2-II-C and 2-II-D). *fus3* cotyledons were thinner and more elongate than *lec1* cotyledons. *fus3* seeds became deflated only during the final stages of desiccation. Trichomes on *fus3* cotyledons were also not as prominent as those produced on *lec1* cotyledons (Figures 2H and 2I). These results suggest that *LEC1* and *FUS3* may perform related but not identical functions during embryogenesis.

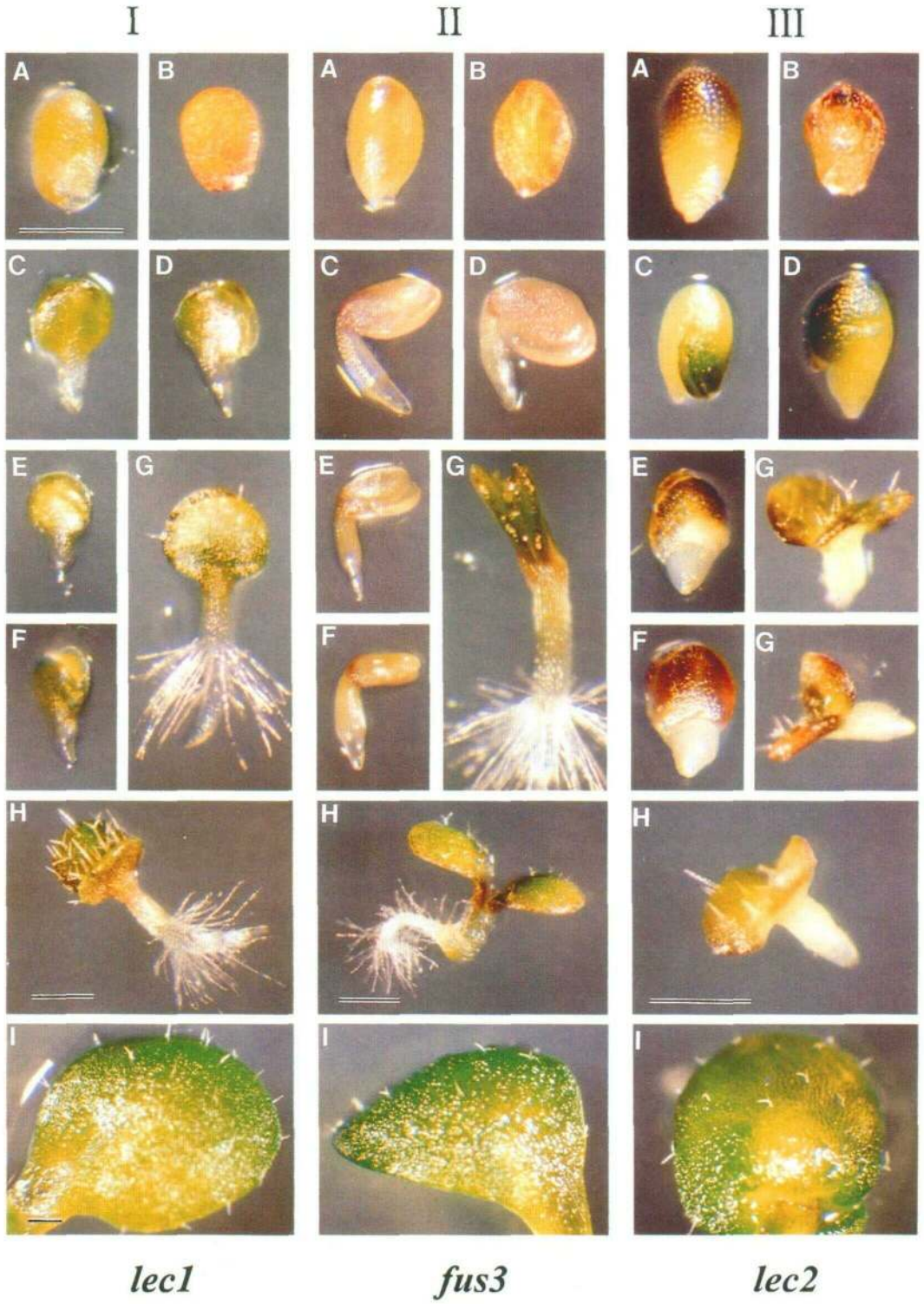

Figure 2. Morphology of Homozygous Mutant Seeds, Embryos, and Rescued Seedlings from *lec1-2* (I), *fus3-1* (II), and *lec2* (III) Parental Lines.

(A) and **(B)** Mutant seeds at the cotyledon stage and at maturity.
(C) to **(F)** Isolated embryos at the cotyledon stage.
(G) Rescued seedlings from embryos cultured 24 hr on basal medium.
(H) Rescued seedlings from embryos cultured 48 hr on basal medium.
(I) Trichomes on cotyledon surface of seedlings after 9 days in culture.
Bars = 0.5 mm.

Morphology of *lec2*

The phenotype of *lec2* clearly differed from that noted above for *lec1* and *fus3*. Similarities were limited to the presence of trichomes on cotyledons (Figures 2G to 2I), the formation of stomata and differentiated vascular elements, enlargement of the shoot apex, and a complex vascular pattern in cotyledons. Anthocyanin was especially prominent in the top half of the mutant cotyledons (Figures 2-III-E and 2-III-F). Mutant seeds were consequently highly pigmented at maturity (Figure 2-III-A). The hypocotyl was either white or pale green and was reduced in some *lec2* embryos (Figures 2-III-D to 2-III-F) but not in others (Figure 2-III-C). Mutant seeds were not viviparous, and mutant embryos did not produce roots during the first 48 hr in culture (Figures 2-III-G and 2-III-H). The resulting seedlings often appeared distorted with an elongated hypocotyl and cotyledons curled down toward the medium. These defects did not appear to be caused by other mutations segregating in this line.

Following desiccation, mutant seeds became deflated only at their tips, corresponding to the distal ends of cotyledons (Figure 2-III-B). The hypocotyl and basal portions of mutant cotyledons were not disrupted. Mutant seeds were usually desiccation tolerant at maturity, although their viability was reduced slightly after several weeks of storage. Dry seeds often germinated to produce seedlings with distorted cotyledons. This defect resulted from degeneration of cotyledon tips prior to germination. The cotyledon surface on seedlings produced from dry seeds lacked trichomes, apparently because any trichomes initiated during embryogenesis did not survive desiccation.

Storage Product Accumulation

Leafy cotyledon mutants exhibited striking differences in storage product accumulation during embryo development. Differences in starch and protein content are illustrated in Figure 3 (light micrographs) and Figure 4 (electron micrographs). Wild-type embryos are normally filled with protein and lipid bodies but contain relatively small amounts of starch at maturity (Figure 4A). The hypocotyl and cotyledons are generally indistinguishable with respect to major storage products (Patton and Meinke, 1990; Mansfield and Briarty, 1992).

Starch was the primary storage product in *lec1* embryos. Plastids with large starch grains were abundant in the mesophyll region of mutant cotyledons. Starch grains stained red with periodic acid-Schiff's reagent (Figure 3A) and were visible in electron micrographs (data not shown). Starch was most pronounced at the tips of mutant cotyledons (Figure 3B). Mature protein bodies similar to those found in wild-type embryos were not present in *lec1* embryos. Electron micrographs revealed that some vacuoles were lined with a small amount of electron-dense material that might correspond to storage protein (data not shown). A similar pattern of protein accumulation was seen in *fus3* embryos. Starch grains were common in *fus3* embryos but were not concentrated at the tips of mutant cotyledons (Figure 3C).

