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The Arabidopsis flowering-time gene *LUMINIDEPENDENS* is expressed primarily in regions of cell proliferation and encodes a nuclear protein that regulates *LEAFY* expression

Milo J. Aukerman[1,†], Ilha Lee[2,†], Detlef Weigel[3] and Richard M. Amasino[1,*]
[1]*Department of Biochemistry, University of Wisconsin, 433 Babcock Drive, Madison, WI 53706–1544, USA*
[2]*Department of Biology and Research Center for Cell Differentiation, Seoul National University, Seoul 151–742, Korea, and*
[3]*Plant Biology Laboratory, Salk Institute for Biological Studies, 10010 North Torrey Pines Road, La Jolla, CA 92037, USA*

Summary

Mutations in the *LUMINIDEPENDENS* (*LD*) gene of *Arabidopsis thaliana* (L.) Heynh. (Arabidopsis) confer a late-flowering phenotype, indicating that *LD* normally functions to promote the floral transition. RNA and protein blot analyses, along with the analysis of transgenic plants containing a fusion between a genomic fragment of *LD* and the reporter gene *uidA* (*GUS*), indicate that *LD* is expressed primarily in apical proliferative regions of the shoot and root, including the shoot apical meristem and leaf primordia. Subcellular localization studies indicate that LD is a nuclear protein, consistent with its previously proposed transcriptional regulatory role. We have also found that in an *apetala1 cauliflower* (*ap1 cal*) background the *ld* mutation converts the reproductive shoot apex to a more vegetative state, a phenotype that is similar to that seen for the *leafy* (*lfy*) mutant. Furthermore, *in situ* hybridization analysis indicates that *LFY* levels are drastically reduced at the apex of *ld ap1 cal* plants after bolting. These data are consistent with the idea that at least one function of *LD* is to participate in the regulation of *LFY*.

Introduction

The transition of the shoot apex from a vegetative to a reproductive mode of growth is a critical developmental switch in the life cycle of a plant. Prior to this floral transition, the shoot apical meristem (SAM) primarily forms leaves, whereas afterwards the SAM produces floral primordia that differentiate into flowers. In order to ensure that flowering occurs at a proper time and thus maximize their reproductive success, many species have evolved mechanisms to regulate the timing of the floral transition in response to developmental cues and certain environmental stimuli (Lang, 1965; Napp-Zinn, 1987; Poethig, 1990). The complexity of these floral timing mechanisms has become increasingly clear from genetic studies which reveal that many genes are involved in the regulation of flowering time (Koornneef *et al.*, 1998; Weller *et al.*, 1997).

Arabidopsis thaliana (L.) Heynh. (Arabidopsis) is a facultative long-day plant which responds to long days by flowering earlier than when grown in short days (Koornneef *et al.*, 1998). More than 20 genes that control flowering time in Arabidopsis have been identified through the analysis of both late and early flowering mutants. The flowering-time mutants have been grouped into several different classes based upon the response of each mutant to changes in photoperiod and temperature (Koornneef *et al.*, 1998). One class of Arabidopsis flowering-time mutants displays a reduced response to changes in photoperiod when compared to wild type and it has been proposed, therefore, that the corresponding genes participate in a photoperiod-regulated pathway. A second class of mutants, while displaying an altered flowering time, are nonetheless unaffected in their response to changes in photoperiod; the genes corresponding to these mutants have thus been placed in an autonomous pathway. A third class, and perhaps a third pathway, is represented by a single mutant, *ga1*, which is deficient in gibberellin and is more responsive to changes in photoperiod than wild-type plants (Wilson *et al.*, 1992).

To date, four flowering-time genes have been cloned: *LUMINIDEPENDENS* (*LD*), *CONSTANS* (*CO*), *FCA* and *FHA* (Guo *et al.*, 1998; Lee *et al.*, 1994; Macknight *et al.*, 1997; Putterill *et al.*, 1995). Mutations in any one of these genes confer a late-flowering phenotype (Koornneef *et al.*, 1991; Redei, 1962), indicating that they normally function to promote the floral transition. Both *CO* and *FHA* belong to the photoperiod-response class, whereas *FCA* and *LD* are in the autonomous flowering pathway (Koornneef *et al.*, 1991; Lee *et al.*, 1994; Redei, 1962). Consistent with these assignments, expression of *CO* is regulated by photoperiod (Putterill *et al.*, 1995), and the *FHA* gene

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*For correspondence (fax +1 608 262 3453; e-mail amasino@biochem.wisc.edu).
[†]Both authors contributed equally to this work.

encodes a blue light receptor that is likely to be involved in photoperiod perception (Guo *et al.*, 1998). The autonomous genes *LD* and *FCA*, on the other hand, do not appear to be regulated by environmental stimuli (Lee *et al.*, 1994; Macknight *et al.*, 1997).

