

# LEAFY COTYLEDON2 encodes a B3 domain transcription factor that induces embryo development

Sandra L. Stone<sup>\*†</sup>, Linda W. Kwong<sup>\*†</sup>, Kelly Matsudaira Yee<sup>\*</sup>, Julie Pelletier<sup>\*</sup>, Loïc Lepiniec<sup>‡</sup>, Robert L. Fischer<sup>§</sup>, Robert B. Goldberg<sup>¶</sup>, and John J. Harada<sup>\*||</sup>

<sup>\*</sup>Section of Plant Biology, Division of Biological Sciences, University of California, One Shields Avenue, Davis, CA 95616; <sup>†</sup>Seed Biology Laboratory, Laboratoire de Biologie des Semences, Institut National de la Recherche Agronomique, Route de St-Cyr, 78026 Versailles, France; <sup>‡</sup>Department of Plant and Microbial Biology, University of California, Berkeley, CA 94720; and <sup>§</sup>Department of Molecular, Cell, and Developmental Biology, University of California, Los Angeles, CA 90024-1606

Contributed by Robert B. Goldberg, August 6, 2001

The *Arabidopsis* *LEAFY COTYLEDON2* (*LEC2*) gene is a central embryonic regulator that serves critical roles both early and late during embryo development. *LEC2* is required for the maintenance of suspensor morphology, specification of cotyledon identity, progression through the maturation phase, and suppression of premature germination. We cloned the *LEC2* gene on the basis of its chromosomal position and showed that the predicted polypeptide contains a B3 domain, a DNA-binding motif unique to plants that is characteristic of several transcription factors. We showed that *LEC2* RNA accumulates primarily during seed development, consistent with our finding that *LEC2* shares greatest similarity with the B3 domain transcription factors that act primarily in developing seeds, *VIVIPAROUS1/ABA INSENSITIVE3* and *FUSCA3*. Ectopic, postembryonic expression of *LEC2* in transgenic plants induces the formation of somatic embryos and other organ-like structures and often confers embryonic characteristics to seedlings. Together, these results suggest that *LEC2* is a transcriptional regulator that establishes a cellular environment sufficient to initiate embryo development.

Embryogenesis in flowering plants begins with the double fertilization event in which the zygote and endosperm are formed after fusion of sperm cells with the egg cell and central cell of the female gametophyte, respectively. The endosperm initially undergoes syncytial development with formation of nuclear-cytoplasmic domains, but later cellularizes (1). The developing embryo is nourished by the endosperm and, in many plants, only the peripheral layer of the endosperm remains in the mature seed. Development of the zygote into the mature embryo can be divided conceptually into two distinct phases. During the early morphogenesis phase, the basic body plan of the plant is established with expression of polarity as a shoot–root axis, specification of morphological domains within the embryo, and formation of embryonic tissue and organ systems (2–4). The morphogenesis phase is followed by a period of maturation in which processes critical for seed formation occur (5, 6). During this late phase, reserves such as storage proteins and lipids are synthesized at high rates and accumulate in the seed. It is also during the maturation phase that the embryo acquires the ability to withstand desiccation at the final stage of seed development. At the end of embryogenesis, the seed consists of a mature, desiccated embryo that is quiescent metabolically. Although many aspects of embryogenesis have been characterized extensively, little is known at a mechanistic level of the processes that initiate embryo development.

The *Arabidopsis* *LEAFY COTYLEDON* (*LEC*) genes, *LEC1*, *LEC2*, and *FUSCA3* (*FUS3*), play key roles in controlling embryo development (7). Unlike most other embryonic regulators that function during specific stages of embryogenesis (8–11), *LEC* genes are unique in that they are required for normal development during both the morphogenesis and maturation phases.

Early in embryogenesis, *LEC* genes are required to specify suspensor cell fate and cotyledon identity (12–16). Late in embryogenesis, *LEC* genes are needed during the maturation phase for the acquisition of desiccation tolerance and the expression of many maturation-specific genes (13–17). Consistent with the finding that conditions that promote maturation suppress germination (6), *lec* mutant embryos prematurely activate the postgermination program (13, 15, 16, 18). Thus, *LEC* genes play a central role in controlling many aspects of embryogenesis, and they are candidates as regulators that coordinate the morphogenesis and maturation phases.

Identification and analysis of two *LEC* genes confirmed their regulatory role in embryogenesis and provided insight into their functions. *LEC1* shares extensive sequence similarity with the HAP3 subunit of CCAAT-binding transcription factor, implicating *LEC1* as a transcriptional regulator (12). Ectopic expression of *LEC1* confers embryonic characteristics to seedlings and results in the formation of embryo-like structures on the surfaces of leaves, suggesting that the gene plays a role in conferring embryogenic competence to cells (12). Thus, we hypothesized that *LEC1* establishes a cellular environment that promotes embryo development and that this environment coordinates the morphogenesis and maturation phases. *FUS3* also encodes a regulatory protein: a B3 domain transcription factor that accumulates primarily during seed development (19). Transient assays showed that *FUS3* is sufficient to activate genes usually expressed during maturation (20). Thus, two *LEC* genes seem to be involved in controlling embryo development by regulating transcription of other genes.

In this article, we focus on the *LEC2* gene to determine its role in embryo development. Because genetic studies suggest that *LEC1* and *LEC2* may have partially redundant functions (12, 15), it is possible that *LEC2* also functions in the initiation and coordination of embryo development. We cloned the *LEC2* gene and showed that it is expressed preferentially during embryogenesis and encodes a protein with similarity to other seed-specific transcription factors. Significant insight into the role of the gene was obtained by showing that transgenic plants expressing the *LEC2* gene ectopically form somatic embryos.

