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RESEARCH ARTICLE

LEAFY COTYLEDON1-LIKE Defines a Class of Regulators Essential for Embryo Development

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Arabidopsis LEAFY COTYLEDON1 (LEC1) is a critical regulator required for normal development during the early and late phases of embryogenesis that is sufficient to induce embryonic development in vegetative cells. *LEC1* encodes a HAP3 subunit of the CCAAT binding transcription factor. We show that the 10 Arabidopsis HAP3 (AHAP3) subunits can be divided into two classes based on sequence identity in their central, conserved B domain. LEC1 and its most closely related subunit, LEC1-LIKE (L1L), constitute LEC1-type AHAP3 subunits, whereas the remaining AHAP3 subunits are designated non-LEC1-type. Similar to *LEC1*, *L1L* is expressed primarily during seed development. However, suppression of *L1L* gene expression induced defects in embryo development that differed from those of *lec1* mutants, suggesting that LEC1 and L1L play unique roles in embryogenesis. We show that *L1L* expressed under the control of DNA sequences flanking the *LEC1* gene suppressed genetically the *lec1* mutation, suggesting that the LEC1-type B domains of L1L and LEC1 are critical for their function in embryogenesis. Our results also suggest that LEC1-type HAP3 subunits arose from a common origin uniquely in plants. Thus, L1L, an essential regulator of embryo development, defines a unique class of plant HAP3 subunits.

INTRODUCTION

The single-celled zygote of a flowering plant undergoes a series of controlled cell divisions and cell differentiation events that lead to the formation of a mature, multicellular embryo that is metabolically quiescent and desiccated. Early in embryogenesis, during the morphogenesis phase, the plant body is formed through the establishment of the shoot-root axis and the formation of the embryonic tissue and organ systems (West and Harada, 1993; Goldberg et al., 1994; Laux and Jurgens, 1997; Jurgens, 2001). Later, during the seed maturation phase, the embryo acquires the ability to withstand desiccation, accumulates storage macromolecules such as lipids and proteins, and becomes metabolically quiescent as a result of desiccation (reviewed by Bewley, 1997; Harada, 1997). Once environmental conditions are favorable, the seed germinates and the vegetative phase of the life cycle begins.

Genetic studies have identified regulatory genes that play critical roles in embryogenesis during either the morphogenesis or the maturation phases. For example, genes such as *WUSCHEL*, *SHOOTMERISTEMLESS*, *SCARECROW*, and *SHORT ROOT* (Dilaurenzio et al., 1996; Long et al., 1996; Mayer et al., 1998; Helariutta et al., 2000) have been shown to be essential for the formation of the shoot and root apical meristems that define the embryonic axis of developing Arabidopsis embryos. A different class of genes, including *ABSCISIC ACID INSENSITIVE3* (*ABI3*), *ABI4*, and *ABI5*, play important roles during the maturation phase of embryogenesis (Giraudat et al., 1992; Finkelstein et al., 1998; Finkelstein and Lynch, 2000), preparing the embryo for desiccation and postgerminative growth.

Another set of genes encoding Arabidopsis LEAFY COTYLEDON (LEC) proteins, LEC1, LEC2, and FUSCA3, are unique in that they are the only known embryonic regulators required for normal development during both the morphogenesis and maturation phases (reviewed by Harada, 2001). For example, LEC1 is required to maintain suspensor cell

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fate, to specify cotyledon identity in the early morphogenesis phase, and to initiate and/or maintain the maturation phase and inhibit precocious germination late in embryogenesis (Meinke, 1992; Meinke et al., 1994; West et al., 1994; Parcy et al., 1997; Lotan et al., 1998; Vicient et al., 2000). Furthermore, ectopic postembryonic expression of *LEC1* is sufficient to confer embryonic characteristics to seedlings and to induce somatic embryo formation from vegetative cells (Lotan et al., 1998). Because LEC1 is required for normal development both early and late during embryogenesis and is sufficient to confer embryogenic competence to vegetative cells, it is a central regulator that acts far upstream in the regulatory hierarchy that controls embryogenesis. We speculate that *LEC1* establishes a cellular environment that promotes embryo development and that this environment coordinates the early and late phases of embryogenesis in flowering plants (Lotan et al., 1998). A major goal of our research is to understand, at a mechanistic level, how LEC1 establishes competence to initiate embryo development.

Given the key role of LEC1 in the control of embryogenesis, we asked if genes related to *LEC1* also encode embryonic regulators. LEC1 shares significant sequence similarity with the HAP3 subunit of CCAAT binding factor (CBF, also known as NF-Y; Lotan et al., 1998). CBFs are eukaryotic transcriptional activators that serve diverse roles in different organisms (Li et al., 1992). In yeast, CBF activates a set of genes involved in mitochondrial respiration (Guarente et al., 1984; Keng and Guarente, 1987; Trueblood et al., 1988; Schneider and Guarente, 1991), whereas mammalian CBFs are thought to act generally to enhance transcription rates, often in combination with other proteins (reviewed by Maity and De Crombrugghe, 1998; Mantovani, 1999). The transcription factor is a hetero-oligomeric complex consisting of at least three subunits, HAP2, HAP3, and HAP5, although yeast possesses a fourth subunit, HAP4 (reviewed by Maity and De Crombrugghe, 1998; Mantovani, 1999). HAP3 subunits are recognized by their central B domain, an ~90–amino acid region of the protein that is conserved across eukaryotic organisms. For example, the LEC1 B domain has 57 and 62% sequence identity with HAP3 subunits from yeast and mammals, respectively. Thus, LEC1 appears to encode a subunit of a transcription factor that regulates the expression of genes required for embryo development.

We used the LEC1 polypeptide sequence to identify other genes encoding Arabidopsis HAP3 (AHAP3) subunits. We show that the subunit most closely related to LEC1, designated LEC1-LIKE (L1L), is required for normal embryo development. L1L and LEC1 have distinct functions in embryogenesis, but L1L can substitute functionally for LEC1 when expressed ectopically. Comparison of the deduced amino acid sequences of L1L and LEC1 identified specific amino acid sequences that appear to be required for the function of these proteins in regulating embryo identity and development.

RESULTS

Arabidopsis HAP3 Proteins Are Encoded by a Gene Family That Can Be Divided into Two Classes

We used the amino acid sequence of LEC1 as a query to identify related Arabidopsis polypeptides. Database searches of the sequenced Arabidopsis genome showed that there are nine genes encoding proteins that share significant sequence identity and that the gene encoding L1L (At5g47670) is related most closely to LEC1. As shown in Figure 1, sequence similarity among the 10 proteins is limited primarily to the central B domain, consistent with comparisons of HAP3 subunits from other organisms (Li et al., 1992). Only two of these putative proteins, At2g37060 and At3g53340, display sequence identity with each other in the N-terminal A domain or the C-terminal C domain. Because the B domain has been shown to underlie HAP3 function in other organisms (Xing et al., 1993; Kim et al., 1996; Sinha et al., 1996) and because all of these Arabidopsis proteins possess residues that are conserved among HAP3 proteins, we conclude that there are 10 AHAP3 subunits.

Close examination of B-domain sequence alignments showed that L1L and LEC1 define a distinct class of AHAP3 subunits. The two proteins share 83% sequence identity with each other but only 52 to 71% identity with the other eight AHAP3 subunits. Furthermore, L1L and LEC1 share the amino acid residues highlighted in red in Figure 1 that differ from the residues that are conserved in the other eight AHAP3 subunits. On the basis of sequence identity within the B domain, we define two classes of AHAP3 subunits: the LEC1-type and the non-LEC1-type. Thus, L1L is the AHAP3 most closely related to LEC1, opening the possibility that L1L also may be an embryonic regulator.

L1L RNA Accumulates Primarily in Developing Embryos

We analyzed *L1L* gene expression to obtain clues about its role during development. *L1L* RNA was detected in RNA gel blot hybridization experiments in developing siliques but not in vegetative organs or in flowers, as shown in Figure 2A. This pattern of RNA accumulation closely resembled that of *LEC1*, which accumulates specifically in seeds and differed substantially from those of non-LEC1-type AHAP3 subunits, whose RNAs are not limited to seed development (Edwards et al., 1998; Lotan et al., 1998; Gusmaroli et al., 2001; M. Kim and J.J. Harada, unpublished results). However, differences between *L1L* and *LEC1* RNA accumulation patterns were observed. Figure 2A shows that *L1L* RNA levels peaked at a later stage of embryogenesis than did *LEC1* levels. Furthermore, sensitive reverse transcriptase–mediated (RT) PCR amplification experiments indicated that *L1L* RNA is present in all vegetative organs, although presumably at low levels, as shown in Figure 2B. By contrast, *LEC1* RNA