The protein product of the *CO* gene (Putterill *et al.*, 1995) displays similarity to the zinc finger class of transcriptional activators and, therefore, promotion of flowering by CO is probably carried out by transcriptional activation of one or more downstream target gene(s). One possible candidate for this is the floral meristem-identity gene *LEAFY* (*LFY*), which is expressed in primordia arising on the flanks of the shoot apical meristem (SAM) (Weigel *et al.*, 1992). Meristem-identity genes such as *LFY* and *APETALA1* (*AP1*) and *CAULIFLOWER* (*CAL*) function to switch these primordia from a vegetative to a floral state (Bowman *et al.*, 1993; Weigel *et al.*, 1992). *LFY* is upregulated as the plant approaches the floral transition, and it is thought that the level of *LFY* reaches some critical threshold at which point the vegetative-to-floral transition occurs (Blazquez *et al.*, 1997). Evidence for regulation of *LFY* by *CO* was provided by Simon *et al.* (1996) who showed that *LFY* transcription is rapidly initiated in response to *CO* expression. It appears, then, that promotion of flowering by *CO* in wild-type plants is accompanied by upregulation of *LFY* at the shoot apex.

The mode of regulation employed by the gene products in the autonomous flowering pathway is less clear. The *FCA* gene product (Macknight *et al.*, 1997) was found to be homologous to a class of RNA-binding proteins, suggesting that *FCA* may promote flowering via a post-transcriptional mechanism. The *LD* gene product (Lee *et al.*, 1994) shows no strong similarity to other proteins, but does have two consensus bipartite nuclear-localization domains (Dingwall and Laskey, 1991), implying that it is a nuclear protein. LD also contains a glutamine-rich region at the carboxy terminus that resembles the glutamine-rich domains found in several transcription factors (Mitchell and Tjian, 1989). These two features, along with a putative divergent homeodomain (Aukerman and Amasino, 1996), suggest that LD may be a transcriptional regulatory protein.

In this report, we have analyzed the spatial expression of *LD* by RNA and protein blots, and by transgenic plant analysis. We have found that *LD* is expressed primarily in regions of the shoot and root apex that contain dividing cells, including the apical meristems. In addition, we demonstrate that the *LD* gene product is targeted to the nucleus, consistent with its proposed transcriptional regulatory function. Finally, we have found that *ld ap1 cal* triple mutants display a drastic reduction of *LFY* expression, leading to the complete inability of these plants to form floral structures. These results suggest that one function of *LD* is to participate in the regulation of the floral meristem-identity gene *LFY*. Our studies also provide support for the notion that both the autonomous and photoperiod-regulated flowering pathways are involved in the regulation of *LFY*.

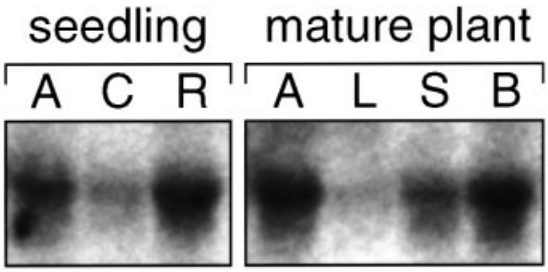

Figure 1. RNA blot analysis of LD expression.
RNA was extracted from seedling shoot apices (A), cotyledons (C), roots (R), mature plant shoot apices (A), leaves (L), stems (S) and floral buds (B). An equal amount of total RNA (20 μg) was loaded in each lane, based upon staining the gels with acridine orange. The probe used for hybridization was a 0.9 kb *Pst*I fragment of the *LD* cDNA (Lee *et al.*, 1994).

Results

Analysis of LD transcript and protein accumulation

We initially determined the spatial distribution of the *LD* transcript by extracting RNA from various organs of wild-type Arabidopsis and performing RNA blot analyses utilizing an *LD*-specific probe. Figure 1 shows that *LD* is expressed in both seedlings and mature plants. The *LD* transcript is most abundant in shoot apices, inflorescence stems, floral buds and roots, and less abundant in cotyledons and leaves.

To determine whether the LD protein exhibits a similar pattern of expression, we utilized a transgenic Arabidopsis line containing a genomic copy of *LD* with six copies of a *c-myc* epitope inserted within the coding region of *LD* (Figure 2a, see Experimental procedures for details). This *LD-myc* construct rescues the late-flowering phenotype when transformed into the *ld-2* mutant background (data not shown), which demonstrates that the LD-myc protein is fully functional, and further suggests that the *LD* promoter in the *LD-myc* construct is driving the expression of the *LD* gene product in a pattern identical to that seen for the endogenous *LD* promoter. Due to the relatively low levels of LD protein present in wild-type Arabidopsis (M. Aukerman, unpublished observations), it was necessary to perform a two-step, immunoprecipitation/immunoblot analysis of the LD protein. Proteins extracted from various organs of transgenic plants containing the *LD-myc* construct were immunoprecipitated with an LD-specific antibody, and the precipitate was resuspended and analyzed by standard immunoblotting using an antibody specific for the myc