Abbreviations: ABI3, ABA INSENSITIVE3; BAC, bacterial artificial chromosome; DIS, distorted trichomes; FUS3, FUSCA3; LEC, LEAFY COTYLEDON; PKL, PICKLE; VP1, VIVIPAROUS1.

Data deposition: Nucleotide sequences reported in this paper have been deposited in the GenBank database (accession nos. AF400123 for the *LEC2* cDNA and AF400124 for the *LEC2* gene).

<sup>†</sup>S.L.S. and L.W.K. contributed equally to this work.

||To whom reprint requests should be addressed. E-mail: jharada@ucdavis.edu.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Together, these results indicate that *LEC2* is sufficient to induce embryogenic competence.

## Materials and Methods

**Plant Material.** *Arabidopsis thaliana* (L.) Heynh. was grown as described (16). *lec2-1* mutant in ecotype Ws-0 was provided by David Meinke (Oklahoma State Univ., Stillwater). Three new *lec2* mutant alleles were identified from a Ws-4 ecotype population mutagenized with T-DNA (21). Genetic tests showed that all three mutants failed to complement the original *lec2-1* mutation. Mutant lines formerly designated CPT9, CUC3, and DLM1 were renamed *lec2-3*, *lec2-4*, and *lec2-5*, respectively. Homozygous *lec2* mutant lines were maintained by immature seed rescue as described (16).

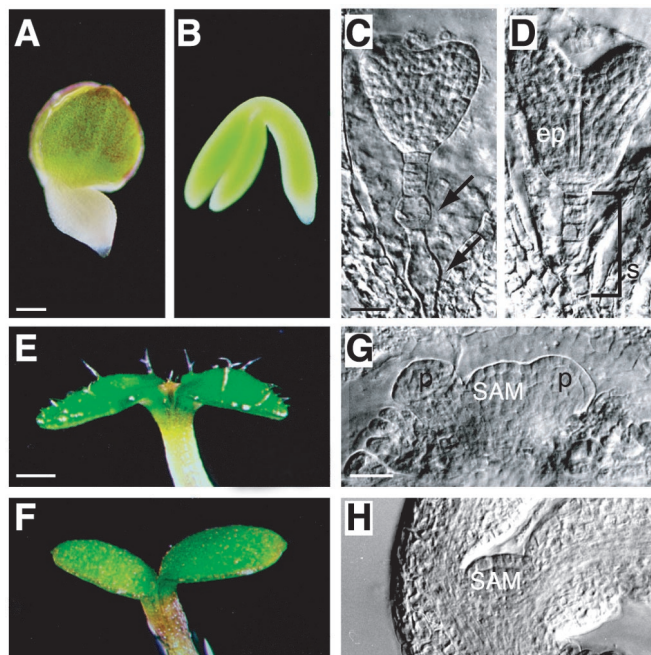
**Gene Cloning.** To enrich for recombination breakpoints near the *LEC2* gene, we crossed homozygous *lec2-1* mutants (Ws-0 ecotype) with either homozygous *distorted trichomes1-1* (*dis1-1*) or *dis2-1* mutants (Ler ecotype). Among F<sub>2</sub> progeny, we selected 381 recombinants homozygous for the *dis1* mutation and heterozygous for *lec2* and 173 that were homozygous for *dis2* and heterozygous for *lec2*. Polymorphisms that distinguish Ws-0 and Ler chromosomes were identified by amplifying chromosomal regions with primers designed from restriction fragment length polymorphism and bacterial artificial chromosome (BAC) clones and sequencing the products. Polymorphic sequences were used to generate PCR-based markers for genetic mapping (22–24).

Cosmid clones for transgene complementation experiments were constructed by inserting DNA fragments from BAC clone F1A10 (GenBank accession no. AL080330) into the plant transformation vector pOCA28 (25). Cosmid clones containing BAC DNA that spanned the *LEC2* gene were transferred into homozygous *lec2-1* mutants as described (12). Clones that suppressed the mutation were identified by using progeny segregation tests and genotyping with PCR-based markers.

**RNA Analysis.** Total RNA was isolated by the method of Wilkins and Smart (26). *LEC2* RNA was detected in tissues by using PCR amplification of reverse transcription products with a primer containing the translation initiation codon, 5'-AAATGGATA-ACTTCTTACCCTTTCC-3', and another immediately upstream of the translation termination codon, 5'-CGGATGAAC-CCACGTACG-3' (27). Potential genomic DNA contaminants of RNA samples were eliminated by digestion with DNase I, *MseI*, and *DdeI* before the reverse transcription step.

*In situ* hybridization experiments were done and cruciferin and oleosin RNA probes were prepared as described (16, 28). To increase the hybridization specificity, *LEC2* antisense RNA from bases -36 to +446 relative to the translation initiation site was used as a probe and RNase was used at a concentration of 100 µg/ml.

**35S::LEC2 Plants.** A *LEC2* cDNA clone containing the complete protein-coding region was constructed, starting with a cDNA clone consisting of *LEC2* sequences from 14 bases downstream of the translation initiation codon to the poly(A) tail. Amplification products of the *LEC2* gene region were fused with this cDNA clone to add 5' sequences to position -36. The sequence of this cDNA corresponded with that of amplified *LEC2* reverse transcription products. This *LEC2* cDNA was inserted between the cauliflower mosaic virus 35S promoter (-1329 to +7 relative to the transcription start site) and the octopine synthase 3' terminator (29), and the 35S::*LEC2*::*ocs* construct was then transferred into pBJ49. *lec2-1* and *lec2-5* homozygous mutant plants and wild-type Ws-0 plants were transformed by standard methods (21). Transgenic seedlings were identified by their resistance to hygromycin and by the presence of *LEC2* transgene-specific sequences as verified by PCR.