The most intriguing pattern of storage product accumulation was found in *lec2* embryos (Figures 3D to 3F and 4B to 4D). The hypocotyl in this case was filled with mature protein bodies, while the cotyledons exhibited a gradient of starch and protein accumulation. Cells in the top half of mutant cotyledons were highly vacuolated, packed with starch grains, and lacked mature protein bodies, whereas cells closer to the hypocotyl contained less starch but many protein bodies. The ultrastructure of mutant cells was therefore dependent on position within the embryo. Examples of large starch grains near the tip of a mutant cotyledon, immature protein bodies in the middle of a mutant cotyledon, and mature protein bodies in the mutant hypocotyl are shown in Figures 4B to 4D. Differences in cell morphology based on position were observed consistently in mutant cotyledons, although the location of transition zones was variable (Figures 3D and 3E).

Leafy cotyledon mutants also differed in the accumulation of lipid bodies during embryogenesis. The distribution of lipid bodies was examined in electron micrographs as shown in Figure 4, and in light micrographs of sections stained with Sudan Black. Examples of *lec1* and *fus3* cotyledon and hypocotyl tissues stained for lipid are shown in Figure 5. Only a few lipid bodies were present in cotyledons of *lec1* and *fus3* embryos at maturity (Figures 5A and 5C). Lipid bodies were slightly more abundant in the *lec1* hypocotyl (Figure 5B), more common yet in the *fus3* hypocotyl (Figure 5D), and highly abundant in the *lec2* hypocotyl (Figure 4D). Each mutant therefore exhibits a distinctive pattern of storage product accumulation in different parts of the developing embryo, and in all three mutants, the cotyledon is more highly disrupted than the hypocotyl with respect to cell ultrastructure.

ABA Response in Culture

The response of immature embryos to exogenous ABA in culture was examined to determine whether *leafy cotyledon* mutants were altered in ABA perception. The similarity of *lec* and *abi3* phenotypes had initially raised the possibility that *leafy cotyledon* mutants might be defective in ABA signal transduction pathways even though *abi3* was fully complemented in allelism tests with *lec1*, *lec2*, and *fus3*. Mutant seeds and isolated embryos from plants heterozygous and homozygous for *lec1*, *lec2*, and *fus3* were placed on agar plates supplemented with 1, 2, or 5 μM ABA. All three mutants were sensitive to these levels of ABA in culture. Results with *lec1* are summarized in Figure 6. We included immature *abi3-3* seeds and embryos as positive controls to show that growth of ABA-insensitive seedlings was not disrupted. Immature wild-type embryos were used as negative controls to demonstrate inhibition of germination by ABA. The most striking effect of ABA on *lec1* and *fus3* embryos was inhibition of root growth. Root elongation and root hair formation were completely inhibited on 1 μM ABA. The response with *lec2* was similar but

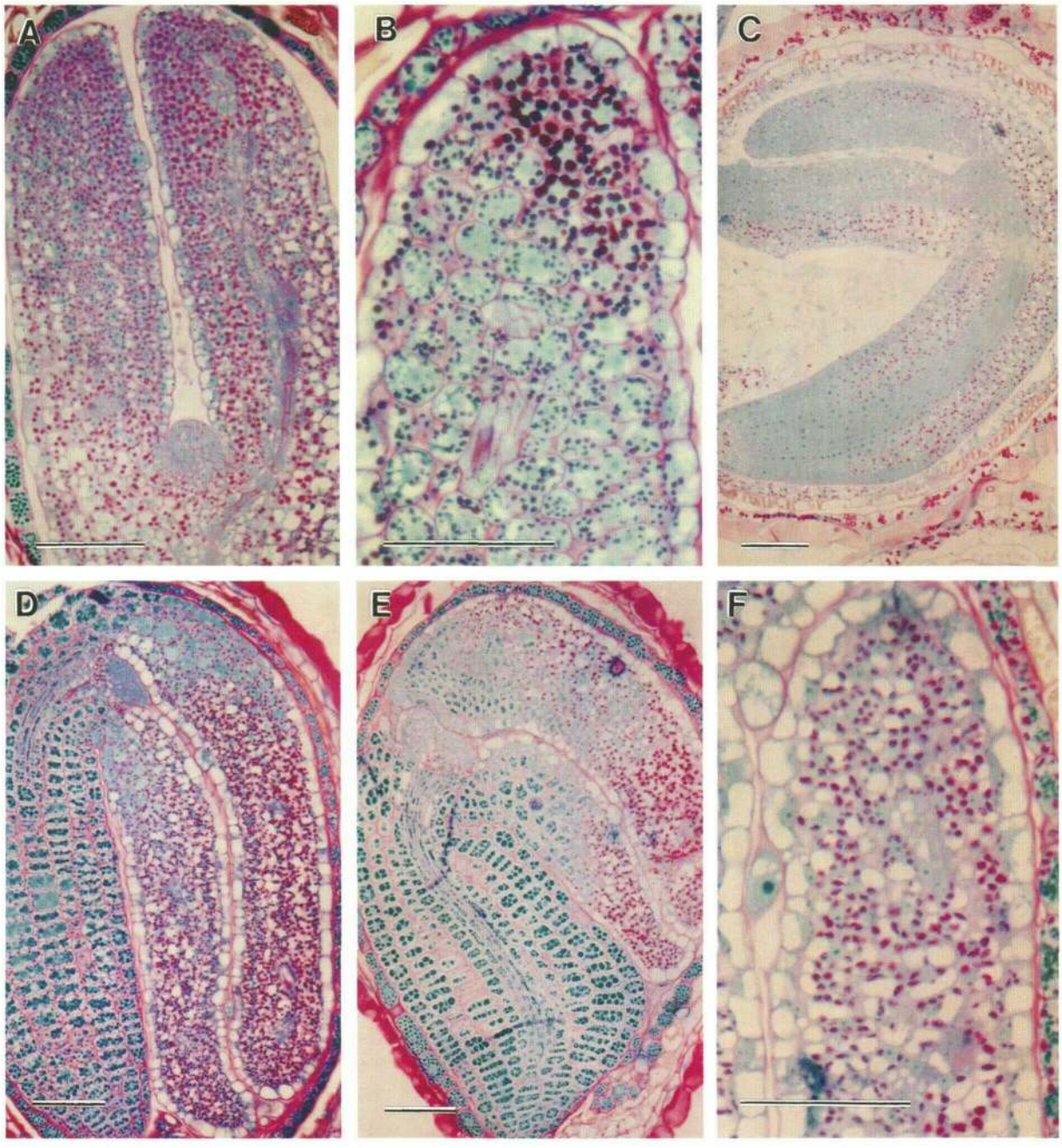

Figure 3. Light Micrographs of Protein and Starch Accumulation in Developing Embryos of *lec2*, *fus3*, and *lec1*.

Mutant seeds were stained with toluidine blue and periodic acid-Schiff's reagent. Nucleoli and protein bodies are dark blue; starch grains are red.
(A) *lec1* embryo at the cotyledon stage.
(B) Enlarged view of starch grains at the tip of a *lec1* cotyledon.
(C) *fus3* embryo at the cotyledon stage.
(D) and **(E)** *lec2* embryos at the cotyledon stage of development. The embryo in **(E)** is at a slightly later stage of development.
(F) Enlarged view of starch grains at the tip of a *lec2* cotyledon.
Bars = 100 μm.