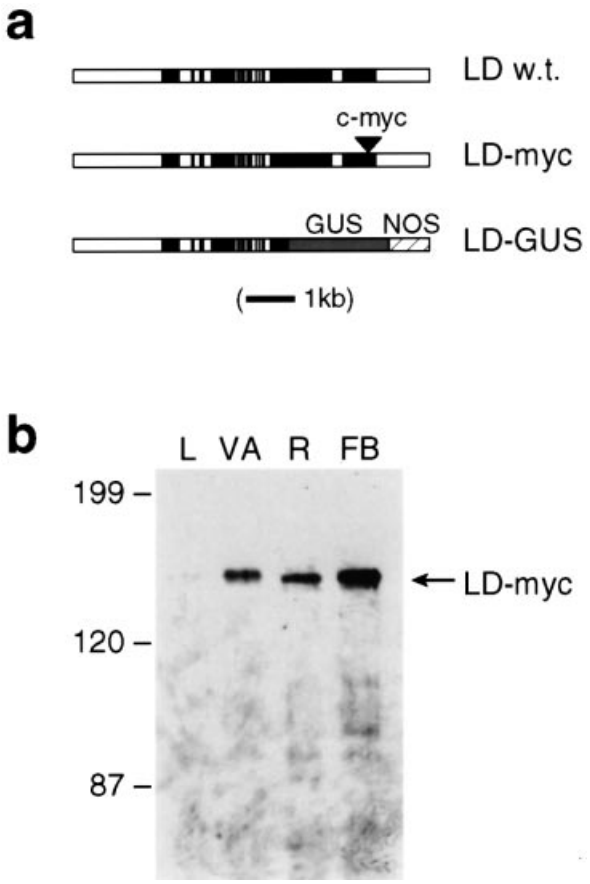

Figure 2. Protein gel blot analysis of LD expression.
(a) *LD-myc* and *LD-GUS* constructs. Top line (*LD* w.t.) shows the 8 kb *Hin*dIII genomic fragment containing the wild-type *LD* gene which has been demonstrated to rescue the *ld* mutant phenotype (Lee *et al.*, 1994). The white regions indicate the 5′ promoter, introns and 3′ untranslated region, while the black regions denote exons. Six copies of a *c-myc* epitope were inserted at codon 942 within the 8 kb genomic *LD* fragment, creating *LD-myc* (middle). For the *LD-GUS* construct (bottom), a 5 kb genomic fragment of *LD* containing 2 kb of promoter region and 3 kb of coding region (including introns) was fused in-frame to the *E. coli* gene encoding β-glucuronidase (GUS), followed by a nopaline synthase terminator (NOS). This construct results in the fusion of the N-terminal 451 amino acids of the LD protein to GUS.
(b) Immunoprecipitation/Immunoblot experiment (see Experimental procedures for details). Protein was extracted from leaves (L), vegetative apices (VA), roots (R) and floral buds (FB) of transgenic plants containing the *LD-myc* construct. Equal protein amounts of each crude extract were immunoprecipitated, based upon SDS-PAGE and Coomassie staining.

epitope. This analysis (Figure 2b) indicates that, as seen for the *LD* transcript, the LD protein accumulates to the highest levels in vegetative shoot apices, roots and floral buds, and is present at much lower levels in leaves.

Analysis of LD-GUS expression

To confirm and extend our findings on the spatial expression of *LD*, we constructed a fusion between a 5 kb genomic fragment of *LD* and the *E. coli* gene *uidA* which encodes β-glucuronidase (GUS) (Jefferson, 1987). As shown in Figure 2a, the *LD-GUS* fusion construct consists of 2 kb of the *LD* promoter region and 3 kb of the *LD* coding region fused in-frame to the GUS gene. This construct generates a protein consisting of the N-terminal 451 amino acids (out of a total of 953) of LD fused to the GUS protein. Staining of transgenic plants containing this construct with X-gluc revealed an accumulation of GUS enzyme in both root and shoot apices (Figure 3a), indicating a preferential expression of the *LD* gene in those regions. *LD* is expressed strongly throughout the SAM (Figure 3d), and young leaf primordia also stain strongly (Figure 3b–d). Consistent with our RNA and protein analyses, cotyledons and fully expanded leaves display very little GUS staining (Figure 3a,c). Thus, rapidly proliferating tissues, including the shoot and root meristems and young leaf primordia, express *LD* at the highest levels.

LD expression remains high after flowering has occurred, as indicated by staining inflorescence sections of transgenic plants (Figure 3e). *LD* is expressed throughout the reproductive SAM and in all whorls of younger flower primordia, for example, the stage 3 flower in Figure 3e (see Bowman, 1994 for discussion of the flower stages). As individual flowers develop, the *LD* expression pattern becomes more restricted, so that at more mature stages only the more recently developed inner whorls corresponding to carpel and stamen primordia express the *LD* gene product. A final restriction of *LD* expression to the inner whorl occurs such that prior to the opening of the flower bud, expression of *LD* appears to be confined primarily to the developing ovules (Figure 3f). Therefore, *LD* expression in the reproductive stage is similar to its expression in the vegetative stage, in that rapidly proliferating and less mature tissues display the highest levels of *LD* expression. We have analyzed three independent transgenic lines containing the LD-GUS construct, and all three display the expression patterns detailed above (I. Lee and M. Aukerman, unpublished observations).