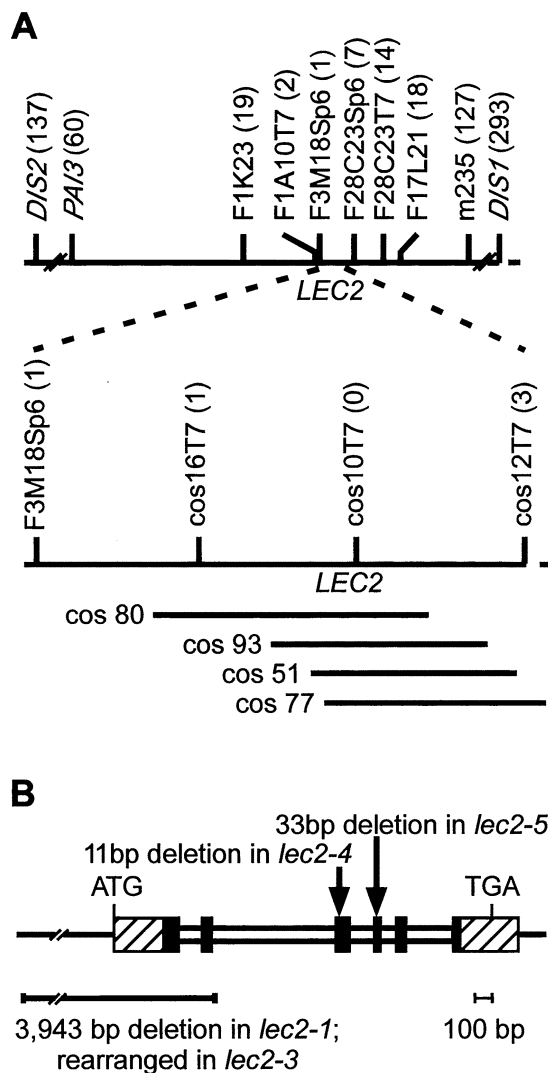
**Fig. 1.** Morphological phenotype of *lec2* mutants. (A) *lec2-5*, (C) *lec2-3*, and (E, G) *lec2-4* mutant embryos. (B, D, F, H) Wild-type embryo. (A and B) Whole-mount photographs of maturing embryos. (C and D) Embryonic suspensors as viewed by using differential interference contrast (DIC) microscopy of cleared seeds. Arrows point to abnormal suspensor cells in *lec2* mutants. (E and F) Cotyledons of seedlings grown for 4–5 days. A *lec2* mutant seedling germinated before desiccation possessed trichomes on the adaxial surface of cotyledons. (G and H) Shoot apices of curled cotyledon-stage embryos seen with DIC optics. The shoot apical meristem of *lec2* mutants is domed and possesses leaf primordia in contrast to the unactivated meristem of wild types. ep, Embryo proper; p, leaf primordium; SAM, shoot apical meristem; s, suspensor. [Bars = 100 µm (A), 20 µm (C, G), 300 µm (E).]

## Results

***LEC2* Gene Isolation.** We identified three new mutant alleles of the *LEC2* gene from T-DNA insertion lines (21) that were designated *lec2-3*, *lec2-4*, and *lec2-5*. Approximately 25% of late-stage embryos from plants heterozygous for these *lec2* mutations were indistinguishable from the original *lec2-1* mutant (15) and differed morphologically from wild-type and *lec1* and *fus3* mutant embryos (Fig. 1 A and B; data not shown). By contrast to wild type, mutant embryos from all lines had abnormal suspensors (Fig. 1 C and D), produced trichomes on cotyledon surfaces (Fig. 1 E and F), and possessed precociously activated shoot apical meristems (Fig. 1 G and H). Unlike *lec1* and *fus3* mutant embryos that die because of their intolerance to desiccation, 50–80% of embryos homozygous for the newly identified *lec2* mutations and the original *lec2-1* mutation were able to germinate from freshly dried seeds. Of those that germinated, 93–97% displayed defective cotyledons in which the distal regions had degenerated to different extents (data not shown; ref. 15). The variable sensitivity of *lec2* mutants to desiccation suggests defects in maturation processes in distal regions of cotyledons.

Although all four *lec2* mutant alleles were derived from plants mutagenized with T-DNA, none of the mutations resulted from an insertion into the gene. Therefore, we cloned the *LEC2* gene based on its position in the genome. *LEC2* was mapped initially between two visual markers, *dis2* and *dis1*, and the molecular markers *PAI3* and m235 on chromosome 1 (30, 31). As shown diagrammatically in Fig. 2A, markers corresponding to the ends of BAC clones that lie between *PAI3* and m235 were mapped by





**Fig. 2.** Genetic mapping and positional cloning of the *LEC2* gene. (A) Diagrammatic representation of the interval on chromosome 1 between *DIS2* and *DIS1*. The position of the *LEC2* gene relative to genetic markers, including the ends of BAC and cosmid clones, are indicated. Numbers in parentheses show recombinant breakpoints observed between the indicated marker and *LEC2*. Positions of cosmid clones that suppress the *lec2* mutation are shown. (B) Representation of the *LEC2* gene. Hatched and shaded boxes indicate exons, and narrow boxes represent introns. Shaded boxes depict the gene region encoding the B3 domain. The position of mutations in specific mutant alleles are indicated. Rearrangement of *lec2-3* was assessed with DNA gel blot hybridization studies.

using plants selected for recombination breakpoints between *LEC2* and *DIS2* and between *LEC2* and *DIS1*. Cosmid clones were prepared from the BAC F1A10 predicted to span the *LEC2* gene. As detailed in Fig. 2A, genetic mapping of selected cosmid clone ends and transgene complementation experiments localized the *LEC2* gene to an 11-kb region equivalent to positions 40,250–51,455 of BAC F3H9 (GenBank accession no. AC021044).