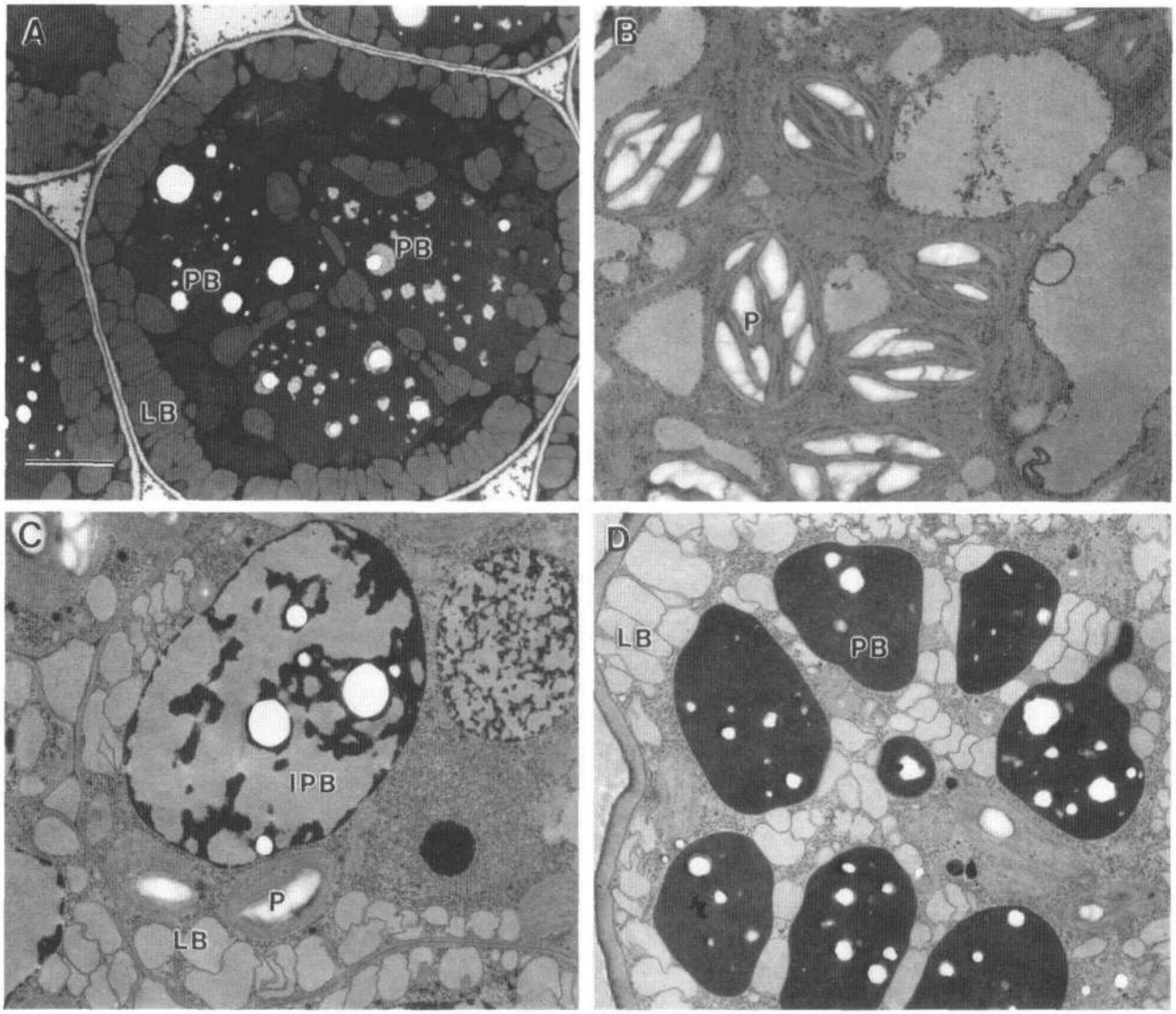

Figure 4. Electron Micrographs of Wild-Type and *lec2* Embryos Showing Position-Dependent Differences in Ultrastructure.

(A) Section through a wild-type cotyledon from an embryo at the cotyledon stage of development. PB, protein body; LB, lipid body.
(B) to **(D)** Sections through different regions of a *lec2* embryo at the cotyledon stage of development. The mesophyll region of the cotyledon is shown in **(B)**, the internal region of the cotyledon is in **(C)**, and the hypocotyl is shown in **(D)**. P, plastid with large starch grains; IPB, immature protein body; LB, lipid body; PB, mature protein body.
Bar = 2 μm.

not as striking because mutant embryos were not viviparous. Mutant cotyledons in each case accumulated anthocyanin, expanded only slightly, and contained few enlarged trichomes. The shoot apex failed to produce any leaves. These results demonstrate that immature embryos from *leafy cotyledon* mutants are not altered in ABA perception.

Analysis of Double Mutants

Double mutant embryos were constructed to study epistatic relationships between five genes with important regulatory functions late in embryogenesis (*lec1*, *lec2*, *fus3*, *abi3*, and *cop1*). Some double mutant embryos were identified by their novel phenotypes in siliques of double heterozygotes. Others were confirmed only after test crosses were performed back to parental lines. Phenotypes of double mutant embryos were noted first in siliques of double heterozygotes and then if possible in siliques of double homozygotes. Maintenance of homozygotes involved rescuing immature embryos in culture, transplanting the resulting seedlings to soil, and screening progeny seeds produced following self-pollination. Novel phenotypes identified in siliques of double mutant plants are shown in Figure 7.

Plants heterozygous for *lec1-1* and *abi3-3* produced double mutant embryos that were resistant to ABA in culture but otherwise exhibited a typical leafy cotyledon phenotype. This additive effect is consistent with the conclusion that *lec1* and *abi3* are altered in different pathways. Homozygous double mutant plants, produced by transplanting ABA-resistant leafy cotyledon seedlings to soil, appeared normal but produced siliques with 100% defective seeds following self-pollination. Double mutant embryos produced by these plants were highly pigmented (Figures 7A to 7C) and extremely viviparous (Figure 7R). When plants were grown in high humidity, almost every seed germinated inside the silique. Some seeds were nearly black from residual anthocyanin and chlorophyll in the embryo. The shoot apex and trichome initials were often larger than in parental lines.

Plants heterozygous for *lec2* and *abi3-3* also produced double mutant embryos with a typical leafy cotyledon phenotype that were resistant to ABA in culture. Both *lec1* and *lec2* are therefore altered in a pathway distinct from ABA signal transduction. Homozygous double mutant plants appeared normal except for the presence of siliques with 100% defective seeds. Immature embryos from these siliques germinated more rapidly in culture than *lec2* controls, but they did not exhibit the striking vivipary characteristic of *lec1 abi3* double mutant embryos (Figure 7S). Occasionally, a mutant seed was found with a short root or enlarged hypocotyl protruding through the seed coat. Double mutant embryos were highly pigmented at maturity and their morphology was often intermediate between *lec1* and *lec2* (Figures 7D and 7E). Many embryos contained a small hypocotyl characteristic of *lec1*. Hypocotyl pigmentation ranged from nearly transparent (*lec1* phenotype) to milky green (*lec2* phenotype). Mutant embryos often contained a large shoot apex and striking trichomes prior to desiccation. Mutant seeds were completely deflated after desiccation (Figure 7F).

Double mutants of *fus3* and *abi3* were not included in this study because identical constructs were being made in other laboratories (Bäumlein et al., 1994; Keith et al., 1994). Based on comparison of published results, it appears that *fus3 abi3* double mutant embryos are less viviparous but otherwise similar in morphology to *lec1 abi3* double mutants. This is consistent with the related phenotypes of *lec1* and *fus3* parental lines. We constructed instead a double mutant between *lec1* and another *fusca* (*cop1*) involved in signal transduction pathways during plant development (Deng et al., 1992; Castle and Meinke, 1994). Siliques heterozygous for *lec1* and *cop1* contained ~6% mutant seeds with a novel phenotype (Figures 7J to 7L). These putative double mutant embryos arrested at the torpedo stage of development, accumulated anthocyanin, and failed to turn green at maturity. Trichomes were not visible on the surface of mutant cotyledons. In this case, homozygous double mutant lines could not be maintained because immature embryos did not respond further in culture. The novel phenotype of these double mutant embryos may result from premature onset of seedling lethality (characteristic of *cop1* null alleles) in leafy cotyledons that have activated germination-specific programs.