Nuclear localization of LD-GUS protein

Closer inspection of the root tips of stained transgenic plants expressing LD-GUS reveals a punctate pattern of X-gluc accumulation (Figure 4, far left), suggesting that the LD-GUS fusion protein resides in the nucleus. Under longer incubation times, the root hairs of *LD-GUS* plants also stain, and the two LD-GUS panels of Figure 4 show a close-up of a root hair cell from an *LD-GUS* plant stained with X-gluc. The staining pattern demonstrates that the LD-GUS fusion protein accumulates exclusively in the nucleus, as indicated by counterstaining the sample with 4′,6-diamidino-2-phenylindole (DAPI). The wild-type GUS protein normally accumulates in the cytoplasm, as indicated by the X-gluc and DAPI staining patterns of a root hair of a transgenic plant expressing GUS alone (Figure 4, GUS panels). Because the *LD-GUS* fusion con-

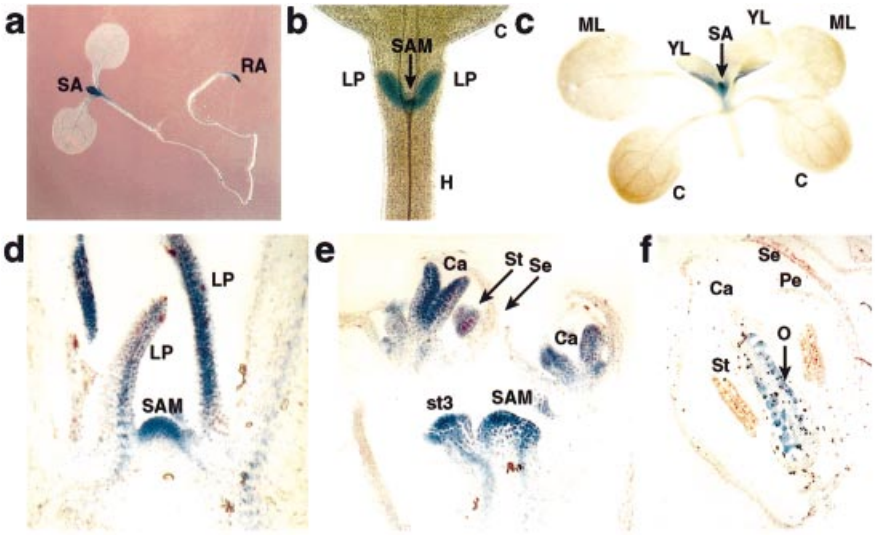

Figure 3. X-gluc staining pattern in LD-GUS plants.
GUS activity is indicated by blue staining. (a) Seedling stage. Both the shoot apex and root apex are stained blue. (b) Enlargement of the shoot apex of seedling. The primordia of two true leaves are stained blue, as is the SAM. The hypocotyl and cotyledons do not show any detectable staining. (c) Rosette-stage plant, with roots removed. The shoot apex is stained dark blue, whereas young leaves are lighter blue. Fully expanded, mature leaves and cotyledons do not exhibit staining above background. (d) Section through shoot apex of rosette-stage plant, showing GUS staining in the SAM and leaf primordia. (e) Section through shoot apex of flowering-stage plant, showing staining throughout the SAM and a stage 3 flower (see Bowman, 1994 for discussion of flower stages). The carpels and stamens of more mature flowers also stain, whereas sepals do not. (f) Section through mature (approximately stage 12) flower. Only developing ovules stain. SA, shoot apex; RA, root apex; SAM, shoot apical meristem; LP, leaf primordia; H, hypocotyl; C, cotyledon; YL, young leaf; ML, mature leaf; st3, stage 3 flower; Ca, carpel; St, stamen; Se, sepal; Pe, petal; O, ovules.

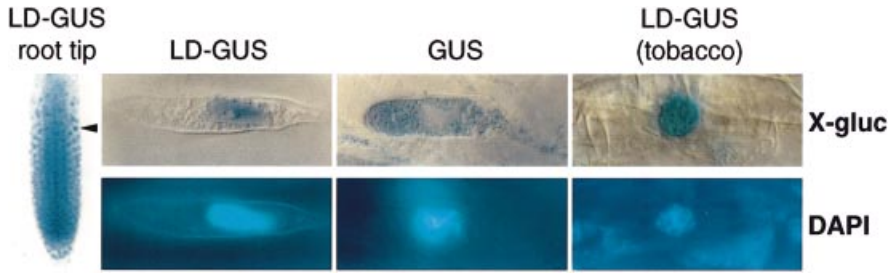

Figure 4. Nuclear localization of LD-GUS protein.
Plants were stained with X-gluc and counterstained with DAPI (4′,6-diamidino-2-phenylindole). Far left panel, a root tip from a plant expressing LD-GUS protein. The arrowhead indicates a representative nucleus. LD-GUS panels, a root hair cell from a plant expressing the LD-GUS protein. GUS panels, a root hair cell from a plant expressing GUS alone. Far right panels, a root cell from a tobacco plant expressing LD-GUS. In each accompanying DAPI panel, the position of the nucleus is indicated by the region of fluorescence.

struct contains the 5′ half of the *LD* coding region fused to GUS, it appears that amino acid sequences within the N-terminal half of LD are sufficient to target the LD-GUS protein to the nucleus. When the LD-GUS fusion protein is expressed in tobacco, X-gluc staining is also confined to the nucleus (Figure 4, far right), indicating that the nuclear localization signals present in the LD protein can function in a heterologous species. We have also fused the entire *LD* coding region to GUS and shown that this full-length LD-GUS protein is also localized to the nucleus in transgenic tobacco (M. Aukerman, unpublished results).