Nucleotide sequences of restriction fragments from the complementing cosmid clones combined with the release of the partial sequence of BAC F3H9 identified a putative gene specifying a single polypeptide in this 11-kb region. Analysis of a near-full-length cDNA clone and amplified reverse transcription products showed that the putative *LEC2* gene consists of six exons as shown diagrammatically in Fig. 2B.

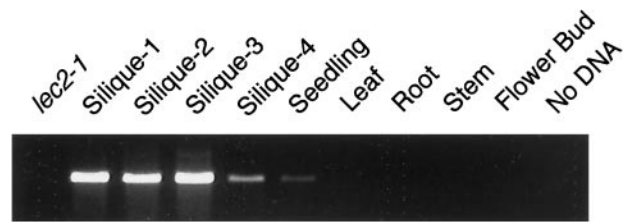
<b>LEC2</b>	<b>QOSTFDNKKLRVLCCEKELKNSDVGSLGRIV</b>	<b>187</b>
<b>FUS3</b>	<b>PARKIDPRKLRFLFOKELKNSDVSSLRMI</b>	<b>107</b>
<b>ABI3</b>	<b>QGWKPE .KNLRFL LQKVLKOSDVGNLGRIV</b>	<b>588</b>
<b>VP1</b>	<b>QGAKAD .KNLRFL LQKVLKOSDVGSLGRIV</b>	<b>533</b>
<b>LEC2</b>	<b>LPKRDAEANLPKLS DKEGIVVQMRDVFVSMQ</b>	<b>217</b>
<b>FUS3</b>	<b>LPKKAEEAHLPALECKEGIPIRMEDLDGFH</b>	<b>137</b>
<b>ABI3</b>	<b>LPKKEAETHLPELEARDGISLAMEDIGTSR</b>	<b>618</b>
<b>VP1</b>	<b>LPKKEAEVHLPELKTRDGISIPMEDIGTSR</b>	<b>563</b>
<b>LEC2</b>	<b>SWSFKYKFWSSNNKSRMYVLENTGEFVKONG</b>	<b>247</b>
<b>FUS3</b>	<b>VWTFKYRWPNNSRMYVLENTGDFVNAHG</b>	<b>167</b>
<b>ABI3</b>	<b>VWNMYRFWPNNSRMYLLENTGDFVKING</b>	<b>648</b>
<b>VP1</b>	<b>VWNMYRFWPNNSRMYLLENTGEFVRSNE</b>	<b>593</b>
<b>LEC2</b>	<b>AEIGDFLTIYEDESKNLYFAMNGNS</b>	<b>272</b>
<b>FUS3</b>	<b>LQLGDFIMVYQDLYSNNYVIQARKA</b>	<b>192</b>
<b>ABI3</b>	<b>LQEGDFIVIYSDVKCGKYLIRGVKV</b>	<b>673</b>
<b>VP1</b>	<b>LQEGDFIVIYSDVKSCKYLIRGVKV</b>	<b>618</b>

**Fig. 3.** *LEC2* contains a B3 domain. Amino acid alignment of residues from the B3 domains of *LEC2*, *FUS3*, *ABI3*, and *VP1*. Residues in black boxes are identical in at least two of the four proteins, and those in shaded boxes share similarity with conserved residues. Numbers in the right column indicate residue numbers in the predicted polypeptides.

In addition to the transgene complementation experiments, analyses of mutant alleles provided support for the identity of the *LEC2* gene. As indicated in Fig. 2B, the *lec2-4* mutation is a deletion of bases 573–583 (11 bp) relative to the translation start codon that creates a truncated protein. The *lec2-5* allele lacks bases 723–755 (33 bp). Both deletions disrupt a part of the encoded protein conserved with other proteins as discussed below. *lec2-1* possesses a deletion that spans 3,048 bp and 895 bp, respectively, upstream and downstream of the translation start codon. DNA gel blot experiments showed that *lec2-3* has a rearrangement or deletion in the 5' region of the gene (data not shown). Thus, all four *lec2* mutant alleles have defects in the same gene. Together, the transgene complementation experiments and analyses of *lec2* mutant alleles demonstrate that we have isolated the *LEC2* gene.

**Predicted *LEC2* Protein Shares Sequence Similarity with Plant Transcription Factors.** The predicted *LEC2* polypeptide consists of 363 amino acid residues with an estimated molecular mass of 41,708 Da. A central region of the protein, whose coding region is indicated diagrammatically in Fig. 2B, shares extensive sequence similarity with the B3 domain, a DNA-binding region found in several plant transcription factors (10, 19, 32–35). The amino- and carboxyl-terminal regions do not share significant similarity with other proteins. *LEC2* B3 domain is most similar to the B3 domains of ABA INSENSITIVE3 (*ABI3*)/VIVIPAROUS1 (*VP1*) and the LEC protein, *FUS3*, sharing 50% and 43% amino acid sequence identity, respectively, with *ABI3* and *FUS3* (Fig. 3; refs. 10, 19, 33). Because all three proteins are transcription factors that function primarily in seeds, we hypothesize that *LEC2* may also serve as a transcriptional regulator of seed development.