The identification of *lec1 lec2* double mutant embryos was more difficult because mapping data indicated linkage between

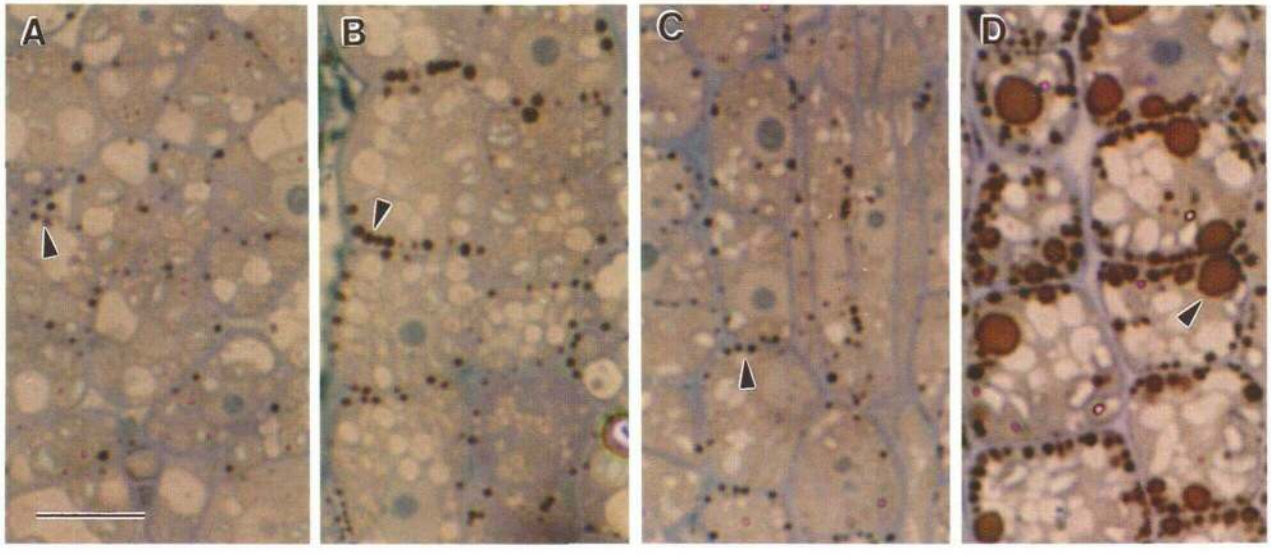

Figure 5. Light Micrographs of Lipid Bodies in *lec1* and *fus3* Cotyledon and Hypocotyl Tissues Stained with Sudan Black.

(A) *lec1* cotyledon with rare lipid bodies (arrowhead).
(B) *lec1* hypocotyl with slightly more abundant lipid bodies (arrowhead).
(C) *fus3* cotyledon with rare lipid bodies (arrowhead).
(D) *fus3* hypocotyl with lipid bodies (arrowhead) more abundant than in *lec1* hypocotyl tissue, but still far less abundant than in wild-type embryos.
Bar = 20 μm.

these two genes (L. Franzmann and E. Yoon, unpublished results). Plants heterozygous for both *lec1* and *lec2* were readily identified based on phenotypes and ratios of progeny seeds, but putative double mutant seeds were not immediately apparent within the silique. Sixteen mutant embryos that were smaller than normal and possibly intermediate in morphology between *lec1* and *lec2* were removed from immature siliques of double heterozygotes and rescued in culture. Siliques of resulting plants were then scored for embryonic defects. Selected plants were subsequently crossed to *lec1* and *lec2* parental lines to confirm genotypes. All 16 plants appeared to be homozygous recessive at one locus and heterozygous at the other. The absence of homozygous double mutant plants in this generation was not surprising if the genes are linked. Siliques on these plants consistently produced 75% mutant seeds with the expected *lec1* or *lec2* phenotype and 25% deflated seeds with embryos blocked at the torpedo stage, earlier than in single mutant lines (Figures 7M to 7O). These putative double mutant embryos exhibited the same range of phenotypes regardless of whether the plant was *lec1/lec1 lec2/LEC2* or *lec2/lec2 lec1/LEC1*. Anthocyanin was often present in the top half of mutant cotyledons, the central region varied from translucent to milky white or pale green, and the hypocotyl tip was often pointed and colorless. Cotyledons were occasionally fused to form a ring similar to that seen in *gnom* or *emb30* mutant embryos (Meinke, 1985; Baus et al., 1986; Mayer et al., 1991, 1993). Double mutant embryos were not viviparous and did not contain a large shoot apex or trichome initials. Immature embryos homozygous for both *lec1* and *lec2* occasionally produced seedlings with an unexpected phenotype in culture (Figures 7P and 7Q). Trichomes on these atypical seedlings formed on both the adaxial and abaxial surfaces of mutant cotyledons. This distribution is not characteristic of single mutant lines. Most examples of misplaced trichomes were obtained with double mutant embryos cultured early in development from *lec2/lec2 lec1/LEC1* parental lines.

Two classes of double mutant embryos (*lec2 fus3* and *lec1 fus3*) have not been identified to date, presumably because they are not easily distinguished in siliques of double heterozygotes. Embryos with a novel phenotype were found in siliques of *lec2 fus3* double heterozygotes (Figures 7G to 7I), but their genotype was inconsistent with being double mutants. Mutant embryos were paler than parental embryos, arrested at the linear cotyledon stage, lacked an enlarged shoot apex and large trichome initials, and when rescued in culture, produced plants with 100% defective seeds with the same phenotype. Test crosses to parental lines revealed that these putative double homozygotes were in fact *fus3/fus3 LEC2/LEC2*; only phenotypically normal seeds were produced from crosses with *lec2* parents. These severe *fus3* embryos may therefore be homozy-

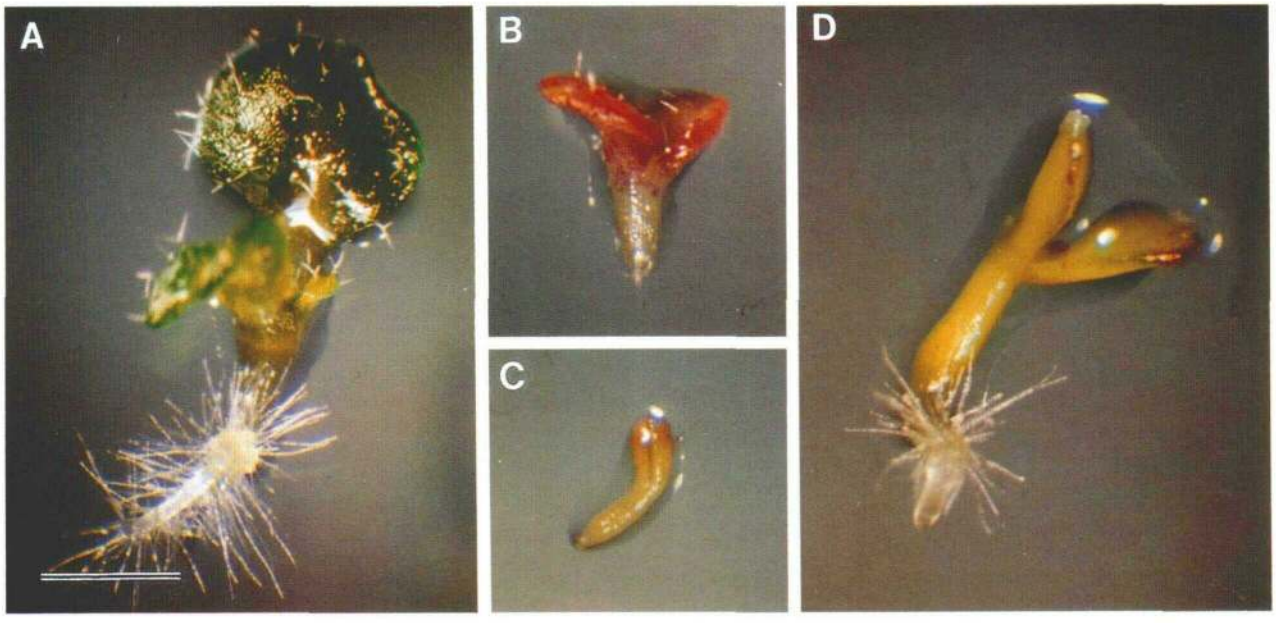

Figure 6. ABA Response of Immature *lec1* Embryos in Culture.