Phenotype of the ld *mutant in an* ap1 cal *background*

The expression pattern of *LD* in the shoot apex overlaps with that of the *LEAFY* (*LFY*) gene, which is expressed in both vegetative and reproductive organ primordia arising on the flanks of the SAM (Blazquez *et al.*, 1997). The floral initiation process is regulated by the *LFY* gene product in combination with the products of other floral meristem-identity genes such as *APETALA1* (*AP1*) and *CAULIFLOWER* (*CAL*). Double and triple mutant combinations of *lfy* with *ap1* and with *ap1 cal* produce a more severe phenotype than that seen in the single mutants (Bowman *et al.*, 1993), indicating that *LFY* and *AP1* have distinct but overlapping functions. The similarities in the expression patterns of *LD* and *LFY* suggest that *ld* might, like *lfy*, display genetic interactions with *ap1* and *cal*. We tested this idea by crossing the *ld* mutant to the *ap1 cal* double mutant and comparing the phenotype of the resultant *ld ap1 cal* plants to that of *ap1 cal* plants. As reported by Bowman *et al.*

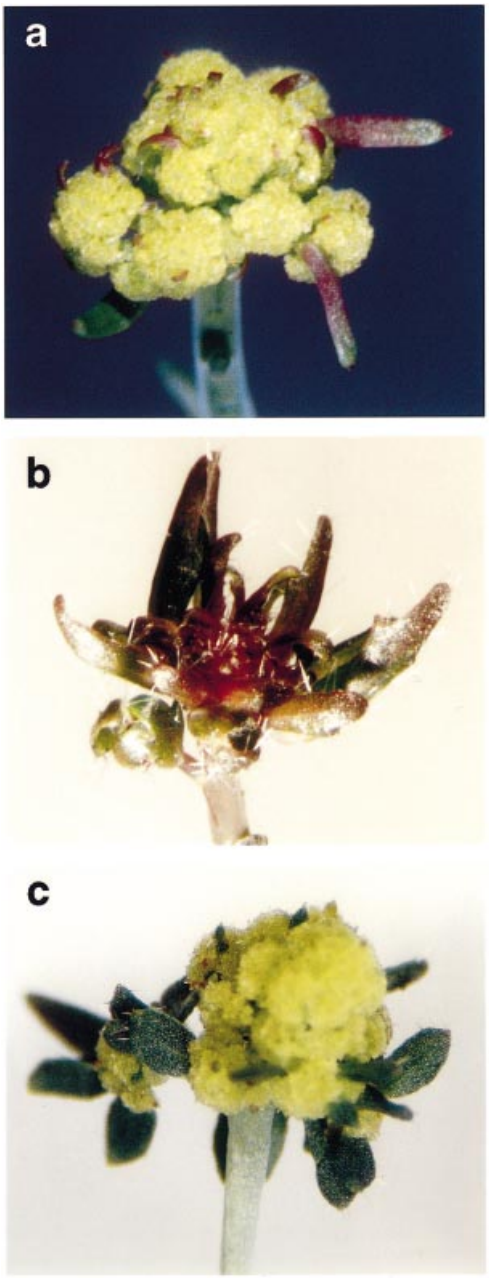

Figure 5. Phenotype of the ld mutant in an ap1 cal background.
(a) Inflorescence apex of *ap1 cal* double mutant grown in continuous light.
(b) Inflorescence apex of *ld ap1 cal* triple mutant grown in continuous light.
(c) Inflorescence apex of *ap1 cal* double mutant grown in short days (8 h light, 16 h dark).

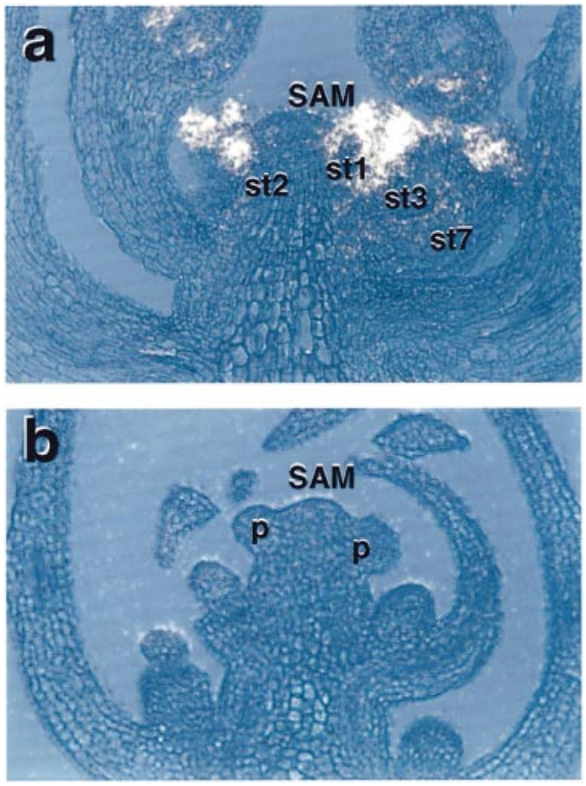

Figure 6. LFY expression in inflorescence apices of *ld* and *ld ap1 cal* mutants.
(a) *In situ* hybridization of *LFY* probe to a longitudinal cross-section of an *ld* mutant inflorescence apex. In stage 1 (st1), stage 2 (st2), and stage 3 (st3) floral primordia, *LFY* expression is detected throughout the primordia. Also shown is a stage 7 (st7) primordia which displays *LFY* expression in the inner whorls. No *LFY* expression is detectable in the shoot apical meristem (SAM).
(b) *In situ* hybridization of *LFY* probe to a longitudinal cross-section of an *ld ap1 cal* mutant inflorescence apex. *LFY* expression is not detectable in the primordia (p) arising on the flanks of the SAM.