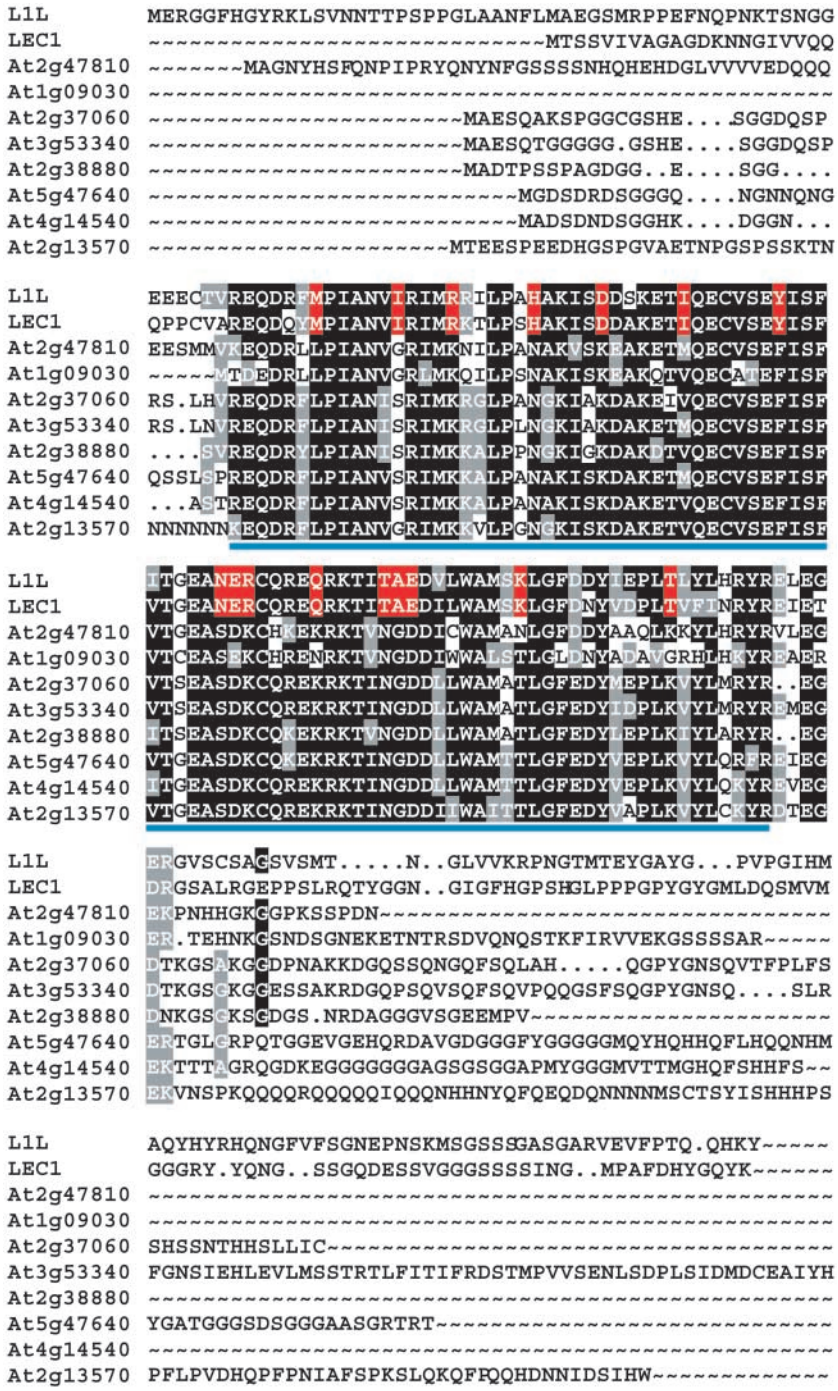

Figure 1. Analysis of Arabidopsis HAP3 Subunits.

Amino acid sequence alignment of AHAP3 proteins. Residues highlighted in black and gray represent identical and similar amino acids, respectively. B-domain residues shared between LEC1 (At1g21970) and L1L (At5g47670) but not with the other proteins are highlighted in red. The B domain is underlined.

was not detected in vegetative organs in parallel experiments. These results suggest that L1L is likely to function primarily during seed development and that it is expressed differently from *LEC1*.

We localized *L1L* RNA using in situ hybridization experiments to determine where it functions in developing siliques. As shown in Figures 3A to 3C and 3E to 3G, *L1L* RNA was detected at low but statistically significant levels in the developing embryo proper, suspensor, and endosperm at early stages, including zygotes (data not shown). During the torpedo stage (Figures 3C and 3G) and the linear cotyledon stage (Figures 3D and 3H), *L1L* RNA became prevalent primarily in the outer cell layers of the embryo, similar to the distribution of *LEC1* RNA (Lotan et al., 1998). *L1L* RNA became evenly distributed throughout the embryo at a high level by the bent-cotyledon stage (Figures 3I, 3J, 3M, and 3N) and was present at low levels in mature-stage embryos (Figures 3K, 3L, 3O, and 3P). This temporal pattern of RNA accumulation corresponds with the results from RNA gel blot analyses. Sense RNA did not bind appreciably with the sections, showing the specificity of the hybridization reactions (data not shown). Together, the RNA accumulation patterns suggest a role for *L1L* in embryogenesis.

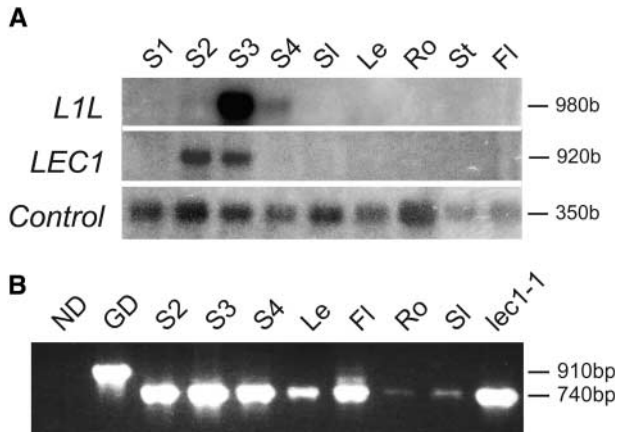

Figure 2. *L1L* RNA Is Detected Predominantly in Developing Siliques.

(A) Analysis of *L1L* and *LEC1* RNA levels with RNA gel blot hybridization experiments. Each lane contained 1 μg of poly(A) RNA from siliques with zygote- to early-globular-stage seeds (S1), siliques with globular- to heart-stage seeds (S2), siliques with torpedo- to bent-cotyledon-stage seeds (S3), siliques with mature green seeds (S4), 2-day-old seedlings (Sl), mature rosette leaves (Le), 3-week-old seedling roots (Ro), stems (St), and unopened floral buds and inflorescences (Fl). Control represents the accumulation of a ribosomal protein RNA.

(B) RT-PCR amplification of *L1L* RNA. Abbreviations are as in **(A)** with the following additions: ND, no DNA; GD, wild-type genomic DNA; and *lec1-1*, mutant siliques with torpedo- to bent-cotyledon-stage embryos.

L1L Is Required for Embryogenesis

Given that *L1L* is expressed primarily during embryogenesis, we used RNA interference (RNAi) experiments to determine if the suppression of *L1L* RNA levels affected embryo development. Because *L1L* shares substantial identity with other AHAP3 subunits in the central B domain, *L1L*-specific nucleotide sequences encoding the C domain were used for targeted suppression (see Methods). Wild-type plants were transformed with the *L1L* RNAi construct under the control of the *35S* promoter, and transgenic plants were recovered. Thirteen of 172 T1 transgenic lines produced defective T2 seeds. More specifically, T1 plants from three independently derived lines segregated 30.1% (n = 1372), 31.9% (n = 1156), and 21.7% (n = 1327) defective T2 seeds. Although the RNAi construct was incompletely penetrant, these results suggest that L1L is required for embryo development. We also found that 4 of 15 lines containing a *35S:L1L* transgene segregated defective seeds. Together, these results suggest that cosuppression of *L1L* gene expression induces defects in embryogenesis (see below).

As shown in Figures 4B to 4D, RNAi mutants arrested at a number of different embryonic stages with a range of morphological phenotypes. Some embryos arrested at the globular stage (Figures 4B and 4C) but had extra cells in the suspensor. Other mutants arrested at later embryonic stages and had reduced cotyledons (Figure 4D). Seeds containing these defective embryos did not germinate, nor did immature seeds collected before desiccation germinate in culture. However, the effects appeared to be limited to seed development, because no defects in the vegetative development of the RNAi transgenic lines were detected. Suppression of *L1L* gene expression induced embryonic defects that differed from those caused by the *lec1* mutation (Lotan et al., 1998).