Photographs were taken 5 days after embryos were placed in culture.
(A) Response of a *lec1* embryo cultured on basal medium.
(B) Response of a *lec1* embryo cultured on medium supplemented with 1 μM ABA.
(C) Response of a wild-type embryo cultured on basal medium.
(D) Response of an *abi3-3* embryo cultured on 1 μM ABA.
Bar = 0.5 mm.

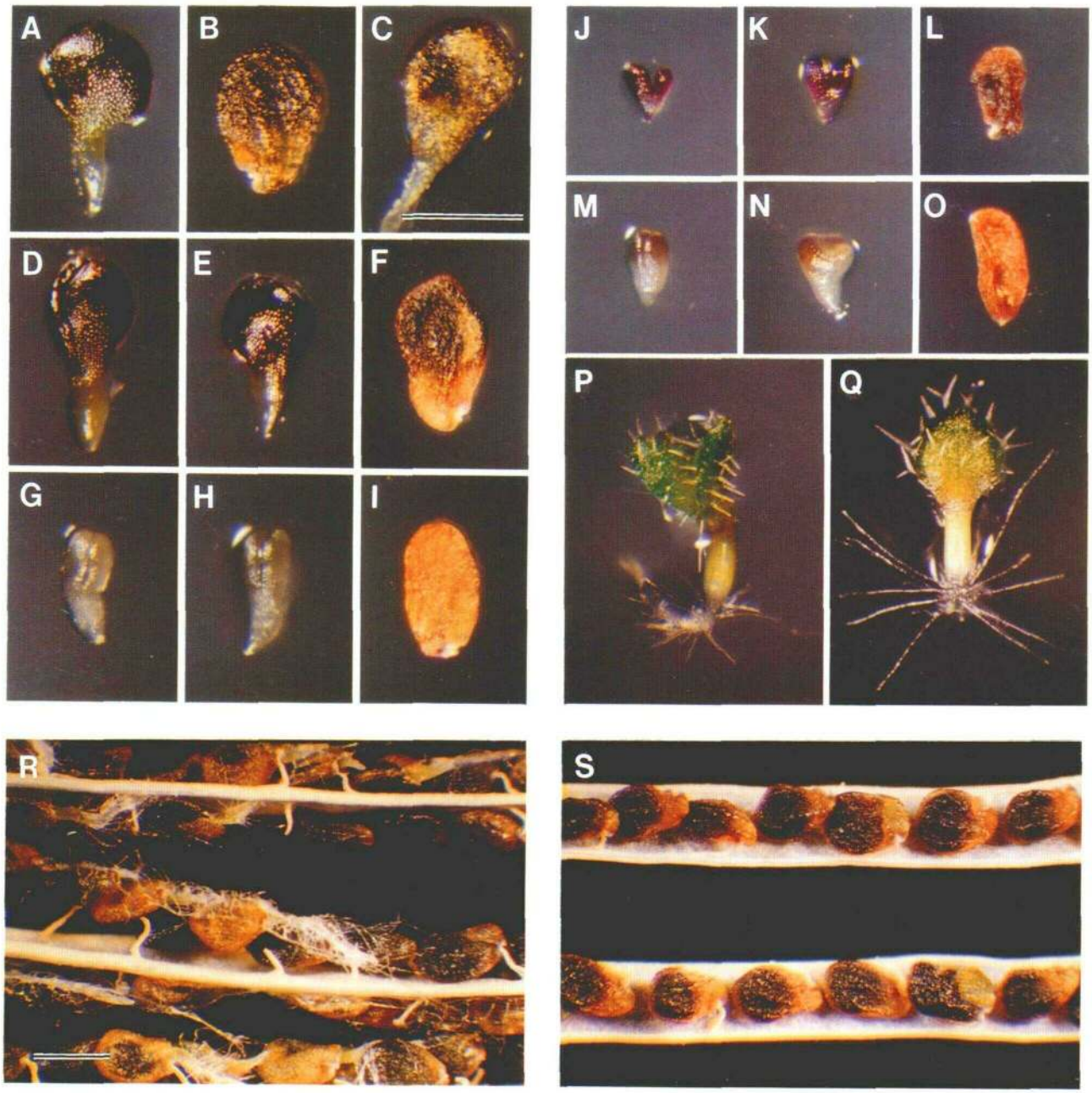

Figure 7. Morphology of Double Mutant Seeds, Embryos, and Rescued Seedlings.

(A) to **(C)** *lec1 abi3* double mutant embryo in **(A)** and dry seeds in **(B)** and **(C)**.
(D) to **(F)** *lec2 abi3* double mutant embryos in **(D)** and **(E)** and dry seed in **(F)**.
(G) to **(I)** Severe *fus3* embryos in **(G)** and **(H)** and dry seed in **(I)**, all identified during construction of *lec2 fus3* double mutant lines.
(J) to **(L)** *lec1 cop1* double mutant embryos in **(J)** and **(K)** and dry seed in **(L)**.
(M) to **(O)** *lec1 lec2* double mutant embryos in **(M)** and **(N)** and dry seed in **(O)**.
(P) and **(Q)** Seedlings produced from *lec1 lec2* putative double mutant embryos rescued in culture. Note the presence of trichomes on both surfaces (abaxial and adaxial) of mutant cotyledons.
(R) Vivipary in mature siliques of *lec1 abi3* double homozygotes.
(S) Absence of vivipary in mature siliques of *lec2 abi3* double homozygotes.
Bars = 0.5 mm.

gous for a recessive enhancer of the *fus3* phenotype present in the *lec2* parental line, or may have acquired a heritable modification within the *fus3* allele itself. Based on segregation of mutant phenotypes in siliques of double heterozygotes, the desired double mutant embryos may have a phenotype similar to *fus3* homozygotes. Embryos homozygous for *lec1* and *fus3* may also have a phenotype not readily distinguished from parent lines because nearly all seeds produced by double heterozygotes had typical phenotypes. Efforts are underway to identify these double mutants by crossing progeny plants to lines with known genotypes.

Prospects for Gene Isolation

Further analysis of *leafy cotyledon* mutants will require molecular isolation of the corresponding genes. Map-based cloning may be required in the case of *FUS3* because no tagged alleles are currently available. This mutant locus has been mapped to chromosome 3 (Keith et al., 1994; Miséra et al., 1994) and chromosome walking has already been initiated (Bäumlein et al., 1994). The *lec2* mutant described here was isolated following seed transformation, but its tagging status remains to be resolved. Unfortunately, only 35% of the embryo-defective mutants in this population of transgenic plants appear to be tagged with a functional T-DNA insert (Castle et al., 1993). Map-based cloning of this locus may require the isolation of additional alleles because *lec2-1* has been difficult to place on the genetic map.