***LEC2* Is Expressed Primarily During Embryo Development.** The effects of the *lec2* mutation are limited to embryogenesis. Therefore, we asked whether *LEC2* RNA accumulates specifically during seed development. Primers with sequences near the predicted *LEC2* translation start and stop codons were used to amplify cDNA



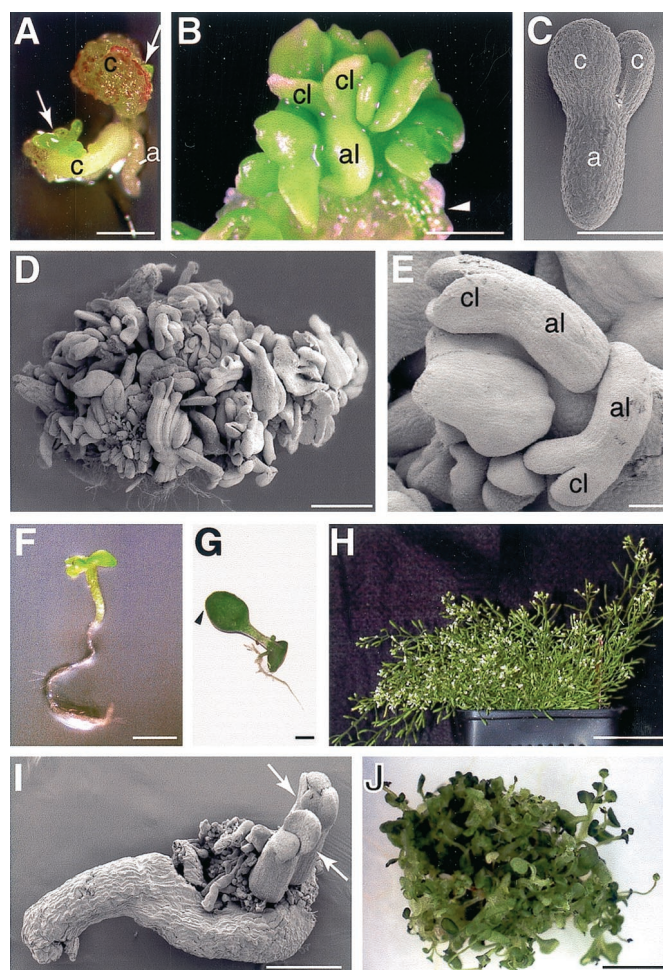
**Fig. 4.** *LEC2* RNA accumulates primarily during seed development. *LEC2* RNA was detected at the indicated stages by amplifying reverse transcription products. Silique stages 1 through 4, respectively, are from siliques containing zygote to early globular-stage embryos, globular-stage to heart-stage embryos, torpedo-stage to curled cotyledon-stage embryos, and mature green embryos. Control experiments showed that a ribosomal protein RNA was amplified with similar efficiency from each reverse transcription reaction.

from wild-type and mutant siliques, seedlings, leaves, roots, stems, and flower buds. As shown in Fig. 4, amplification products were detected primarily in early- and middle-stage siliques and occasionally but not consistently in seedlings, leaves, roots, and stems, suggesting that the RNA may be present at very low levels in vegetative organs. This result suggests that *LEC2* is expressed primarily during seed development.

**Ectopic *LEC2* Expression Induces Somatic Embryo Formation.** Because *LEC2* RNA accumulates primarily during seed development, we expressed the gene postembryonically to obtain additional clues about its role in embryo development. We transferred a *LEC2* cDNA clone under the transcriptional control of the cauliflower mosaic virus 35S promoter into *lec2* mutant plants. Unlike *lec2* mutants with defective cotyledons, transgenic seedlings had complete cotyledon margins, indicating that the transgene had suppressed the mutant phenotypes. However, transgenic seedlings exhibited a range of morphological phenotypes.

At one extreme, represented in Fig. 5*A*, transgenic seedlings possessed embryonic characteristics as exemplified by their small and fleshy cotyledons, short hypocotyls, and unextended roots. Callus-like growth formed primarily on adaxial cotyledon surfaces of seedlings cultured on medium. Remarkably, somatic embryo-like structures, as shown in Figs. 5*A* and *B* and 6*A*, developed from these regions, although cotyledon-like and leaf-like structures and shoots also were observed (data not shown). Occasionally, somatic embryo-like structures emerged over the entire surface of an embryo-like seedling (Fig. 5*D*). Similar results were obtained with wild-type seedlings containing the 35S::*LEC2* transgene.

We believe that these structures are somatic embryos for several reasons. First, Fig. 5*E* shows that somatic embryo-like structures share morphological similarities with zygotic embryos, possessing cotyledon-like and embryonic axis-like organ systems typical of wild-type linear cotyledon-stage zygotic embryos (Fig. 5*C*). Structures similar in appearance to mature green-stage zygotic embryos were observed occasionally (Fig. 5*B*). Second, analyses of tissue sections suggest that somatic embryo-like structures possess axes in that both shoot and root apical meristems were formed (data not shown). Roots of somatic embryos “germinated” readily on medium or attached to plants, indicating that the root apical meristem was functional (Fig. 5*F*). Finally, somatic embryo-like structures expressed embryo-specific genes. Fig. 6*A*, *C*, and *D* shows that RNAs encoding the storage protein cruciferin A and the lipid body protein oleosin accumulate in the somatic embryos and in regions of the underlying cotyledons of embryo-like seedlings. Significantly, *LEC2* RNA was distributed similarly with the embryo-specific RNAs in seedlings giving rise to somatic embryos (Fig. 6*B*), indicating a correspondence between *LEC2* gene expression and