(1993), the reproductive shoot apex of an *ap1 cal* mutant plant grown in long days consists of a proliferation of undifferentiated inflorescence meristems, with occasional leaf-like structures emerging (Figure 5a). Eventually, flowers with a typical *ap1* phenotype will form in lateral positions (Bowman *et al.*, 1993). In contrast, the reproductive shoot apex of the *ld ap1 cal* triple mutant grown in long days is almost entirely converted to leaf-like structures (Figure 5b); furthermore, flowers or flower-like organs never formed on *ld ap1 cal* plants, even after several months of growth. In *ld ap1 cal* plants, lateral inflorescence branches develop in the positions on the main stem where flowers would normally develop. The leaves which proliferate at the shoot apex (Figure 5b) are associated with these lateral branches, and thus they become separated from the shoot apex as elongation of the main stem occurs (M. Aukerman, unpublished observations).

To establish whether the *ld ap1 cal* phenotype is specifically caused by the absence of *LD* function or by a more general delay of flowering, we grew *ap1 cal* plants in short-day conditions (8 h light, 16 h dark), causing them to bolt at approximately the same time as *ld ap1 cal* mutants grown in long days. As seen in Figure 5c, the shoot apex of an *ap1 cal* plant grown in short days consists mostly of the proliferating inflorescence meristem also seen in *ap1 cal* grown in long days (Figure 5a), but with a slight increase in the number of leaf-like structures emerging from it. This increase in vegetative character in short-day grown *ap1*

cal plants is very mild in comparison to the almost complete conversion to vegetative growth seen in long-day grown *ld ap1 cal* plants (Figure 5b). Furthermore, unlike long-day grown *ld ap1 cal* plants, short-day grown *ap1 cal* plants will eventually form flowers (M. Aukerman, unpublished observations). This experiment indicates that the phenotype conferred to *ap1 cal* plants by the *ld* mutation cannot be mimicked by a general delay of flowering, and therefore the phenotype is more likely due to the loss of a specific function associated with the *LD* gene product.

LFY expression in the ld ap1 cal *mutant*

It has been shown that *LFY* expression in the inflorescence of the *ap1 cal* mutant is partially reduced but not absent, and remains at wild-type levels in those primordia that are likely to develop into flowers (Bowman *et al.*, 1993). The fact that no flowers or flower-like structures were ever observed in *ld ap1 cal* plants suggests that *LFY* levels in these plants might be even lower than those seen in *ap1 cal* alone. We tested this by examining *LFY* expression by *in situ* hybridization of inflorescences of both *ld* mutant plants and *ld ap1 cal* mutant plants. As shown in Figure 6a, *LFY* expression in *ld* mutants within the reproductive apex is normal, reaching high levels in stage 1 and 2 flower primordia. In contrast, the *ld ap1 cal* triple mutant displayed no detectable expression of *LFY* in the inflorescence (Figure 6b). Because *LFY* levels are only partially reduced in *ap1 cal* alone (Bowman *et al.*, 1993), this result indicates that the *ld* mutation causes a further reduction of *LFY* expression in this background.

Discussion

Using several independent methods, we have described the spatial expression pattern of the flowering-time gene *LUMINIDEPENDENS*. RNA and protein blot analyses indicated that the *LD* gene product accumulates to higher levels in shoot apices, roots and floral buds than in mature leaves. Further work utilizing an *LD-GUS* transgenic line allowed us to observe high levels of *LD* expression within the SAM, the root apex, young leaf primordia, and the inflorescence. In general, *LD* expression is highest in younger tissues where cells are still rapidly dividing. As these tissues mature and differentiate, *LD* expression declines. Interestingly, the pattern of *LD* expression in the shoot apex closely mirrors that of the photoperiod response pathway gene *CO* (Simon *et al.*, 1996), which is consistent with the idea that the autonomous and photoperiod responsive flowering pathways converge at the shoot apex. Although the expression patterns of *LD* and *CO* overlap, to date there is no evidence that the *LD* and *CO* gene products directly interact.

The late-flowering phenotype of *ld* (Lee *et al.*, 1994; Redei, 1962) indicates that the *LD* gene plays an important role in the floral transition, and our studies on the *LD* expression pattern have provided some clues as to what that role might be. Classical physiological studies have indicated that diffusible signals, thus far uncharacterized, travel from the leaves to the shoot apex to stimulate the flowering response (Zeevaart, 1984). Given that *LD* is expressed at high levels in the SAM, one possibility is that *LD* functions in the SAM to somehow promote competency to flower in response to a stimulus from the leaves. *LD* is also expressed in leaf primordia and, in light of recent studies in maize indicating that leaf primordia are sufficient to provide the flowering signal to the apex (Colasanti *et al.*, 1998; Irish and Jegla, 1997), it is also possible that *LD* plays a role in generating or transmitting the flowering signal. There does not appear to be any role for *LD* in leaf development itself, however, since *ld* mutants do not display any obvious leaf abnormalities. Likewise, although we observe expression of *LD* in the root apex and in developing ovules, the *LD* gene product does not appear to play a prominent role in the development of those organs because *ld* mutations do not produce any obvious root phenotype or adversely affect fertility (S. Sanda and M. Aukerman, unpublished observations). Nonetheless, we cannot conclude from the experiments described herein that the *LD* gene product in roots plays no role in flowering. Although it seems unlikely that *LD* produced in the root could affect the vegetative-to-reproductive conversion in the SAM, it has been suggested that certain flowering signals emanate from the roots (McDaniel *et al.*, 1996). We are currently attempting both grafting experiments and transgenic experiments to determine whether the *LD* gene product expressed in roots can affect flowering at the shoot apex.