An unusual pattern of inheritance was uncovered when the tagging status of *lec1-2* was examined. Although results obtained to date are consistent with tagging, we have been unable to recover a subfamily that lacks other inserts. This phenomenon has not been encountered previously in our extensive analysis of tagged embryo-defective mutants (Errampalli et al., 1991; Castle et al., 1993). Plants heterozygous for a tagged allele without additional inserts should produce a 2:1 ratio of kanamycin-resistant to kanamycin-sensitive (Kan^r/Kan^s) progeny. All nopaline-positive (Nop^+) plants should be heterozygotes, and all nopaline-negative (Nop^-) plants should be wild type. Two *lec1-2* heterozygotes identified in the initial screen produced a 5:1 ratio of Kan^r/Kan^s progeny, indicative of the presence of more than one insert. Ninety-two progeny plants from these lines were scored for nopaline and the presence of mutant seeds. As expected, two-thirds of these plants were heterozygous for the mutation. All 59 heterozygotes were Nop^+, 14 wild-type plants were Nop^-, and 19 wild-type plants were Nop^+. These results suggested that two inserts were present in this family, one responsible for the mutation and the other distantly linked to the mutant locus.

We then attempted to identify a subfamily with a low ratio of Kan^r/Kan^s progeny following loss of the second insert. When progeny seeds from 50 Nop^+ heterozygotes were plated on kanamycin, 17 subfamilies gave all resistant seedlings, as expected for plants homozygous for a second insert, but the ratio of Kan^r/Kan^s seedlings for the other 33 subfamilies was again 5:1. No plant produced a 2:1 ratio of Kan^r/Kan^s progeny. Similar results were obtained when subfamilies with the lowest ratios were examined in the next generation. Wild-type plants homozygous for an insert not associated with the mutation are therefore often recovered in this line, but some factor is preventing recovery of heterozygotes without this other insert.

One model is that a chromosomal aberration is responsible for our inability to recover the desired class of progeny. Chromosomal translocations are common in this population of transgenic plants (Castle et al., 1993). Inversions may be present as well and could potentially lead to elimination of a desired class of recombinants. One problem with this model is that chromosomal aberrations are often associated with semisterility. Surprisingly, pollen and ovule development in *lec1-2* appear normal. Alternatively, desired recombinants may be lost through association with a linked pollen factor that prevents fertilization while leaving pollen morphology unchanged. If chromosomal rearrangements have scrambled the *lec1-2* region, map-based cloning may be required to facilitate gene isolation. To this end, we have mapped *lec1-1* relative to linked visible markers, collected valuable recombinants in this region, and identified a restriction fragment length polymorphism (RFLP) molecular marker that appears to be within 1 centimorgan based on DNA gel blot analysis of pools of *LEC1/LEC1* F_2 plants (E. Yoon and D. Meinke, unpublished data). By combining these complementary approaches to gene isolation, we may soon be able to extend the analysis of *leafy cotyledon* mutants to the molecular level.

DISCUSSION

We describe in this report a class of mutants with fundamental defects in plant embryogenesis. This *leafy cotyledon* class defines at least three different genes with distinct but related functions during plant development (*LEC1*, *LEC2*, and *FUS3*). We believe that *LEAFY COTYLEDON* genes in Arabidopsis encode regulatory factors that activate a wide range of embryo-specific programs beginning at the heart stage of development. Loss of normal gene function causes mutant cotyledons to revert to a primitive developmental state characterized by partial or complete replacement of embryo-specific traits (storage products, desiccation tolerance, and simple vascular pattern) with features normally restricted to vegetative leaves (trichomes, sensitivity to desiccation, and complex vascular pattern). According to this model, precocious germination of mutant embryos results from loss of embryo-specific inhibition of germination during seed development. The basal state of wild-type cotyledons may therefore be leaflike, just as the default condition for developing flowers appears to be leaflike (Coen and Meyerowitz, 1991). The alternative model that *leafy cotyledon* mutants are altered only in developmental timing and prematurely activate germination-specific programs fails to explain why leafy traits consistently appear on mutant

cotyledons when they are not characteristic of wild-type cotyledons either before or after germination. This heterochronic interpretation, recently elaborated by Keith et al. (1994) in their discussion of the *fus3-3* phenotype, also fails to explain the leafy cotyledon appearance of *lec2* embryos, which do not exhibit precocious germination.

Previous studies on the regulation of late embryogenesis in higher plants have emphasized the role of ABA in modulating the appearance of embryo-specific programs (Hughes and Galau, 1991; Finkelstein, 1993; Paiva and Kriz, 1994). This has contributed to a false impression that ABA is the primary regulator of late embryogenesis. We demonstrate in this report that normal perception of ABA by *ABI3* is necessary but not sufficient for many critical events associated with embryogenesis in Arabidopsis. A separate but related group of regulatory factors is encoded by *LEAFY COTYLEDON* genes. A revised model for the regulation of late embryogenesis in Arabidopsis that incorporates the results of studies with *leafy cotyledon* and *ABA-insensitive* mutants is presented in Figure 8. According to this model, *ABI3* and *LEC* genes (*LEC1, LEC2, FUS3*) operate through different pathways to ensure that developing seeds prepare for desiccation and dormancy. This model is consistent with the observation that the most complete elimination of embryo-specific programs was seen in double mutants between *abi3* and mutants of the *leafy cotyledon* class. *LEC1* and *FUS3* are placed close to each other in this model because their mutant phenotypes are most closely related. This may reflect similar functions or physical interactions between their gene products. *LEC1* is placed upstream of *FUS3* because it exhibits the greatest number of defects in embryo development. *LEC2* appears to act further downstream in this network because loss of *LEC2* gene function does not eliminate as many features of normal embryogenesis. Our failure to identify *lec1 fus3* double mutant embryos with a novel phenotype is consistent with the view that these two genes perform closely related functions. Our results with *lec2 fus3* double mutants are more difficult to explain because the model appears to predict that double mutant embryos should exhibit a *lec1* phenotype. This may be an indication that these gene products are not simply associated with a linear pathway, but rather are part of a complex network that includes other regulatory factors that remain to be identified.

The *lec2* phenotype is particularly intriguing because it allows several related aspects of late embryogenesis (desiccation tolerance, storage product accumulation, and inhibition of precocious germination) to be separated from inhibition of trichome formation. *Leafy cotyledon* mutants are therefore not necessarily viviparous, desiccation-intolerant during seed development, and defective in storage products throughout the embryo. The gradient of storage products observed in *lec2* embryos suggests that despite their morphological similarities in wild-type embryos, cells of the hypocotyl and cotyledon can differ in their response to developmental signals. Embryos from this mutant could be useful in future studies of storage protein and lipid biosynthesis and acquisition of desiccation tolerance during plant development.

The desiccation tolerance of *lec2* seeds at maturity raises the possibility that additional mutants with a leafy cotyledon phenotype may be included among mutants with altered vegetative morphology. Unfortunately, leafy traits may be difficult to identify in cotyledons of mutant seedlings derived from dry seeds. Several lines of evidence suggest that trichomes initiated during embryogenesis will not survive desiccation: (1) the scarcity of trichomes on cotyledons of *fus3* seedlings produced from green seeds planted in soil; (2) the lack of trichomes on cotyledons of *lec2* seedlings produced from dry seeds germinated in culture; (3) the lack of previous reports of mutant seedlings with trichomes on cotyledons despite large-scale screens of mutagenized populations derived from dry seeds; and (4) the observation that severe *fus3* seedlings derived from *lec2 fus3* lines exhibit trichomes on cotyledons only if very young mutant embryos are cultured prior to initial

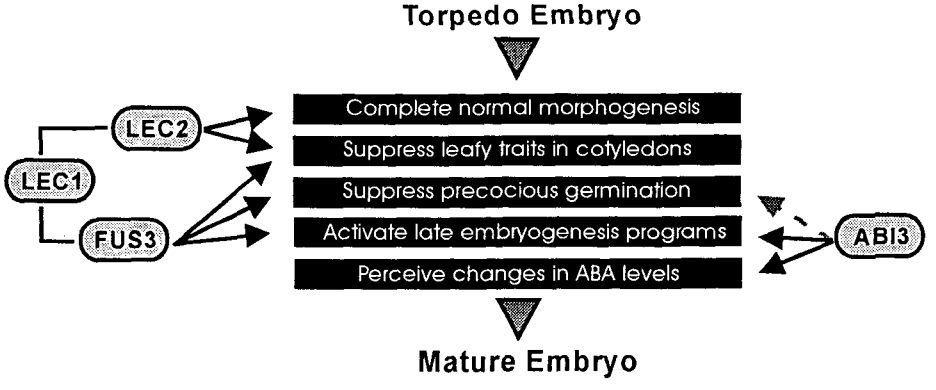

Figure 8. Model of Genetic Regulation of Late Embryogenesis in Arabidopsis Based on Phenotypes of *leafy cotyledon* and *ABA-insensitive* Mutants.