To confirm that defects in embryo development resulted from the silencing of the *L1L* gene, we analyzed *L1L* RNA levels in transgenic lines using in situ hybridization experiments with *L1L*-specific probes that excluded sequences encoding the C domain. *L1L* RNA was not detected at significant levels in 60 of 62 embryos with a mutant phenotype (Figures 4E and 4F) from four independent transgenic lines and was present only at a low level compared with the wild type in the 2 other mutant embryos. Of embryos that segregated with a wild-type phenotype, 31% (n = 352) possessed high levels of *L1L* RNA, similar to embryos with a wild-type genotype (Figures 4G and 4H), whereas the remainder had only intermediate levels. We interpret these results to indicate that very low levels of *L1L* RNA do not support embryo development but intermediate levels are sufficient for normal embryogenesis. The incomplete penetrance and variable expressivity of RNAi suppression of *L1L* gene expression probably allowed us to recover viable progeny containing the transgene. We also demonstrated the specificity of gene silencing by showing that *LEC1*, oleosin, and cruciferin storage protein RNAs were detected in

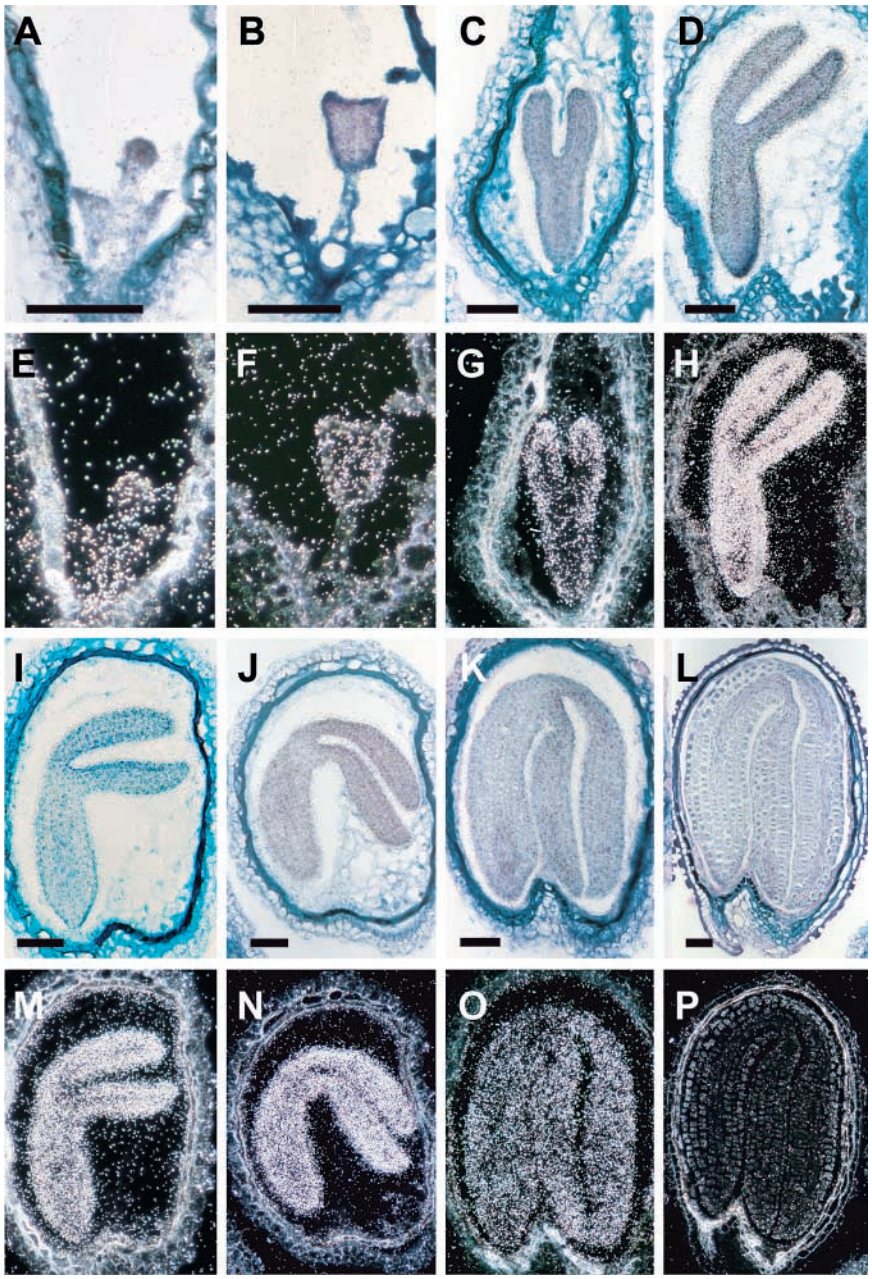

Figure 3. In Situ Detection of *L1L* RNA in Developing Embryos.

Wild-type seed sections were hybridized with an *L1L*-specific antisense probe. All sections were exposed for 10 days. **(A)** to **(D)** and **(I)** to **(L)** show bright-field micrographs, and **(E)** to **(H)** and **(M)** to **(P)** show dark-field micrographs. The sense RNA control did not bind appreciably with the sections. Bars = 50 μm.

(A) and **(E)** Globular-stage embryo.
(B) and **(F)** Heart-stage embryo.
(C) and **(G)** Linear cotyledon-stage embryo.
(D) and **(H)** Early bent-cotyledon-stage embryo.
(I) and **(M)** Bent-cotyledon-stage embryo.
(J) and **(N)** Late bent-cotyledon-stage embryo.
(K) and **(O)** Mature green-stage embryo.
(L) and **(P)** Mature yellowing-stage embryo.

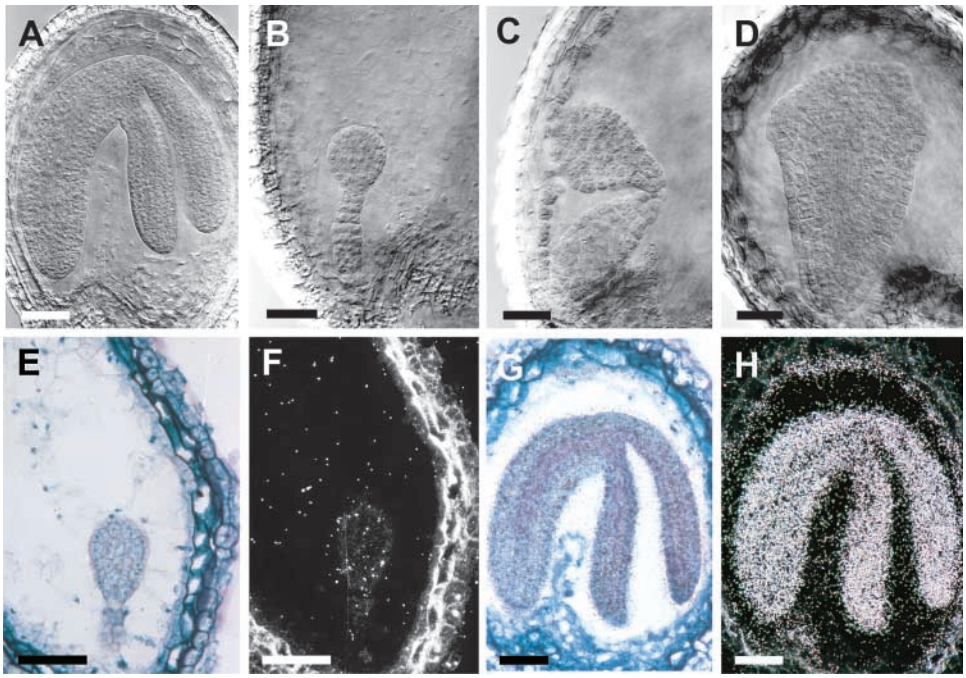

Figure 4. RNAi Suppression of *L1L* Gene Expression Induces Embryo Defects.

(A) Seed with a wild-type embryo at the bent-cotyledon stage. The seed was cleared and viewed with Nomarski optics.
(B) to **(D)** Cleared seeds containing defective embryos from lines containing the *L1L* RNAi constructs. Progeny segregating with a wild-type phenotype in the same silique were at the bent-cotyledon stage.
(E) to **(H)** *L1L* RNA accumulation in defective embryos. Sections were hybridized with an antisense *L1L* probe and exposed for 10 days.
(E) and **(F)** Bright- and dark-field micrographs of a defective embryo from a line containing the *L1L* RNAi construct.
(G) and **(H)** Bright- and dark-field micrographs of a wild-type embryo at the mature green stage.
Bars = 50 μm in **(A)**, **(D)**, **(E)**, and **(H)** and 25 μm in **(B)** and **(C)**.

RNAi embryos exhibiting a mutant phenotype as they were in wild-type embryos (data not shown). Together, these data suggest strongly that *L1L* is essential for embryo development.

Ectopically Expressed *L1L* Can Function in Place of *LEC1*

To examine the functional relationship between L1L and LEC1, we asked if *L1L* could suppress the *lec1* mutation when expressed ectopically. *L1L* RNA was detected in *lec1-1* null mutants (Figure 2B), indicating that the endogenous *L1L* gene cannot substitute completely for the *LEC1* gene. Although the spatial distribution of *L1L* RNA was similar to that of *LEC1*, there were temporal differences in accumulation during embryogenesis (Figures 2 and 3) (Lotan et al., 1998). Therefore, we fused the *L1L* coding region with 1997 and 774 bp of sequence 5′ and 3′, respectively, of the *LEC1* coding region and transferred the chimeric gene into *lec1-1* null mutants. The *lec1* mutation causes embryos to become intolerant of desiccation, and no mutant seeds germinate (Meinke, 1992; West et al., 1994; Lotan et al., 1998). As shown in Table 1 and Figure 5A, transgenic *lec1-1* mutant seeds that were dried extensively (see Methods) produced viable seedlings, indicating that *L1L* expressed under the control of *LEC1* flanking DNA sequences was able to rescue the desiccation intolerance of *lec1* mutants. Moreover, no embryonic or postembryonic abnormalities were detected in *lec1* mutant plants containing the *L1L* transgene, and T1 plants segregated progeny with wild-type and mutant phenotypes at ratios indicating the presence of one, two, or multiple transgenes (data not shown). By contrast, the expression of two genes encoding non-LEC1-type AHAP3 subunits, At4g14540 and At3g53340, under the control of *LEC1* 5′ and 3′ flanking sequences did not rescue the desiccation intolerance of the *lec1* mutants significantly (Table 1). Together, these results suggest that *L1L* but not non-LEC1-type *AHAP3* genes can function in place of *LEC1* when expressed ectopically.