The *LFY* gene is expressed in a subset of the tissues in which *LD* is expressed and, given the central role that *LFY* plays in flower initiation, it seemed likely that *LD* might participate in its regulation. This indeed appears to be the case, as the apex of the *ld ap1 cal* triple mutant proliferates leaf-like structures (Figure 5b) that are similar to those seen for *lfy ap1 cal* (Weigel *et al.*, 1992). This phenotype is probably due to a specific loss of *LD* function rather than to a general late-flowering effect because *ap1 cal* plants grown in non-inductive short days do not display this phenotype (Figure 5c). Furthermore, *LFY* levels are drastically reduced in *ld ap1 cal* plants (Figure 6b) and the reduction appears more severe than that reported for *ap1 cal* mutants (Bowman *et al.*, 1993). This last observation is consistent with the proposed role of *LFY* in flower initiation because *ld ap1 cal* plants never form any floral structures, whereas *ap1 cal* plants do. Nevertheless, the loss of *LFY* activity alone cannot account for the severity of the phenotype seen in *ld ap1 cal* because *lfy ap1 cal* plants make a limited number of floral structures (Bowman *et al.*, 1993;

Weigel *et al.*, 1992). This suggests that *LD* regulates other genes in the floral meristem pathway in addition to *LFY*, for example *APETALA2* or *UNUSUAL FLORAL ORGANS* (Bowman *et al.*, 1993; Lee *et al.*, 1997). It is important to emphasize, however, that because *ld* single mutant plants do not display a meristem-identity phenotype, regulation of the meristem-identity pathway by *LD* appears to be masked by the more prominent meristem-identity functions of genes such as *AP1* and *CAL*.

Other investigators who have constructed mutant combinations between flowering-time mutants and meristem-identity mutants have reported a similar enhancement of meristem-identity mutant phenotypes by flowering-time mutations (Putterill *et al.*, 1995; Ruiz-Garcia *et al.*, 1997). From these studies, it is becoming clear that different flowering-time genes can have effects on different genes within the meristem-identity pathway. *CO*, for example, has been demonstrated to control *LFY* expression (Simon *et al.*, 1996), whereas activation of *AP1* appears to require an additional pathway. This additional pathway is likely to contain the flowering-time genes *FT* and *FWA*, since these seem to primarily regulate *AP1* and *CAL* instead of *LFY* (Ruiz-Garcia *et al.*, 1997). Furthermore, recent studies by Nilsson *et al.* (1998) support the idea that *CO* operates upstream of *LFY*, whereas *FT* and *FWA* primarily affect the response to *LFY* activity. The observation that *LFY* expression is normal in *ld* single mutants could be interpreted as evidence that *LD* acts in parallel with *LFY* or downstream of *LFY* to affect floral meristem identity, in a manner similar to *FT* and *FWA*. However, the loss of *LFY* expression in *ld ap1 cal* plants suggests instead that *LD* regulates *LFY*, and thus is more similar to *CO* in terms of its regulatory properties. Interestingly, *CO* and *LD* belong to different environmental response classes (photoperiod response and autonomous, respectively), and yet they share a common regulatory target, *LFY*. This is consistent with the idea that *LFY* expression is not only regulated by photoperiod signals, as has been demonstrated previously (Blazquez *et al.*, 1997), but also by autonomous signals, including gibberellins (Blazquez *et al.*, 1998).

Although our work suggests that one function of the *LD* gene product is to regulate *LFY* expression at the shoot apex, it is still not clear how many transduction steps lie between *LD* and *LFY*, or what biochemical mode of action *LD* employs in regulating downstream target genes. One approach to obtain clues about the biochemical function of a protein is to determine its subcellular localization and, in this report, we have demonstrated that an LD-GUS fusion protein is localized to the nucleus thus indicating that LD itself is a nuclear protein. The N-terminal half of LD (Figure 2a, LD-GUS), which contains one bipartite nuclear localization consensus motif, is sufficient for nuclear localization. There does not appear to be any tissue-specificity to the nuclear localization or any regulation by photoperiod because the LD-GUS fusion protein accumulates in the nuclei of both the shoot apex and the root apex and under various photoperiodic treatments (M. Aukerman and I. Lee, unpublished observations).