Black rectangles highlight major events that occur during normal development. Events are not arranged sequentially. Arrows show which wild-type genes are required to complete each event, based on loss-of-function mutant phenotypes. *LEC2* may activate late embryogenesis programs in some parts of the cotyledon. *ABI3* is involved to a limited extent in suppressing vivipary (dotted line).

stages of desiccation. Thus, trichome initials do not appear to contain sufficient levels of cellular protectants to survive desiccation.

If all *leafy cotyledon* mutants exhibit both inappropriate accumulation of anthocyanin and defects in morphogenesis, few additional mutants of this class are likely to be found because extensive screens for this phenotype have already been performed (Müller, 1963; Müller and Heidecker, 1968; Miséra et al., 1994). The best candidate for a new *lec* allele would be *fus10*, the other known *fusca* mutant with a defective seed phenotype (Miséra et al., 1994). Alternatively, if leafy cotyledons can be produced in the absence of excess anthocyanin, then additional *LEC* genes may be present. The most definitive screen would require germination of immature mutant embryos in culture followed by examination of the cotyledon surface. We cannot predict whether all *leafy cotyledon* mutants should accumulate anthocyanin during embryo development because the precise factors responsible for altered pigmentation in *fusca* mutants in general remain to be identified (Castle and Meinke, 1994). The amount of anthocyanin produced is also sensitive to plant growth conditions, which could make saturation mutagenesis for this phenotype difficult. Nevertheless, the important role played by these genes in plant development may justify further attempts to identify related members of this regulatory network.

METHODS

Plant Materials and Culture Conditions

Several different ecotypes of Arabidopsis were included in this study: Wassilewskija (*leafy cotyledon* mutants *lec1-1*, *lec1-2*, and *lec2*; *constitutive photomorphogenic* mutant *cop1*); Dijon (*fusca* mutant *fus3-1*); and Columbia (*fus3-3*; abscisic acid (ABA)-insensitive mutant *abi3-3*). Seeds of *fus3-1* were obtained from H. Bäumlein (Institute of Plant Genetics and Crop Plant Research, Gatersleben, Germany). Seeds of *abi3-3* and *fus3-3* were obtained from P. McCourt (University of Toronto, Canada). Allelism between *fus3-3* and *fus3-1* was demonstrated by crosses performed in our laboratory. Mutant lines were maintained by growing plants in pots at 23 ± 3°C under 40-W cool-white fluorescent lights on 16-hr-light/8-hr-dark cycles (Meinke, 1985; Heath et al., 1986). Heterozygotes were identified by screening immature siliques for the presence of 25% defective seeds following self-pollination. Homozygous mutant plants were produced by culturing immature embryos on a basal nutrient medium containing the inorganic salts of Murashige and Skoog (1962), 3% glucose, and 0.8% purified agar, prepared as described by Baus et al. (1986). Rescued seedlings were transplanted to soil to complete the life cycle. Double mutants were constructed by crossing homozygous mutant plants grown from rescued seedlings. The chromosomal location of *lec2* was determined by crossing mutant plants with visible markers as described previously by Patton et al. (1991). Responses of mutant embryos to ABA were analyzed on basal media supplemented with (±) *cis-trans* ABA (Sigma). Kanamycin-resistant (Kan^r) *lec1-2* seedlings were identified on media supplemented with 50 mg/L kanamycin sulfate (Sigma). Nopaline assays were performed on leaf tissue as outlined by Errampalli et al. (1991).

Light and Electron Microscopy

Seeds prepared for light microscopy were fixed in 2% glutaraldehyde and 2% paraformaldehyde in 0.05 M phosphate buffer, pH 7.2, for 24 hr at 4°C, dehydrated for 24 hr in methyl Cellosolve (Sigma) followed by two changes of 100% ethanol (24 hr each), infiltrated and embedded with Historesin (Leica Canada, Toronto), and sectioned (2 to 3 μm thick) on a rotary microtome (model No. 2040; Reichert-Jung, Leica Canada) as described by Yeung and Law (1987). Sections were stained with periodic acid-Schiff's reagent to reveal total carbohydrate and counterstained with amido black 10B or toluidine blue O (Yeung, 1984). Lipid bodies were visualized in thick sections prepared for electron microscopy as described below, stained with Sudan Black B (Bronner, 1975), and counterstained with Mallory's reagent. Nomarski images of cleared seeds and photographs of isolated embryos were obtained as described by Meinke (1994).

Seeds prepared for electron microscopy were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, for 2 to 4 hr at 4°C, postfixed for 2 hr in 1% OsO_4 followed by three rinses in cacodylate buffer, dehydrated in an ethanol series, transferred through propylene oxide, infiltrated and embedded in Spurr's resin (Polysciences, Warrington, PA), sectioned on a MT-6000 ultramicrotome (Sorvall/DuPont, Wilmington, DE), stained with uranyl acetate, and observed on a transmission electron microscope (100cx; JEOL, Peabody, MA). Seeds prepared for both light and electron microscopy were punctured during fixation to facilitate penetration.

ACKNOWLEDGMENTS

We thank Ken Feldmann for the invitation to screen transgenic plants for embryo-defective mutations; Peter McCourt and Helmut Bäumlein for providing mutant seeds and sharing data prior to publication; John Harada for discussions about a *lec* mutant (*lec1-2*) being studied in his laboratory; Elizabeth Yoon for generating RFLP mapping data for *lec1-1*; Sandra Law for assistance in sectioning for light microscopy; Ginger Baker at the Oklahoma State University Electron Microscopy lab for assistance with electron microscopy; and Aynsley Kealiher, Jennifer Guertin, and Jennifer Pollack for technical assistance and plant maintenance. This research was supported by grants from the National Science Foundation (EPSCoR and Developmental Biology Programs) and Samuel Roberts Noble Foundation (Plant Biology Division). E.C.Y. has been funded by the National Sciences and Engineering Research Council of Canada.

Received April 4, 1994; accepted June 10, 1994.

REFERENCES

Bäumlein, H., Miséra, S., Luersen, H., Kölle, K., Horstmann, C., Wobus, U., and Müller, A.J. (1994). The *FUS3* gene of *Arabidopsis thaliana* is a regulator of gene expression during late embryogenesis. Plant J., in press.

Baus, A.D., Franzmann, L., and Meinke, D.W. (1986). Growth *in vitro* of arrested embryos from lethal mutants of *Arabidopsis thaliana*. Theor. Appl. Genet. **72,** 577–586.

Bronner, R. (1975). Simultaneous demonstration of lipids and starch in plant tissues. Stain Technol. **50,** 1–4.

Castle, L.A., and Meinke, D.W. (1994). A *FUSCA* gene of Arabidopsis encodes a novel protein essential for plant development. Plant Cell **6,** 25–41.

Castle, L.A., Errampalli, D., Atherton, T.L., Franzmann, L.H., Yoon, E.S., and Meinke, D.W. (1993). Genetic and molecular characterization of embryonic mutants identified following seed transformation in *Arabidopsis.* Mol. Gen. Genet. **241,** 504–514.

Coen, E.S., and Meyerowitz, E.M. (1991). The war of the whorls: Genetic interactions controlling flower development. Nature **353,** 31–37.