We also showed that L1L reproduced effects caused by LEC1 when both were expressed postembryonically. We fused the *L1L* coding region with the *35S* promoter from

Table 1. Transgene Suppression of the *lec1-1* Mutation

Gene[a]	Total Seeds Screened	Desiccation-Tolerant Seeds[b]	Percentage of Viable Seeds[c]
LEC1	13,600	82	0.60
L1L	10,885	71	0.65
At4g14540	12,800	0	0
At3g53340	12,800	7	0.05

[a] Genes were fused with the *LEC1* promoter and terminator and transferred into *lec1-1* mutants.
[b] Number of seeds that germinated after drying for 2 weeks at 28°C.
[c] Percentage of viable seeds reflects both transformation efficiency and the ability of the construct to suppress the *lec1* mutation.

Cauliflower mosaic virus (Odell et al., 1985) and transferred the construct into *lec1-1* mutants. Plants containing *35S:L1L* produced viable seedlings, indicating that the transgene could rescue the desiccation intolerance of *lec1* mutants. However, the seedlings did not resemble wild-type. Rather, as shown in Figure 5B, transgenic seedlings developed thick and fleshy cotyledons and hypocotyls. Between 25 and 59% of the transgenic seedlings developed multiple pairs of fleshy, cotyledon-like structures at positions normally occupied by leaves, whereas the other seedlings remained arrested in their development, with only two cotyledons. Seedlings with a wild-type genotype transformed with the *35S:L1L* construct displayed similar phenotypes (data not shown). These morphological defects reproduced those observed in transgenic seedlings overexpressing *LEC1* (Lotan et al., 1998), although we did not detect somatic embryos on *35S:L1L* seedlings.

We used in situ hybridization experiments to determine if these fleshy seedlings express embryonic programs of development. As shown in Figures 5C and 5D, RNAs encoding cruciferin storage protein and oleosin lipid body protein, which normally accumulate specifically during embryo development, were detected in these transgenic seedlings. Thus, ectopic expression of *L1L* reproduces the effects of *LEC1* overexpression by creating an environment sufficient to induce embryonic characteristics in vegetative organs. By contrast, expression of a non-LEC1-type *AHAP3*, At4g14540, under the control of the *35S* promoter did not induce detectable developmental abnormalities (data not shown). Together, these results demonstrate that L1L can confer embryonic characteristics to seedlings and support the idea that the LEC1-type B domain may underlie L1L and LEC1 function in embryogenesis.

L1L Genes Are Present in Other Plants

Because L1L and LEC1 both are required for embryo development, we determined the extent to which LEC1-type AHAP3 subunits are present in other organisms. As part of a study to obtain ESTs from the embryo proper of globular-stage scarlet runner bean embryos at 6 days after pollination (A.Q. Bui, K. Weterings, and R.B. Goldberg, unpublished results), cDNA clones encoding a HAP3 subunit with a LEC1-type B domain were identified. Because sequence analysis revealed that the predicted protein shared 94% sequence identity with the Arabidopsis L1L B domain and only 85% identity with that from LEC1, we named the cDNA *PcL1L*.

To obtain clues about *PcL1L* function, we examined its temporal and spatial patterns of RNA accumulation. Figure

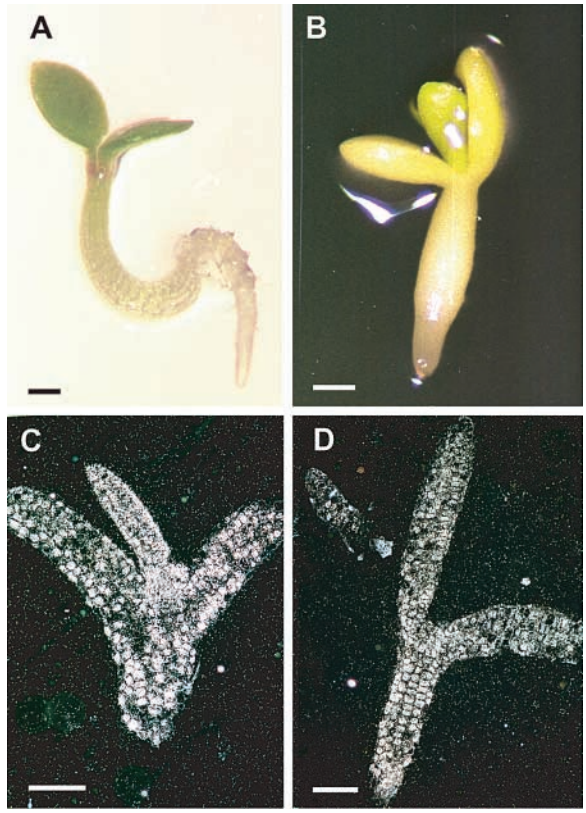

Figure 5. Suppression of the *lec1* Mutation by *L1L*.

(A) A representative *lec1-1* seedling containing the *LEC1:L1L:LEC1* transgene that has survived seed desiccation. The transgene confers desiccation tolerance to *lec1* mutant embryos.
(B) A *lec1-1* seedling transformed with *35S:L1L*. The transgene allows *lec1-1* mutant embryos to withstand desiccation and confers embryonic characteristics to the seedling, including a lack of cotyledon expansion, failure of hypocotyls and roots to extend, and production of cotyledon-like organs at the positions of leaves.
(C) and **(D)** Hybridization of cruciferin storage protein and oleosin probes, respectively, with embryo-like *35S:L1L* seedlings. Sections were exposed for 2 days.
Bars = 300 μm in **(A)** and **(B)** and 100 μm in **(C)** and **(D)**.

6A shows that *PcL1L* RNA was detected only in developing embryos by RNA gel blot analysis. However, RT-PCR analysis showed that *PcL1L* RNA was present, presumably at low levels, at all developmental stages tested, including in vegetative organs, inflorescences, and unfertilized ovules (Figure 6B). Thus, the pattern of *PcL1L* RNA accumulation is more similar to that of *L1L* than that of *LEC1* in Arabidopsis (Figure 2). In situ hybridization analysis showed that *PcL1L* RNA accumulated at high levels in the embryo proper and suspensor of preglobular-stage (5 days after pollination [DAP]) and globular-stage (7 DAP) embryos but at very low levels in the endosperm of the seeds and integuments of the unfertilized ovules (Figures 6C to 6E). *PcL1L* RNA was present at the highest levels in the epidermal and subepidermal layers of the embryo proper at the globular stage (Figure 6D). This pattern is similar to the distribution of Arabidopsis *L1L* RNA observed at the torpedo and bent-cotyledon stages (Figures 3C and 3D). The finding that scarlet runner bean appears to possess a protein with a LEC1-type B domain and that RNA encoding this protein accumulates with a pattern similar to that of Arabidopsis *L1L* suggests that the two *L1L* genes are orthologous.

Through database searches, we extended this analysis by identifying 12 other HAP3 subunits from other plants with conserved amino acid residues characteristic of the LEC1-type B domain. Most were identified from embryo or seed-derived cDNA libraries, although sequences from pine pollen cone and lotus root nodule cDNA libraries were identified. As shown in Figure 7A, alignment of the B domains of these 15 plant HAP3 subunits revealed 17 amino acid residues that are shared between LEC1-type B domains but that differ from residues conserved in non-LEC1-type B domains. By contrast, no conserved amino acid residues were detected in the A and C domains of these 15 proteins, although some similarities were observed in these regions between L1L, PcL1L, and some of the other plant L1L proteins (data not shown). The phylogenetic tree obtained by maximum parsimony analysis (PAUP 4.0) shown in Figure 7B indicates that the L1L proteins constitute a well-supported, monophyletic clade. Based on their sequence identity within the B domain, their origin in seed RNA populations, and the ability of L1L to suppress the *lec1* mutation when expressed ectopically, we speculate that some of these *L1L* genes also may play roles in embryogenesis.