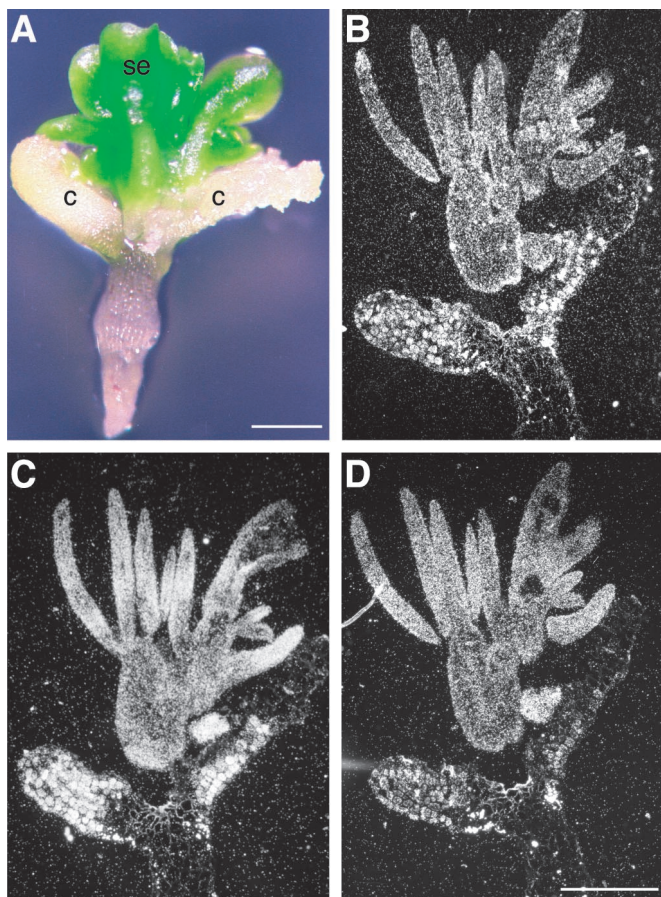


**Fig. 5.** *LEC2* induces somatic embryo development. Seeds from *lec2-1* and *lec2-5* plants transformed with the 35S::*LEC2* gene were germinated, and their subsequent development was monitored. (A) Formation of somatic embryo-like clusters (arrows) on the cotyledons of an embryo-like 35S::*LEC2* seedling. (B) Somatic embryo-like structures emerging from the cotyledon (arrowhead) of a 35S::*LEC2* seedling. (C) Scanning electron microscopy (SEM) photograph of a wild-type linear cotyledon-stage zygotic embryo. (D) A mass of somatic embryo-like structures covering a 35S::*LEC2* seedling as observed by SEM. (E) SEM photograph of somatic embryo-like structures on a 35S::*LEC2* seedling. (F) Seedling resulting from the germination of a 35S::*LEC2* somatic embryo formed on a wild-type transgenic seedling. (G) A 35S::*LEC2* seedling with a wild-type phenotype. Arrowhead shows that the distal region of the cotyledon is not defective. (H) A short, bushy plant grown on soil that developed from a 35S::*LEC2* seedling such as that shown in *G*. (I) Somatic embryos (arrows) emerging from leaf-like organ dissected from a plantlet mass. (J) A mass of plantlets formed from a single 35S::*LEC2* seedling such as that shown in *G*. a, Embryonic axis; al, embryonic axis-like; c, cotyledon; cl, cotyledon-like. [Bars = 1 mm (A, B, D, F, G), 100  $\mu$ m (C, E), 500  $\mu$ m (I), 1 cm (J), and 5 cm (H).]

development of embryonic characteristics. Although *LEC2*-induced somatic embryos were larger than zygotic embryos, similar size differences are observed with *Arabidopsis* somatic embryos generated by other methods (36).

As shown in Fig. 5*G*, seedlings at the other morphological extreme had expanded cotyledons and resembled wild type (compare Fig. 1*F*). Seedlings of this type with extended roots typically developed into small, bushy plants when grown on soil, as shown in Fig. 5*H*. When 35S::*LEC2* seedlings were cultured on medium, we were initially surprised to find that these seedlings gave rise to masses of vegetatively growing plantlets (Fig. 5*J*), given the apparent role of *LEC2* in promoting embryo development. However, further analysis showed that plantlets





**Fig. 6.** *LEC2*-induced somatic embryos express embryo-specific genes. (A) Whole-mount photograph of a *35S::LEC2* seedling with somatic embryo-like structures on its cotyledons. Sections of the same seedling were hybridized with antisense probes for *LEC2* RNA (B), cruciferin A storage protein RNA (C), and oleosin lipid body protein RNA (D). After autoradiography, sections were photographed with use of dark-field optics. A sense-strand probe did not hybridize appreciably with the sections (data not shown). Regions of the seedlings in B–D that did not hybridize lacked cellular contents and were likely dead. c, Cotyledon; se, somatic embryo. (Bars = 0.5 mm.)

seem to have developed from somatic embryos, as shown in Fig. 5I. Taken together, these results suggest that *LEC2* is sufficient to induce somatic embryo development.

## Discussion

The pleiotropic effects of *lec* mutations, represented in Fig. 1, suggest that the *LEC* genes serve as central regulators of embryo and seed development. This prediction was confirmed by showing that *LEC1* and *FUS3* encode putative transcription factors that regulate critical embryonic processes (12, 19, 20). Here we present evidence suggesting that *LEC2* likely encodes a transcriptional regulator with a significant role in embryogenesis.

### **LEC2 Possesses a B3 Domain Characteristic of Transcription Factors.**

Our findings that the *LEC2* gene encodes a B3 domain protein (Fig. 3) and is expressed primarily during embryogenesis (Fig. 4) suggest that it, like *LEC1* and *FUS3*, is a transcriptional regulator of seed development. The B3 domain is an  $\approx 120$ -amino acid residue region defined originally as the third basic region of maize VP1, which shares the largest contiguous block of sequence identity with its ortholog, *Arabidopsis* ABI3 (10). Subsequently, this domain was identified in other plant proteins, such as AUXIN RESPONSE FACTOR1 (34) and RELATED

TO ABI3/VP1 (32). Many proteins containing B3 domains, including ABI3/VP1, *FUS3*, and AUXIN RESPONSE FACTOR1, function as transcription factors (20, 33, 34). The B3 domain is responsible, at least in part, for the DNA-binding activity of ABI3/VP1 and RELATED TO ABI3/VP1 (32, 37, 38). To our knowledge, the B3 DNA-binding domain is unique to plants.