Deng, X.-W., Matsui, M., Wei, N., Wagner, D., Chu, A.M., Feldmann, K.A., and Quail, P.H. (1992). *COP1*, an Arabidopsis regulatory gene, encodes a protein with both a zinc-binding motif and a G-β homologous domain. Cell **71,** 791–801.

Errampalli, D., Patton, D., Castle, L., Mickelson, L., Hansen, K., Schnall, J., Feldmann, K., and Meinke, D. (1991). Embryonic lethals and T-DNA insertional mutagenesis in *Arabidopsis.* Plant Cell **3,** 149–157.

Feldmann, K.A. (1991). T-DNA insertion mutagenesis in *Arabidopsis*: Mutational spectrum. Plant J. **1,** 71–82.

Finkelstein, R.R. (1993). Abscisic acid-insensitive mutations provide evidence for stage-specific signal pathways regulating expression of an *Arabidopsis* late embryogenesis-abundant (Lea) gene. Mol. Gen. Genet. **238,** 401–408.

Finkelstein, R.R., and Somerville, C.R. (1990). Three classes of abscisic acid (ABA)-insensitive mutations of *Arabidopsis* define genes that control overlapping subsets of ABA responses. Plant Physiol. **94,** 1172–1179.

Giraudat, J., Hauge, B.M., Valon, C., Smalle, J., Parcy, F., and Goodman, H.M. (1992). Isolation of the Arabidopsis *ABI3* gene by positional cloning. Plant Cell **4,** 1251–1261.

Hattori, T., Vasil, V., Rosenkrans, L., Hannah, L.C., McCarty, D.R., and Vasil, I.K. (1992). The *Viviparous-1* gene and abscisic acid activate the *C1* regulatory gene for anthocyanin biosynthesis during seed maturation in maize. Genes Dev. **6,** 609–618.

Heath, J.D., Weldon, R., Monnot, C., and Meinke, D.W. (1986). Analysis of storage proteins in normal and aborted seeds from embryo-lethal mutants of *Arabidopsis thaliana.* Planta **169,** 304–312.

Hughes, D.W., and Galau, G.A. (1991). Developmental and environmental induction of *Lea* and *LeaA* mRNAs and the postabscission program during embryo culture. Plant Cell **3,** 605–618.

Keith, K., Kraml, M., Dengler, N.G., and McCourt, P. (1994). *fusca3*: A heterochronic mutation affecting late embryo development in Arabidopsis. Plant Cell **6,** 589–600.

Koornneef, M., Reuling, G., and Karssen, C.M. (1984). The isolation and characterization of abscisic acid-insensitive mutants of *Arabidopsis thaliana.* Physiol. Plant. **61,** 377–383.

Koornneef, M., Hanhart, C.J., Hilhorst, H.W.M., and Karssen, C.M. (1989). *In vivo* inhibition of seed development and reserve protein accumulation in recombinants of abscisic acid biosynthesis and responsiveness mutants in *Arabidopsis thaliana.* Plant Physiol. **90,** 463–469.

Mansfield, S.G., and Briarty, L.G. (1992). Cotyledon cell development in *Arabidopsis thaliana* during reserve deposition. Can. J. Bot. **70,** 151–164.

Mayer, U., Torres Ruiz, R.A., Berleth, T., Miséra, S., and Jürgens, G. (1991). Mutations affecting body organization in the *Arabidopsis* embryo. Nature **353,** 402–407.

Mayer, U., Buttner, G., and Jürgens, G. (1993). Apical-basal pattern formation in the *Arabidopsis* embryo: Studies on the role of the *gnom* gene. Development **117,** 149–162.

McCarty, D.R., Hattori, T., Carson, C.B., Vasil, V., and Vasil, I.K. (1991). The *viviparous-1* developmental gene of maize encodes a novel transcriptional activator. Cell **66,** 895–905.

Meinke, D.W. (1985). Embryo-lethal mutants of *Arabidopsis thaliana*: Analysis of mutants with a wide range of lethal phases. Theor. Appl. Genet. **69,** 543–552.

Meinke, D.W. (1991). Perspectives on genetic analysis of plant embryogenesis. Plant Cell **3,** 857–866.

Meinke, D.W. (1992). A homoeotic mutant of *Arabidopsis thaliana* with leafy cotyledons. Science **258,** 1647–1650.

Meinke, D.W. (1994). Seed development in *Arabidopsis thaliana.* In *Arabidopsis*, E. Meyerowitz and C. Somerville, eds (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press), in press.

Meurs, C., Basra, A.S., Karssen, C.M., and van Loon, L.C. (1992). Role of abscisic acid in the induction of desiccation tolerance in developing seeds of *Arabidopsis thaliana.* Plant Physiol. **98,** 1484–1493.

Miséra, S., Müller, A.J., Weiland-Heidecker, U., and Jürgens, G. (1994). The *FUSCA* genes of *Arabidopsis*: Negative regulators of light responses. Mol. Gen. Genet., in press.

Müller, A.J. (1963). Embryonentest zum Nachweis rezessiver Letalfaktoren bei *Arabidopsis thaliana.* Biol. Zentralbl. **82,** 133–163.

Müller, A.J., and Heidecker, U. (1968). Lebensfähige und letale *fusca*-Mutanten bei *Arabidopsis thaliana. Arabidopsis* Inf. Serv. **5,** 54–55.

Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant. **15,** 493–497.

Nambara, E., Naito, S., and McCourt, P. (1992). A mutant of *Arabidopsis* which is defective in seed development and storage protein accumulation is a new *abi3* allele. Plant J. **2,** 435–441.

Neill, S.J., Horgan, R., and Parry, A.D. (1986). The carotenoid and abscisic acid content of viviparous kernels and seedlings of *Zea mays* L. Planta **169,** 87–96.

Ooms, J.J.J., Léon-Kloosterziel, K.M., Bartels, D., Koornneef, M., and Karssen, C.M. (1993). Acquisition of desiccation tolerance and longevity in seeds of *Arabidopsis thaliana.* Plant Physiol. **102,** 1185–1191.

Paiva, R., and Kriz, A.L. (1994). Effect of abscisic acid on embryo-specific gene expression during normal and precocious germination in normal and *viviparous* maize (*Zea mays*) embryos. Planta **192,** 332–339.

Patton, D.A., and Meinke, D.W. (1990). Ultrastructure of arrested embryos from lethal mutants of *Arabidopsis thaliana.* Am. J. Bot. **77,** 653–661.

Patton, D.A., Franzmann, L.H., and Meinke, D.W. (1991). Mapping genes essential for embryo development in *Arabidopsis thaliana.* Mol. Gen. Genet. **227,** 337–347.

Pepper, A., Delaney, T., Washburn, T., Poole, D., and Chory, J. (1994). *DET1*, a negative regulator of light-mediated development and gene expression in *Arabidopsis*, encodes a novel nuclear-localized protein. Cell **78,** 109–116.

Robertson, D.S. (1955). The genetics of vivipary in maize. Genetics **40,** 745–760.

Robichaud, C., and Sussex, I.M. (1986). The response of *viviparous-1* and wild-type embryos of *Zea mays* to culture in the presence of abscisic acid. J. Plant Physiol. **126,** 235–242.

Rock, C.D., and Zeevaart, J.A.D. (1991). The *aba* mutant of *Arabidopsis thaliana* is impaired in epoxy-carotenoid biosynthesis. Proc. Natl. Acad. Sci. USA **88,** 7496–7499.

Yeung, E.C. (1984). Histological and histochemical staining procedures. In Cell Culture and Somatic Cell Genetics of Plants. Vol. 1, Laboratory Procedures and Their Applications, I.K. Vasil, ed (Orlando, FL: Academic Press), pp. 689–697.

Yeung, E.C., and Law, S.K. (1987). Serial sectioning techniques for a modified LKB historesin. Stain Technol. **62,** 147–153.