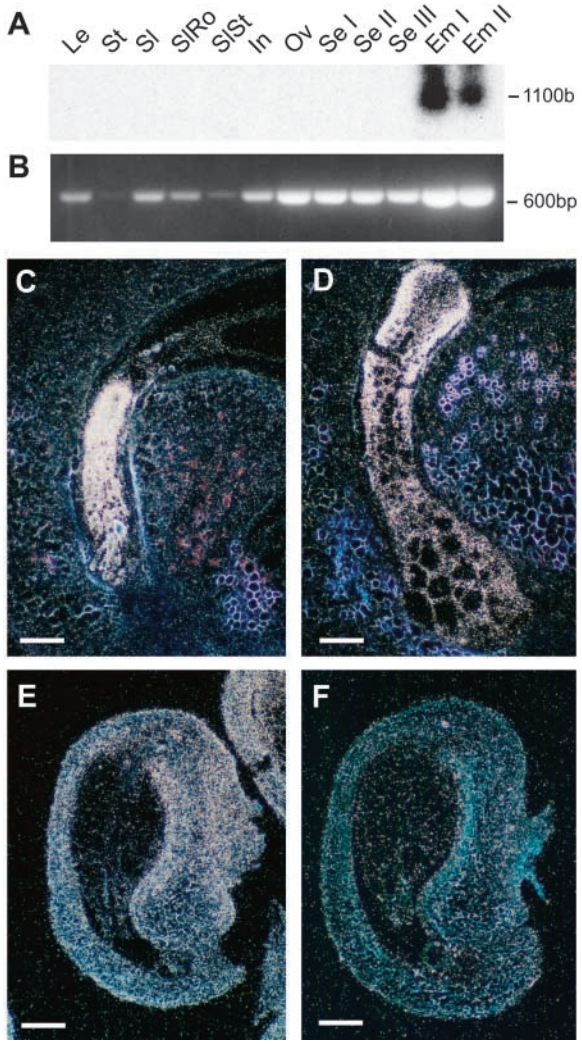

Figure 6. *PcL1L* RNA Is Present Primarily in Developing Seeds.

(A) Gel blot analysis of *PcL1L* RNA accumulation. Twenty-five micrograms of total RNA was analyzed from leaves (Le), stems (St), 2-week-old seedling leaves (Sl), 2-week-old seedling roots (SlRo), 2-week-old seedling stems (SlSt), inflorescences (In), ovules (Ov), 2-DAP seeds (Se I), 4- to 5-DAP seeds (Se II), 6-DAP seeds (Se III), 12- to 14-DAP embryos (Em I), and 19- to 21-DAP embryos (Em II).
(B) RT-PCR analysis of *PcL1L* RNA accumulation. Each lane corresponds to the RNA gel blot sample in **(A)**.
(C) to **(F)** Distribution of *PcL1L* RNA. Sections were hybridized with a *PcL1L* antisense probe **(C)** to **(E)** or a sense RNA control **(F)**. **(C)** and **(D)** were exposed for 4 days, whereas **(E)** was exposed for 47 days.
(C) Preglobular-stage seed. *PcL1L* RNA is high in the embryo proper and suspensor.
(D) Globular-stage seed. *PcL1L* RNA is at its highest levels in outer tissue layers of the embryo.
(E) Unfertilized ovule. *PcL1L* RNA is present at low levels throughout the ovule.
(F) Unfertilized ovule that does not bind sense RNA probe.
Bars = 100 μm.

DISCUSSION

L1L and LEC1 Constitute a Subclass of HAP3 Subunits

L1L and LEC1 display substantial sequence identity with HAP3 subunits of CBFs. Although a CBF from plants has not been isolated, several lines of evidence suggest that L1L and LEC1 function as part of a CBF that regulates embryogenesis. First, in addition to HAP3, paralogs of the other two

CBF subunits required for DNA binding activity, HAP2 and HAP5, have been identified in plants (Li et al., 1992; Albani and Robert, 1995; Edwards et al., 1998; Kusnetsov et al., 1999; Gusmaroli et al., 2001). Unlike yeast and mammals, which possess single genes for each subunit, plants possess families of subunits. For example, there are 6, 10, and 8 genes encoding the AHAP2, AHAP3, and AHAP5 subunits, respectively, in Arabidopsis, opening the possibility that different combinations of subunits may regulate diverse processes (Edwards et al., 1998; Gusmaroli et al., 2001; M. Kim, H. Lee, R.W. Kwong, and J.J. Harada, unpublished results). Second, an AHAP2 gene has been shown to complement a yeast HAP2 mutation, indicating that the Arabidopsis protein can function in a CBF (Edwards et al., 1998). Third, an AHAP5 subunit has been shown to interact with other nuclear proteins, presumably AHAP2 and AHAP3, to form a complex that binds a double-stranded oligonucleotide containing a CAAT box (Kusnetsov et al., 1999). Fourth, loss-of-function mutations of *L1L* and *LEC1*, two HAP3 paralogs, have severe consequences on plant development, suggesting that these subunits play essential roles.

Our results show that there are at least two distinct classes of AHAP3 subunits that differ in several respects. LEC1-type and non-LEC1-type HAP3 subunits differ by 16 amino acid residues that serve as signatures of their B domains (Figure 1) (Gusmaroli et al., 2001). Phylogenetic analysis suggests that HAP3 subunits possessing these signature residues have a common evolutionary origin (Figure 7). Residues at corresponding positions of yeast and mammalian HAP3 subunits are more similar to non-LEC1-type than to LEC1-type AHAP3 subunits. This finding opens the possibility that L1L and LEC1 represent novel HAP3 subunits of CBF. Next, sequence diversity between the two types of subunits appears to underlie the functional differences, because *L1L* but not two other genes that encode non-LEC1-type AHAP3 subunits suppressed the *lec1* mutation when expressed under the control of *LEC1* flanking sequences (Table 1). Similarly, a non-LEC1-type *AHAP3* did not induce embryonic characteristics in seedlings when fused with the *35S* promoter, as did *L1L* and *LEC1* (Table 1, Figure 5). Finally, genes encoding L1L and LEC1 are expressed predominately or exclusively during seed development (Figure 2) (Lotan et al., 1998), whereas the non-LEC1-type *AHAP3* genes generally are expressed at high levels in nonembryonic tissues (Edwards et al., 1998; Gusmaroli et al., 2001; M. Kim and J.J. Harada, unpublished results). In this regard, *LEC1*, *L1L*, and *PcL1L* exhibit similar spatial patterns of RNA accumulation in developing embryos (Figures 3 and 6) (Lotan et al., 1998).

The B Domain Underlies the Function of LEC1-Type HAP3 Subunits in Embryogenesis

We present strong evidence that the B domain of LEC1-type HAP3 subunits underlies their function in embryogenesis. Sequence similarity between L1L and LEC1 was observed exclusively in the B domain and not in the A and C domains (Figure 1). Suppression of the *lec1* mutation by *L1L* (Figure 5, Table 1) suggests that some or all of the 16 residues unique to LEC1-type B domains account for the ability of L1L to substitute functionally for LEC1 when expressed ectopically. Most HAP3 subunits from other plants that possess LEC1-type B domains (Figure 7) are present in embryos or seeds, consistent with the expression patterns of L1L and LEC1. This finding opens the possibility that other L1Ls play important roles in seed development. However, two L1Ls are present in pollen cones and root nodules, suggesting that the LEC1-type HAP3 subunit may function at other developmental stages. This class of HAP3 subunit is present in gymnosperms and monocotyledonous and dicotyledonous angiosperms, but it has not been detected in nonplant organisms, suggesting that the LEC1-type B domain evolved uniquely in plants.

The central B domain of HAP3 subunits serves critical roles in CBF function. Studies of yeast and mammalian HAP3s show that the B domain contains amino acid residues that account for its ability to interact with HAP2 and HAP5 subunits and for the CBF to bind DNA (Xing et al., 1993; Kim et al., 1996; Sinha et al., 1996). One explanation for the differences in the activities of L1L and LEC1 versus non-LEC1-type AHAP3 subunits is that LEC1-type B domains may mediate interactions with specific AHAP2 and AHAP5 subunits to form a CBF that activates the genes required for embryo development. Because AHAP2 and AHAP5 are encoded by six and eight genes, respectively, and most are expressed in nonseed tissues (Gusmaroli et al., 2001; H. Lee, M. Kim, and J.J. Harada, unpublished results), the possibility exists that defined combinations of AHAP subunits confer specific functions to the transcription complex. However, this interpretation requires that the specific AHAP2 and AHAP5 subunits present in embryos also are present in vegetative tissues, because ectopic expression of *L1L* and *LEC1* confers embryonic characteristics to vegetative tissues (Figure 5) (Lotan et al., 1998).

A second possibility is that the LEC1-type B domain may recruit other transcription factors to the CBF that confer unique specificity to the complex. CBFs have been shown to interact with other transcription factors to activate specific sets of genes. For example, activation of the MHC class II gene promoter requires the binding of CBF and an X-box binding factor, and activation of the 3-hydroxy-3-methylglutaryl-CoA synthase gene requires the binding of both CBF and sterol regulatory element binding proteins (Wright et al., 1994; Linhoff et al., 1997; Dooley et al., 1998). In addition, no Arabidopsis paralog of the HAP4 subunit that provides a transcriptional activation domain to the yeast CBF has been identified, and transcriptional activation domains are not apparent in the HAP2 and HAP5 subunits as they are in their mammalian counterparts (Forsburg and Guarente, 1989; Coustry et al., 1996). Thus, a protein with transcriptional