Maximum parsimony analysis (39) of the B3 domains of 48 predicted *Arabidopsis* proteins suggests that *LEC2*, *FUS3*, and *ABI3* constitute one class, perhaps indicating their common origin (S.L.S. and J.J.H., unpublished results). All three genes are expressed primarily during embryogenesis and are required genetically for the completion of normal seed development. Although they all play roles in seed development, their specific functions have diverged. *ABI3* is a transcription factor that operates primarily during the maturation phase, whereas *LEC2* and *FUS3* are LEAFY COTYLEDON proteins required for processes during both the morphogenesis and maturation phases.

### **Role of LEC2 in Seed Development.**

Two consequences of expressing the *LEC2* gene postembryonically provide insight into its role in embryo development and strongly support the conclusion that *LEC2* is a key regulator of embryogenesis. First, although seedlings expressing the *35S::LEC2* gene exhibited a range of morphologies (Fig. 5), approximately one-third possessed embryonic rather than postgerminative characteristics. Cotyledons of these seedlings remained fleshy and did not expand, and their roots and hypocotyls did not extend, suggesting that ectopic *LEC2* expression can extend embryonic development. *35S::LEC2* seedlings at the other end of the phenotypic spectrum initially resembled wild type. The reason for this range of phenotypes is not clear, but one possibility is that it relates to differences in transgene expression levels. Second, regardless of their initial morphology, *35S::LEC2* seedlings gave rise to somatic embryos as indicated by their morphological similarities with zygotic embryos, their expression of embryo-specific genes, and their establishment of functional apical meristems (Figs. 5 and 6). Together, these results suggest that ectopic *LEC2* expression is sufficient to establish an embryonic environment that promotes somatic embryo formation. In this regard, it is possible that *LEC2* RNA accumulation is regulated posttranscriptionally, thereby accounting for the ability of somatic embryos to “germinate” and, in some cases, give rise to masses of vegetatively growing plantlets.

Transgenic seedlings ectopically expressing a different *LEC* gene, *LEC1*, have embryonic characteristics that are substantially similar to those of *35S::LEC2* embryo-like seedlings (12). Moreover, postembryonic expression of *LEC1* is sufficient to induce somatic embryogenesis in transgenic plants as occurs with *35S::LEC2* seedlings, although somatic embryo formation is more robust with *LEC2*. Recessive mutations in the *Arabidopsis* PICKLE (*PKL*) gene also induce somatic embryogenesis in postgerminative roots cultured on hormone-free medium (40). However, *LEC1* is expressed in the roots of *pkl* mutant but not wild-type seedlings (41). This result suggests that the chromo-domain protein PKL acts normally to repress *LEC1* in postgerminative roots, and therefore that *LEC1*, at least in part, mediates somatic embryo formation in *pkl* mutants. Given our results, we hypothesize that *LEC2* may also be derepressed in *pkl* mutants.

The ability of *LEC2* and *LEC1* to induce somatic embryogenesis suggests a role for the genes in the establishment of embryogenic competence. Somatic plant cells generally must be induced to become competent for somatic embryogenesis (42–44). Embryogenic competence is often induced by culturing cells with the hormone auxin and, sometimes cytokinin, although completion of somatic embryogenesis usually requires removal

of the hormone(s). *LEC2* and *LEC1* obviate the need for hormone treatments in the acquisition of embryogenic competence, suggesting the two *LEC* transcription factors activate genes that play roles in the initiation of somatic embryogenesis. Our finding that both *LEC2* and *LEC1* RNAs are detected at the earliest embryonic stages tested (Fig. 4; ref. 12) opens the possibility that both genes are involved in establishing embryogenic competence during zygotic embryogenesis. Thus, the role of *LEC2* and *LEC1* in somatic embryogenesis may reflect their function in zygotic embryogenesis. Similarities in the expression patterns and overexpression phenotypes of *LEC2* and *LEC1* suggest that they may have partially overlapping roles early in zygotic embryogenesis to induce embryo formation. This interpretation is consistent with analyses showing that *lec1 lec2* double mutants arrest at an earlier embryonic stage than either single mutant, which indicates partial genetic redundancy (12, 15).

Although *LEC1* and *LEC2* are each sufficient to induce

embryogenic competence in somatic cells, they have similar but not identical functions. Mutations in each gene result in distinct phenotypes, and the double mutant displays a synergistic phenotype (12, 15, 16). Furthermore, the vast majority of *35S::LEC1* seedlings arrest as embryo-like seedlings and fail to develop further, although cotyledon-like organs sometimes form in place of the first true leaves (12). By contrast, *35S::LEC2* embryo-like seedlings continued to proliferate, producing callus, cotyledon-like and leaf-like organs in addition to somatic embryos. Thus, *LEC1* and *LEC2* may have complementary but partially redundant functions in embryo formation. The precise roles of *LEC1* and *LEC2* in embryo development remain to be determined.

We thank David Meinke for providing the *lec2-1* mutant, the *Arabidopsis* Biological Resource Center for BAC clones and mutant lines, Sara Wortley and Julia Little for technical assistance, and Chuck Gasser and John Bowman and members of their labs for plasmids and advice. This work was supported by grants from Ceres, Inc., and the Department of Energy (to J.J.H.).