The observation that LD is nuclear localized has spurred us to investigate the biochemical nature of its role within the nucleus. Two clues regarding LD biochemical function have come from inspection of the primary sequence (Lee *et al.*, 1994). First, there is a glutamine-rich region towards the C-terminus of LD which, by analogy to other proteins that contain such a region, could function in transcriptional regulation. Second, a region near the N-terminus has limited similarity to homeodomains which suggests that LD may be a DNA-binding protein (Aukerman and Amasino, 1996). Although we have observed non-sequence-specific binding by LD to calf thymus DNA *in vitro*, we have been unable to detect sequence-specific DNA binding by LD (M. Aukerman and Y. Noh, unpublished observations). It is possible that the LD homeodomain-like region serves as an RNA-binding motif, similar to the homeodomain in the Drosophila protein Bicoid (Dubnau and Struhl, 1996). This possibility is especially intriguing since the *FCA* gene encodes an RNA-binding protein and, like *LD*, is a member of the autonomous flowering pathway (Macknight *et al.*, 1997). It may also be that LD requires other proteins in addition to itself to form a specific complex with DNA or RNA. In this regard, it is tantalizing to speculate that LD directly interacts with the protein product(s) specified by one or more of the other flowering-time genes in the autonomous pathway. Such putative interactions can be more easily investigated as more flowering-time genes are cloned and their protein products become amenable to study.

Experimental procedures

Plant strains, genetics and growth conditions

The Arabidopsis ecotype Wassilewskija (WS) was used for RNA analyses. Plant transformations were performed either with the *ld-2* mutant (in the WS background) or with the RLD ecotype, as described below. To generate the *ld ap1 cal* triple mutant, the *ld-3* mutant was crossed to the *ap1-1 cal-1* mutant (Bowman *et al.*, 1993), and late-flowering F2 plants that displayed the enhanced floral meristem phenotype were selected. Growth conditions in all experiments consisted of continuous light from a mixture of fluorescent and incandescent bulbs, unless otherwise noted.

RNA and protein analyses

For RNA blot analysis, plant tissue was ground in liquid nitrogen and RNA was extracted using an RNeasy Plant RNA kit (Qiagen). Twenty μg of each RNA was electrophoresed and blotted, and the blot was hybridized as described previously (Aukerman *et al.*, 1991), using a 0.9 kb *Pst* fragment of the *LD* cDNA as a probe.

Staining of the gel before transfer indicated that equal amounts of RNA were loaded in each lane (data not shown). Immunoblot analysis utilized an *LD-myc* transgenic line that was generated as follows. Specific oligonucleotides were used in a PCR reaction to amplify the insert from plasmid pMT6, which contains six tandem copies of a DNA sequence encoding a *c-myc* epitope (Roth *et al.*, 1991). The six myc copies were inserted into a *Bam*HI site that was introduced by site-directed mutagenesis at codon 942 in a genomic DNA fragment containing the *LD* gene. The *LD-myc* genomic fragment was inserted into the plant transformation vector pCGN1547 (McBride and Summerfelt, 1990) and this construct was introduced into *ld-2* mutant plants by standard Agrobacterium-mediated transformation as described by Lee *et al.* (1994). Transgenic plants containing this construct were analyzed by a combination of immunoprecipitation and immunoblotting as follows. Transgenic *LD-myc* plant tissue was ground in liquid nitrogen, transferred to a centrifuge tube and proteins were immunoprecipitated with an LD-specific antibody essentially as described by Vierstra and Quail (1982), except that protein A-Sepharose (Pharmacia) was used for the immunoprecipitation step. The polyclonal LD-specific antibody used for immunoprecipitation was raised against an LD fusion protein expressed in *E. coli*. The immunoprecipitate was loaded onto a 6% SDS/PAGE gel, electrophoresed and transferred to Hybond ECL membrane (Amersham), following standard protocols (Harlow and Lane, 1988). Subsequent immunodetection followed the ECL protocol (Amersham) and utilized the 9E10 monoclonal antibody to *c-myc* (a gift from M. Sussman, University of Wisconsin) and a goat anti-mouse secondary antibody conjugated to horseradish peroxidase (Amersham).

LD-GUS construction, transformation and staining

To generate the *LD-GUS* fusion construct, a 5 kb *Hin*dIII/*Bam*HI genomic fragment of *LD* was inserted into a shuttle vector containing the *uidA* gene (GUS) from *E. coli* (Jefferson, 1987) and a nopaline synthase terminator. The resulting *LD-GUS* construct contains 3 kb of *LD* promoter and 2 kb of *LD* coding region fused to the GUS gene, and generates a predicted polypeptide that consists of the N-terminal 451 amino acids of *LD* fused to the N-terminus of the GUS polypeptide (see Figure 2a). A *Hin*dIII fragment containing this entire *LD-GUS* fusion construct was excised from the shuttle vector and inserted into the *Hin*dIII site of pCGN1578. This construct was introduced into the Arabidopsis ecotype RLD and *Nicotiana tabacum* by Agrobacterium-mediated transformation (Lee *et al.*, 1994). Fixation, staining and clearing of *LD-GUS* transgenic plants with X-gluc was performed as described previously (An *et al.*, 1996). Stained plants were viewed and photographed through a Leica MZ6 dissecting microscope. For nuclear localization, stained roots were mounted on a slide in 50 mM phosphate buffer, pH 7.2, 20 μg ml^{-1} 4′,6-diamidino-2-phenylindole (DAPI) and photographed through a Nikon microscope using Nomarski optics. For the analysis of *LFY* expression in *ld* and *ld ap1 cal* plants, *in situ* hybridization was performed as previously described (Weigel *et al.*, 1992).

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