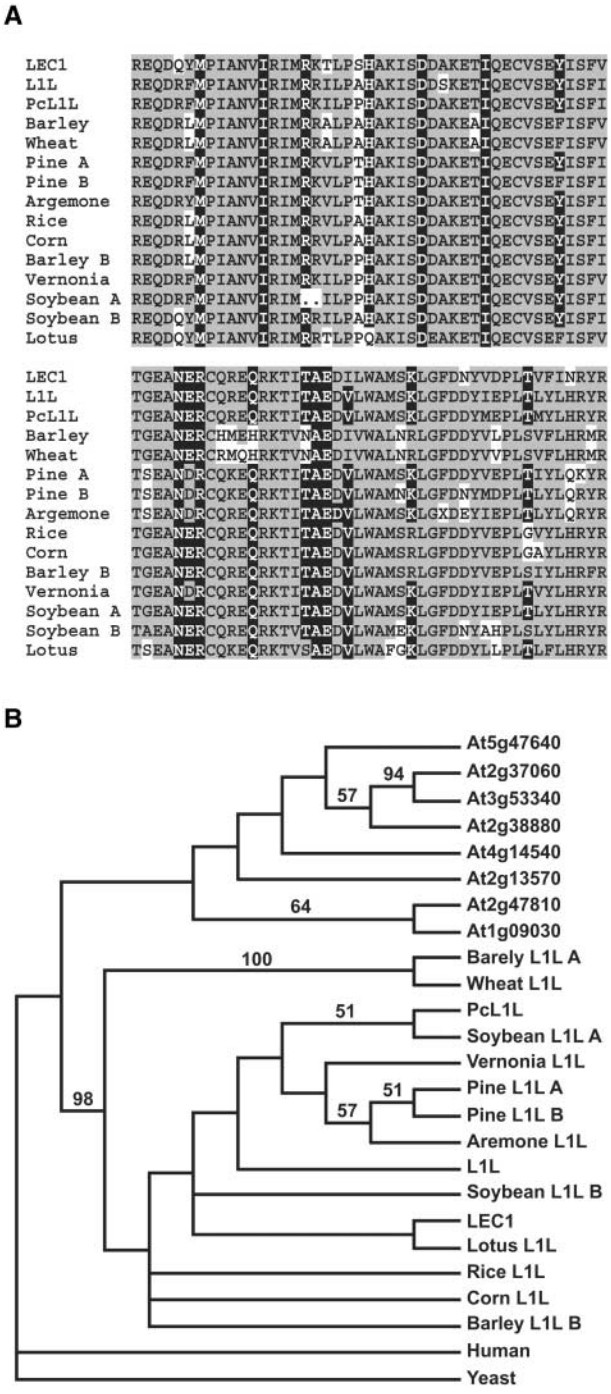

Figure 7. Identification of L1L Proteins from Other Plants.

(A) Amino acid sequence alignment of the B domains of plant L1L proteins. Conserved amino acid residues are highlighted in gray, and residues unique to L1L proteins are highlighted in black. Accession numbers are given at the end of Methods.

(B) Phylogenetic relationships between L1L and non-LEC1-type-HAP3 subunits. The cladogram illustrates the most parsimonious consensus pattern of relationships obtained using maximum parsimony analysis. Bootstrap values generated with 1000 replicates are indicated before the nodes. Nodes with bootstrap scores of <50% are not shown. The high bootstrap values provide strong support for the monophyletic L1L clade.

activation function may be recruited to the complex by L1L and LEC1.

A final alternative is that B domain residues unique to LEC1-type HAP3 subunits may confer a novel DNA binding specificity to the CBF that differs from that afforded by non-LEC1-type AHAP3 subunits. Thus, CBFs containing L1L and LEC1 would bind and modulate the transcription of genes required for embryo development, whereas non-LEC1-type AHAP3s would not. This is the simplest alternative, because there is no need to invoke novel interactions with other proteins. However, to our knowledge, no HAP3 subunit has been identified that alters the binding specificity of CBFs in other organisms.

L1L and LEC1 Have Distinct Functions during Embryogenesis

Although our studies have shown that *L1L* can substitute for *LEC1* if expressed ectopically (Table 1, Figure 5), other evidence suggests that L1L and LEC1 normally have distinct functions during embryogenesis. The first and most compelling argument is that monogenic, loss-of-function mutations in either *L1L* or *LEC1* cause defects in embryo development. These results show that the endogenous genes cannot substitute for one another, although we cannot exclude the possibility that the genes have partial overlaps in function. Consistent with this interpretation is the finding that the suppression of *L1L* and *LEC1* gene expression induces different embryonic phenotypes. *lec1* mutants arrest at a late stage of embryo development, with complete though misshapen cotyledons and embryonic axes, and mutant embryos can be rescued before desiccation to produce viable seedlings (reviewed by Harada, 2001). By contrast, RNAi suppression of *L1L* caused embryos to arrest in their development as early as the globular stage, and mutant embryos cannot be rescued to produce postembryonic plants (Figure 4). We conclude that although *L1L* clearly is required for embryo development, it appears to play a fundamentally different role in embryogenesis than *LEC1*.

With regard to the L1L mutant phenotype, RNAi suppression of *L1L* gene expression is characterized by incomplete penetrance and variable expressivity. Not all embryos containing the construct exhibit a mutant phenotype, and those that do arrest at a number of different embryonic stages with a variety of defects (Figure 4). However, transgenic embryos displaying a mutant phenotype possessed low to undetectable levels of *L1L* RNA, whereas those with a wild-

type phenotype had intermediate to high RNA levels (Figure 4). Therefore, defects in embryo development appear to result from the suppression of *L1L* expression. Although we have not yet identified an insertional mutation of *L1L*, we note the possibility that the RNAi suppression of *L1L* may not produce a mutant phenotype as severe as that of a genetic null mutation. The RNAi construct is controlled by the *35S* promoter, and we have shown that this promoter does not become active detectably during embryogenesis until the globular stage (J. Pelletier and J.J. Harada, unpublished results). Thus, *L1L* RNA may accumulate early during embryogenesis in RNAi lines, albeit at a very low level, and decline only after the globular stage. Despite these qualifications, it is unlikely that a null *l1l* mutant would share similar characteristics with the *lec* class of mutants, because *l1l* mutant embryos arrest earlier in embryogenesis than do *lec1* mutants.

Three other lines of evidence support the conclusion that *L1L* and *LEC1* have distinct endogenous functions. First, *L1L* RNA accumulates later in embryogenesis than does *LEC1* RNA (Figure 2). Second, *L1L* RNA is present in developing seeds and at low levels in vegetative organs, whereas *LEC1* RNA is detected only in developing seeds (Lotan et al., 1998) (Figure 2). Third, *lec1* mutants display an abnormal phenotype even though *L1L* RNA is detected in the mutant seeds, indicating that the endogenous *L1L* gene is not sufficient to completely prevent defects induced by the *lec1* mutation (Figure 2).

There are several potential explanations to reconcile the findings that the endogenous *L1L* and *LEC1* genes do not act redundantly, yet *L1L* can be made to substitute functionally for *LEC1*. One hypothesis is that the specific pattern of *LEC1* gene expression is critical for its function. Although the distribution of *LEC1* and *L1L* RNAs in embryos is similar, there are differences in the timing of their accumulation (Figures 2 and 3) (Lotan et al., 1998). Similar situations have been described for two Arabidopsis MYB genes, *WEREWOLF* and *GLABROUS1*. Genes encoding these functionally equivalent proteins play different roles in plant development because they are transcribed in distinct cell types (Lee and Schiefelbein, 2001). Alternatively, increased dosage of the *L1L* gene and, by inference, increased *L1L* RNA levels in transgenic *lec1* mutants containing the *LEC1*:*L1L*:*LEC1* transgene may account for the suppression of the mutation. Dosage suppression has been described in microorganisms (Puziss et al., 1994). A third possibility is that because the accumulation of *LEC1* and *L1L* RNA does not differ substantially, RNA sequences in the *LEC1* 5′ and/or 3′ untranslated regions, which are included in the *LEC1*:*L1L*:*LEC1* gene (see Methods), may regulate LEC1 function at the translational level. Additional information is needed to distinguish between these possibilities.

In conclusion, we have shown that L1L, the AHAP3 subunit most closely related to LEC1, is a regulator of embryo development. *L1L* is expressed predominately during embryo development, and it is required for the completion of embryogenesis. The ability of L1L but not non-LEC1-type AHAP3 subunits to function in place of LEC1 when expressed ectopically implicates the B domain as the region of L1L and LEC1 that is critical for their function. Mutagenesis studies are needed to define which of the 16 amino acid residues of LEC1-type AHAP3 subunits differentiate their functions from non-LEC1-type subunits. Although L1L can function redundantly with LEC1 when expressed ectopically, the two subunits have distinct functions during embryogenesis. Thus, L1L is a novel regulatory protein that plays an essential role during embryogenesis.

METHODS

Plant Materials and Manipulations

lec1-1 mutants and wild-type plants of *Arabidopsis thaliana* (ecotype Wassilewskija) were grown as described previously (West et al., 1994). Seeds of the day-neutral scarlet runner bean (*Phaseolus coccineus* cv Hammond's Dwarf Red Flower) were grown in the greenhouse as described by Weterings et al. (2001). Seeds were germinated in vermiculite to obtain seedlings. Flowers were pollinated and collected at specific days after pollination (DAP) as described previously (Weterings et al., 2001).

Approximately 500 unfertilized runner bean ovules were collected from young, open flowers. Approximately 90, 66, and 50 seeds were collected from 2-DAP, 4- to 5-DAP, and 6-DAP pods, respectively. Approximately 100 cotyledon-stage embryos were isolated from seeds of 12- to 14-DAP and 19- to 21-DAP pods. Seed and embryo stages were according to Weterings et al. (2001). Small young leaves, stems, and inflorescences were collected from lateral branches of flowering plants. True leaves, roots, and stems were collected from 2-week-old seedlings. Upon collection, tissues were frozen immediately in liquid nitrogen and stored at −80°C until use.