- Olsen, O. A. (2001) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **52**, 241–267.
- Goldberg, R. B., de Paiva, G. & Yadegari, R. (1994) *Science* **266**, 605–614.
- West, M. A. & Harada, J. J. (1993) *Plant Cell* **5**, 1361–1369.
- Jurgens, G. (2001) *EMBO J.* **20**, 3609–3616.
- Bewley, J. D. (1997) *Plant Cell* **9**, 1055–1066.
- Harada, J. J. (1997) *Advances in Cellular and Molecular Biology of Plants*, eds. Larkins, B. A. & Vasil, I. K. (Kluwer, Dordrecht, The Netherlands), Vol. 4, pp. 545–592.
- Harada, J. J. (2001) *J. Plant Physiol.* **158**, 405–409.
- Di Lorenzo, L., Wysocka-Diller, J., Malamy, J. E., Pysh, L., Helariutta, Y., Freshour, G., Hahn, M. G., Feldmann, K. A. & Benfey, P. N. (1996) *Cell* **86**, 423–433.
- Long, J. A., Moan, E. I., Medford, J. I. & Barton, M. K. (1996) *Nature (London)* **379**, 66–69.
- Giraudat, J., Hauge, B. M., Valon, C., Smalle, J., Parcy, F. & Goodman, H. M. (1992) *Plant Cell* **4**, 1251–1261.
- Finkelstein, R. R. & Lynch, T. J. (2000) *Plant Cell* **12**, 599–609.
- Lotan, T., Ohto, M., Matsudaira Yee, K., West, M. A. L., Lo, R., Kwong, R. W., Yamagishi, K., Fischer, R. L., Goldberg, R. B. & Harada, J. J. (1998) *Cell* **93**, 1195–1205.
- Keith, K., Kraml, M., Dengler, N. G. & McCourt, P. (1994) *Plant Cell* **6**, 589–600.
- Meinke, D. W. (1992) *Science* **258**, 1647–1650.
- Meinke, D. W., Franzmann, L. H., Nickle, T. C. & Yeung, E. C. (1994) *Plant Cell* **6**, 1049–1064.
- West, M. A. L., Matsudaira Yee, K., Danao, J., Zimmerman, J. L., Fischer, R. L., Goldberg, R. B. & Harada, J. J. (1994) *Plant Cell* **6**, 1731–1745.
- Baumlein, H., Misera, S., Luerben, H., Kollé, K., Horstmann, C., Wobus, U. & Muller, A. J. (1994) *Plant J.* **6**, 379–387.
- Nambara, E., Hayama, R., Tsuchiya, Y., Nishimura, M., Kawaide, H., Kamiya, Y. & Naito, S. (2000) *Dev. Biol.* **220**, 412–423.
- Luerben, H., Kirik, V., Herrmann, P. & Misera, S. (1998) *Plant J.* **15**, 755–764.
- Reidt, W., Wohlfarth, T., Ellerstroem, M., Czihal, A., Tewes, A., Ezcurra, I., Rask, L. & Baumlein, H. (2000) *Plant J.* **21**, 401–408.
- Bechtold, N., Ellis, J. & Pelletier, G. (1993) *C. R. Acad. Sci. Paris* **316**, 1194–1199.
- Neff, M. M., Neff, J. D., Chory, J. & Pepper, A. E. (1998) *Plant J.* **14**, 387–392.
- Konieczny, A. & Ausubel, F. M. (1993) *Plant J.* **4**, 403–410.
- Bell, C. & Ecker, J. R. (1994) *Genomics* **19**, 137–144.
- Olszewski, N. E., Martin, F. B. & Ausubel, F. M. (1988) *Nucleic Acids Res.* **16**, 10765–10782.
- Wilkins, T. A. & Smart, L. B. (1996) *A Laboratory Guide to RNA: Isolation, Analysis and Synthesis*, ed. Krieg, P. (Wiley-Liss, New York), pp. 21–41.
- Kawasaki, E. S. (1990) *PCR Protocols: A Guide to Methods and Applications*, ed. Innis, M. A. (Academic, San Diego), pp. 21–27.
- Dietrich, R. A., Maslyar, D. J., Heupel, R. C. & Harada, J. J. (1989) *Plant Cell* **1**, 73–80.
- Gleave, A. P. (1992) *Plant Mol. Biol.* **20**, 1203–1207.
- Feenstra, W. J. (1978) *Arabidopsis Inf. Serv.* **15**, 35–38.
- Bender, J. & Fink, G. R. (1995) *Cell* **83**, 725–734.
- Kagaya, Y., Ohmiya, K. & Hattori, T. (1999) *Nucleic Acids Res.* **27**, 470–478.
- McCarty, D. R., Hattori, T., Carson, C. B., Vasil, V., Lazar, M. & Vasil, I. K. (1991) *Cell* **66**, 895–906.
- Ulmasov, T., Hagen, G. & Guilfoyle, T. J. (1997) *Science* **276**, 1865–1868.
- Hardtke, C. S. & Berleth, T. (1998) *EMBO J.* **17**, 1405–1411.
- Mordhorst, A. P., Voerman, K. J., Hartog, M. V., Meijer, E. A., van Went, J., Koornneef, M. & de Vries, S. C. (1998) *Genetics* **149**, 549–563.
- Ezcurra, I., Wycliffe, P., Nehlin, L., Ellerstrom, M. & Rask, L. (2000) *Plant J.* **24**, 57–66.
- Suzuki, M., Kao, C. Y. & McCarty, D. R. (1997) *Plant Cell* **9**, 799–807.
- Hershkovitz, M. A. & Leipe, D. D. (1998) *Bioinformatics: A Practical Guide to the Analysis of Genes and Proteins*, ed. Baxevanis, A. D. (Wiley-Liss, New York), pp. 189–230.
- Ogas, J., Cheng, J.-C., Sung, Z. R. & Somerville, C. (1997) *Science* **277**, 91–94.
- Ogas, J., Kaufmann, S., Henderson, J. & Somerville, C. (1999) *Proc. Natl. Acad. Sci. USA* **96**, 13839–13844.
- Mordhorst, A. P., Toonen, M. A. J. & de Vries, S. C. (1997) *Crit. Rev. Plant Sci.* **16**, 535–576.
- Zimmerman, J. L. (1993) *Plant Cell* **5**, 1411–1423.
- Dudits, D., Bogre, L. & Gyorgyey, J. (1991) *J. Cell Sci.* **99**, 475–484.