Agrobacterium tumefaciens strain GV3101 containing transformation constructs was infiltrated into *lec1-1* and wild-type plants (Bechtold et al., 1993). Seeds from T0 plants were germinated on medium containing 60 μg/mL glufosinate ammonium to select for transgenic plants (Finale; AgrEvo Environmental Health, Montvale, NJ). Plant genotypes were verified in PCR amplification experiments.

Isolation and Preparation of cDNA and Genomic Clones

PCR was used to amplify the genomic fragments containing *AHAP3* genes. Primers that flanked the putative *L1L* open reading frame with the addition of BamHI and XbaI sites for subcloning purposes were used (BAMMNJ7-5, 5′-AGGATCCATGGAACGTGGAGGCTTCCAT-3′; and 3-MNJ7XBA, 5′-ATCTAGATCAGTACTTATGTTGTTGAGTCG-3′). The AHAP3 genes At4g14540 (3-224) and At3g53340 (3-180) were amplified using primer combinations 3-224-F/3-224-R (5′-CCTATCTCGAGATGGCGGATTCGGACAACGATTC-3′/5′-CCCGGTCTAGATTAAGAAAAATGATGGGAAAATTGATGTCC-3′) and AH3-180-F/AH3-180-R (5′-CCCGGGGAGATCTATGGCGGATACGCCTTCGAGCCCAGC-3′/5′-GGGCCCCTAGGCTTTTACCAGCTCGGCATTTCTTCACC-3′), respectively. Nucleotide sequences of the genomic clones were verified.

L1L, At4g14540, and At3g53340 genomic clones were inserted

between the *LEC1* promoter and terminator within the plant transformation vector BJ49 (Gleave, 1992). The *LEC1* promoter/terminator cassette consists of 1992 bp of DNA 5′ of the *LEC1* translation start codon plus 770 bp 3′ of the *LEC1* stop codon (H. Lee and J.J. Harada, unpublished results). The *L1L* gene was fused with the *35S* promoter from *Cauliflower mosaic virus* and the octopine synthase terminator of the plasmid pART7 and transferred into the binary transformation vector pMLBART (Gleave, 1992).

cDNA clone pPCEP112 was identified from a scarlet runner bean cDNA library by EST sequencing analysis. This cDNA library was constructed with total RNA isolated from the embryo proper of 6-DAP seeds using the SMART PCR cDNA Library Construction Kit (Clontech, Palo Alto, CA) (A.Q. Bui, K. Weterings, and R.B. Goldberg, unpublished results).

Protein Sequence Analysis

Amino acid sequences were aligned with the PileUp program (Seqweb version 2.0.2; Accelrys, Burlington, MA), and alignments were prepared with BOXSHADE (ch.EMBnet.org). Database searches were performed with the LEC1 protein sequence as a query (http://www.ncbi.nlm.nih.gov/blast/ and http://www.arabidopsis.org/Blast/ [as of August 5, 2002]). The analysis identified the following AHAP3 genes: At2g47810, At1g09030, At2g37060, At3g53340, At2g38880, At5g47640, At4g14540, At2g13570, and At5g47670 [the last of which we renamed LEC1-LIKE].

Parsimony trees of the B domains of HAP3 subunits were generated with CLUSTAL X (version 1.8; Thompson et al., 1997) and the heuristic search algorithm of the PAUP program (version 4.0 beta; Swofford et al., 1996). One hundred replicates were used for weighted analysis in generated consensus parsimony trees. For maximum parsimony analysis, 1000 iterations were used to create bootstrap percentages.

RNA Analyses

Arabidopsis RNA was isolated as described previously (Stone et al., 2001). Total RNA from scarlet runner bean was isolated using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA) and treated with RNase-free DNase I (Boehringer Mannheim, Indianapolis, IN) according to the protocol of Ausubel et al. (1995). Approximately 25 μg of RNA was loaded on a formaldehyde gel, and RNA gel blot analysis was performed as described previously (Harada et al., 1988). In situ hybridization experiments with Arabidopsis and scarlet runner bean tissues were performed as described previously (Dietrich et al., 1989; Weterings et al., 2001).

The presence of *L1L* RNA in an organ system was assessed using nonquantitative reverse transcriptase–mediated PCR analysis. For Arabidopsis, first-strand cDNA was generated from 5 μg of each RNA in a 20-μL reaction volume using the Thermal Script reverse transcriptase system (Invitrogen, Carlsbad, CA). One microliter of each reaction was amplified in a 20-μL reaction volume according to the manufacturer's specifications using primers for *L1L* (see above), *LEC1* (LP/UP, 5′-GACATACAACACTTTTCCTTAAAG-3′/5′-CAGCAACAACCCACCCCCAATG-3′), and a ribosomal protein gene (TIN1/TIN2, 5′-TTTGGTGGATGCCCCTGATA-3′/5′-TAATTTCCGAATCCAAAATC-3′) (T. Lotan and J.J. Harada, unpublished results). Amplification products were fractionated by agarose gel electrophoresis.

For scarlet runner bean RNA, first-strand cDNA was generated from 2 μg of each total RNA in a 20-μL reaction using Superscript II Reverse Transcriptase according to the manufacturer's specifications (Gibco BRL, Rockville, MD). PCR amplification was performed using the primers PcL1L-F (5′-AGATTCTTCCTCCACATGCCAAGAT-3′) and PcL1L-R (5′-CCTTAATCCCATCCATCCCCTTAAT-3′) with 2 μL of each reverse transcriptase reaction in a 50-μL reaction volume.

RNA Interference Suppression of *L1L*

The primer combination 3LEFTXX (5′-TCTAGACTCGAGCTTAGCTGCAGTGCTGGG-3′) and 3RIGHTBAM (5′-GGATCCTTGAACCAAGACGCATTACG-3′) was used to amplify a 500-bp fragment unique to the C domain of *L1L*. The fragment was placed in both orientations into the RNA interference vector pRNA69, which contains the *35S* promoter (J.F. Emery and J.L. Bowman, unpublished results). This construct then was placed into the pMLBART binary vector.

Upon request, all novel materials described in this article will be made available in a timely manner for noncommercial research purposes.

Accession Numbers

Accession numbers for the CCAAT binding factor HAP3 subunits shown in Figure 7 are as follows: LEC1, AF036684; L1L, AY138461; PcL1L, AF533650; barley (A), AL506199 and AL509098; wheat, AY058921; pine A, AW754604; pine B, AW981729; Argemone, AY058920; rice, AU088581; maize, AF410176; barley B, BE603222; Vernonia, AY058919; soybean A, AY058917; soybean B, AY058918; and lotus, AW719547 and AW720671.

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REFERENCES

Albani, D., and Robert, L.S. (1995). Cloning and characterization of a *Brassica napus* gene encoding a homologue of the B subunit of a heteromeric CCAAT-binding factor. Gene **167,** 209–213.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., and Smith, J.A. (1995). Short Protocols in Molecular Biology, 3rd ed. (New York: John Wiley & Sons).

Bechtold, N., Ellis, J., and Pelletier, G. (1993). In planta Agrobacte-

rium mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants. C. R. Acad. Sci. Paris **316,** 1194–1199.

Bewley, J.D. (1997). Seed germination and dormancy. Plant Cell **9,** 1055–1066.

Coustry, F., Maity, S.N., Sinha, S., and de Crombrugghe, B. (1996). The transcriptional activity of the CCAAT-binding factor CBF is mediated by two distinct activation domains, one in the CBF-B subunit and the other in the CBF-C subunit. J. Biol. Chem. **272,** 14485–14491.

Dietrich, R.A., Maslyar, D.J., Heupel, R.C., and Harada, J.J. (1989). Spatial patterns of gene expression in *Brassica napus* seedlings: Identification of a cortex-specific gene and localization of messenger RNA encoding isocitrate lyase and a polypeptide homologous to proteinases. Plant Cell **1,** 73–80.

Dilaurenzio, L., Wysocka-Diller, J., Malamy, J.E., Pysh, L., Helariutta, Y., Freshour, G., Hahn, M.G., Feldmann, K.A., and Benfey, P.N. (1996). The *SCARECROW* gene regulates an asymmetric cell division that is essential for generating the radial organization of the Arabidopsis root. Cell **86,** 423–433.

Dooley, K.A., Millinder, S., and Osborne, T.F. (1998). Sterol regulation of 3-hydroxy-3-methylglutaryl-coenzyme A synthase gene through a direct interaction between sterol regulatory element binding protein and the trimeric CCAAT-binding factor/nuclear factor Y. J. Biol. Chem. **273,** 1349–1356.

Edwards, D., Murray, J.A.H., and Smith, A.G. (1998). Multiple genes encoding the conserved CCAAT-box transcription factor complex are expressed in Arabidopsis. Plant Physiol. **117,** 1015–1022.

Finkelstein, R.R., and Lynch, T.J. (2000). The Arabidopsis abscisic acid response gene *ABI5* encodes a basic leucine zipper transcription factor. Plant Cell **12,** 599–609.

Finkelstein, R.R., Wang, M.L., Lynch, T.J., Rao, S., and Goodman, H.M. (1998). The Arabidopsis abscisic acid response locus ABI4 encodes an APETALA 2 domain protein. Plant Cell **10,** 1043–1054.

Forsburg, S.L., and Guarente, L. (1989). Identification and characterization of HAP4: A third component of the CCAAT-bound HAP2/HAP3 heteromer. Genes Dev. **3,** 1166–1178.

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.

Gleave, A.P. (1992). A versatile binary vector system with a T-DNA organisational structure conducive to efficient integration of cloned DNA into the plant genome. Plant Mol. Biol. **20,** 1203–1207.

Goldberg, R.B., de Paiva, G., and Yadegari, R. (1994). Plant embryogenesis: Zygote to seed. Science **266,** 605–614.

Guarente, L., Lalonde, B., Gifford, P., and Alani, E. (1984). Distinctly regulated tandem upstream activation sites mediate catabolite repression of the CYC1 genes of *S. cerevisiae*. Cell **32,** 317–321.

Gusmaroli, G., Tonelli, C., and Mantovani, R. (2001). Regulation of the CCAAT-binding NF-Y subunits in *Arabidopsis thaliana*. Gene **264,** 173–185.

Harada, J.J. (1997). Seed maturation and control of germination. In Advances in Cellular and Molecular Biology of Plants, Vol. 4, Cellular and Molecular Biology of Seed Development, B.A. Larkins and I.K. Vasil, eds (Dordrecht, The Netherlands: Kluwer Academic Publishers), pp. 545–592.

Harada, J.J. (2001). Role of Arabidopsis LEAFY COTYLEDON genes in seed development. J. Plant Physiol. **158,** 405–409.

Harada, J.J., Baden, C.S., and Comai, L. (1988). Spatially regulated genes expressed during seed germination and postgerminative development are activated during embryogeny. Mol. Gen. Genet. **212,** 466–473.

Helariutta, Y., Fukaki, H., Wysocka-Diller, J., Nakajima, K., Jung, J., Sena, G., Hauser, M.T., and Benfey, P.N. (2000). The SHORT-ROOT gene controls radial patterning of the Arabidopsis root through radial signaling. Cell **101,** 555–567.

Jurgens, G. (2001). Apical-basal pattern formation in Arabidopsis embryogenesis. EMBO J. **20,** 3609–3616.

Keng, T., and Guarente, L. (1987). Constitutive expression of the yeast *HEM1* gene is actually a composite of activation and repression. Proc. Natl. Acad. Sci. USA **84,** 9113–9117.

Kim, I.S., Sinha, S., de Crombrugghe, B., and Maity, S.N. (1996). Determination of functional domains in the C subunit of the CCAAT-binding factor (CBF) necessary for formation of a CBF-DNA complex: CBF-B interacts simultaneously with both the CBF-A and CBF-C subunits to form a heterotrimeric CBF molecule. Mol. Cell. Biol. **16,** 4003–4013.

Kusnetsov, V., Landsberger, M., Meurer, J., and Oelmuller, R. (1999). The assembly of the CAAT-box binding complex at a photosynthesis gene promoter is regulated by light, cytokinin, and the stage of the plastids. J. Biol. Chem. **274,** 36009–36014.

Laux, T., and Jurgens, G. (1997). Embryogenesis: A new start in life. Plant Cell **9,** 989–1000.

Lee, M.M., and Schiefelbein, J. (2001). Developmentally distinct MYB genes encode functionally equivalent proteins in Arabidopsis. Development **128,** 1539–1546.

Li, X.-Y., Mantovani, R., Hooft Van Huijsduijnen, R., Andre, I., Benoist, C., and Mathis, D. (1992). Evolutionary variation of the CCAAT-binding transcription factor NF-Y. Nucleic Acids Res. **20,** 1087–1091.

Linhoff, M.W., Wright, K.L., and Ting, J.P.-Y. (1997). CCAAT-binding factor NF-Y and RFX are required for in vivo assembly of a nucleoprotein complex that spans 250 base pairs: The invariant chain promoter as a model. Mol. Cell. Biol. **17,** 4589–4596.

Long, J.A., Moan, E.I., Medford, J.I., and Barton, M.K. (1996). A member of the knotted class of homeodomain proteins encoded by the STM gene of Arabidopsis. Nature **379,** 66–69.

Lotan, T., Ohto, M., Matsudaira Yee, K., West, M.A.L., Lo, R., Kwong, R.W., Yamagishi, K., Fischer, R.L., Goldberg, R.B., and Harada, J.J. (1998). *Arabidopsis* LEAFY COTYLEDON1 is sufficient to induce embryo development in vegetative cells. Cell **93,** 1195–1205.

Maity, S.N., and De Crombrugghe, B. (1998). Role of the CCAAT-binding protein CBF/NF-Y in transcription. Trends Biochem. Sci. **23,** 174–178.

Mantovani, R. (1999). The molecular biology of the CCAAT-binding factor NF-Y. Gene **239,** 15–27.

Mayer, K.F.X., Schoof, H., Haecker, A., Lenhard, M., Juergens, G., and Laux, T. (1998). Role of *WUSCHEL* in regulating stem cell fate in the Arabidopsis shoot meristem. Cell **95,** 805–815.

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

Meinke, D.W., Franzmann, L.H., Nickle, T.C., and Yeung, E.C. (1994). *leafy cotyledon* mutants of Arabidopsis. Plant Cell **6,** 1049–1064.

Odell, J.T., Nagy, F., and Chua, N.-H. (1985). Identification of DNA sequences required for activity of cauliflower mosaic virus 35S promoter. Nature **313,** 810–812.

Parcy, F., Valon, C., Kohara, A., Misera, S., and Giraudat, J. (1997). The ABSCISIC ACID-INSENSITIVE3, FUSCA3, and LEAFY

COTYLEDON1 loci act in concert to control multiple aspects of Arabidopsis seed development. Plant Cell **9,** 1265–1277.

Puziss, J.W., Hardy, T.A., Johnson, R.B., Roach, P.J., and Hieter, P. (1994). MDS1, a dosage suppressor of an mck1 mutant, encodes a putative yeast homolog of glycogen synthase kinase 3. Mol. Cell. Biol. **14,** 831–839.

Schneider, J.C., and Guarente, L. (1991). Regulation of the yeast *CYT1* gene encoding cytochrome c_1 by HAP1 and HAP2/3/4. Mol. Cell. Biol. **11,** 4934–4942.

Sinha, S., Kim, I.S., Sohn, K.-Y., de Crombrugghe, B., and Maity, S.N. (1996). Three classes of mutations in the A subunit of the CCAAT-binding factor CBF delineate functional domains involved in the three-step assembly of the CBF-DNA complex. Mol. Cell. Biol. **16,** 328–337.

Stone, S.L., Kwong, L.W., Yee, K.M., Pelletier, J., Lepiniec, L., Fischer, R.L., Goldberg, R.B., and Harada, J.J. (2001). LEAFY COTYLEDON2 encodes a B3 domain transcription factor that induces embryo development. Proc. Natl. Acad. Sci. USA **98,** 11806–11811.

Swofford, D.L., Olsen, G.J., Waddell, P.J., and Hills, D. (1996). Phylogenic inference. In Molecular Systematics, 2nd ed, C. Moritz, D.M. Hills, and B.K. Mable, eds (Sunderland, MA: Sinauer and Associates), pp. 407–514.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., and Giggins, D.G. (1997). The CLUSTALX Windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. **25,** 4876–4882.

Trueblood, C.E., Wright, R.M., and Poyton, R.O. (1988). Differential regulation of the two genes encoding *Saccharomyces cerevisiae* cytochrome oxidase subunit V by heme and *HAP2* and *REO1* genes. Mol. Cell. Biol. **8,** 4537–4540.

Vicient, C.M., Bies-Etheve, N., and Delseny, M. (2000). Changes in gene expression in the leafy cotyledon1 (lec1) and fusca3 (fus3) mutants of *Arabidopsis thaliana* L. J. Exp. Bot. **51,** 995–1003.

West, M.A.L., and Harada, J.J. (1993). Embryogenesis in higher plants: An overview. Plant Cell **5,** 1361–1369.

West, M.A.L., Matsudaira Yee, K.L., Danao, J., Zimmerman, J.L., Fischer, R.L., Goldberg, R.B., and Harada, J.J. (1994). *LEAFY COTYLEDON1* is an essential regulator of late embryogenesis and cotyledon identity in Arabidopsis. Plant Cell **6,** 1731–1745.

Weterings, K., Apuya, N.R., Bi, Y., Fischer, R.L., Harada, J.J., and Goldberg, R.B. (2001). Regional localization of suspensor mRNAs during early embryo development. Plant Cell **13,** 2409–2425.

Wright, K.L., Vilen, B.J., Itoh-Lindstrom, Y., Moore, T.L., Li, G., Criscitiello, M., Cogswell, P., Clarke, J.B., and Ting, J.P.-Y. (1994). CCAAT box binding protein NF-Y facilitates in vivo recruitment of upstream DNA binding transcription factors. EMBO J. **13,** 4042–4053.

Xing, Y., Fikes, J.D., and Guarente, L. (1993). Mutations in yeast HAP/HAP3 define a hybrid CCAAT box binding domain. EMBO J. **12,** 4647–4655.