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Isolation of *LUMINIDEPENDENS*: A Gene Involved in the Control of Flowering Time in Arabidopsis

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Plants have evolved the ability to regulate flowering in response to environmental signals such as temperature and photoperiod. The physiology and genetics of floral induction have been studied extensively, but the molecular mechanisms that underlie this process are poorly understood. To study this process, we isolated a gene, *LUMINIDEPENDENS (LD)*, that is involved in the timing of flowering in Arabidopsis. Mutations in this gene render Arabidopsis late flowering and appear to affect light perception. The late-flowering phenotype of the *ld* mutation was partially suppressed by vernalization. Genomic and cDNA clones of the *LD* gene were characterized. The predicted amino acid sequence of the LD protein contains 953 residues and includes two putative bipartite nuclear localization signals and a glutamine-rich region.

INTRODUCTION

The initiation of flowering, the conversion of shoot apical meristems from vegetative to reproductive development, is a critical event in the life cycle of higher plants. Factors controlling the onset of flowering vary among plant species. In some species, the perception of environmental signals has a major influence on flowering time, allowing the plant to coordinate flowering with specific seasons. Two common environmental cues that affect flowering time are exposure to low temperatures (vernalization) and changes in daylength (photoperiodism). There are different response types among plants that exhibit photoperiodism, including species in which flowering is promoted by long nights (short-day plants) or long days (long-day plants). In other plant species, flowering is largely independent of environmental changes and is therefore controlled by developmental signals.

Although different plant species exhibit a wide range of flowering responses to environmental and developmental signals, physiological and genetic studies indicate that some of the basic mechanisms controlling flowering time may be conserved. For example, grafting studies have shown that translocatable promoters and inhibitors of flowering produced in one photoperiod response type can affect flowering time in plants of a different photoperiod response type (e.g., Lang, 1989). Genetic analyses have revealed that single gene differences can alter the flowering response type; for example, mutations that introduce or eliminate an effect of photoperiod on flowering have been described in tobacco (Allard, 1919), maize (Galinat and Naylor, 1951), pea (Murfet, 1989), sorghum (Quinby and Karper, 1945), and Arabidopsis (Rédei, 1962; Koornneef et al., 1991; Zagotta et al., 1992). A mutation in gibberellin biosynthesis has recently been shown to inhibit the ability of Arabidopsis to flower in noninductive photoperiods (Wilson et al., 1992).

The control of flowering time has been extensively studied in Arabidopsis. Flowering in most ecotypes of Arabidopsis is promoted by long-day photoperiods and by vernalization (Napp-Zinn, 1985; Martínez-Zapater and Somerville, 1990; Koornneef et al., 1991). Because inductive photoperiods and vernalization are not absolutely required for flowering in Arabidopsis, these processes can be studied genetically in this species. Two major classes of mutations that affect flowering time in Arabidopsis have been described; they are the late-flowering mutations that result in a delay of flowering (e.g., Rédei, 1962; Koornneef et al., 1991) and early-flowering mutations that accelerate flowering (Goto et al., 1991; Sung et al., 1992; Zagotta et al., 1992). Koornneef et al. (1991) have placed most of the late-flowering mutants into 12 complementation groups. The majority of the early-flowering mutants have not been as extensively characterized, except for the long hypocotyl *hy3* mutant, which has been shown to result from a lesion in the phytochrome B gene (Reed et al., 1993). Other genes controlling flowering time have been identified by analyzing naturally occurring variation in flowering time among Arabidopsis ecotypes (e.g., Karlovska, 1974; Napp-Zinn, 1985; Lee et al., 1993). For example, the *FLA* locus is responsible for the late-flowering habit of certain ecotypes and is distinct from any genes identified by induced mutations in flowering time (Lee et al., 1993).

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Physiological and genetic analyses of flowering time in Arabidopsis and other species, such as pea and tobacco, indicate that flowering is controlled by multiple interacting pathways that are subject to both positive and negative regulation (Lang, 1989; Murfet, 1989; Martínez-Zapater and Somerville, 1990; Koornneef et al., 1991). Phytochrome and gibberellins appear to be involved in these pathways; however, the biochemical basis of these pathways is not known. To contribute to the understanding of floral induction, we have isolated and characterized a gene, *LUMINIDEPENDENS (LD)*, that is involved in the regulation of flowering time in Arabidopsis. Mutations in *LD* delay flowering, as first described by Rédei (1962). Here, we describe the structure of the *LD* gene and the physiological effects of *ld* mutations.

RESULTS

Identification of a Late-Flowering Mutant Caused by T-DNA Insertion

We screened the T-DNA insertion lines generated by Agrobacterium seed transformation of Arabidopsis (Feldmann, 1991) for mutations that resulted in late flowering under inductive photoperiods. The criterion for selecting a late-flowering mutant is that the mutant is developmentally late flowering; that is, the shoot apical meristem forms more leaves before converting to reproductive development but is phenotypically normal in other respects such as growth rate (Rédei, 1962; Koornneef et al., 1991). One line showed cosegregation of the T-DNA insert with the late-flowering phenotype in a simple Mendelian fashion. In the T_3 population (see Feldmann, 1991 for nomenclature), the late-flowering phenotype segregated as a single recessive mutation (40 mutant and 109 wild-type plants; $\chi^2 = 0.31$, $P > 0.5$). In this population, all of the late-flowering plants were homozygous with respect to the T-DNA insertion because the progeny resulting from self-pollination of these mutant plants were uniformly kanamycin resistant. The progeny of early-flowering (wild-type) plants from this population were either all kanamycin sensitive (no T-DNA) or segregated 3:1 for kanamycin resistance to sensitivity (hemizygous for T-DNA). The cosegregation of the T-DNA insert and the mutant phenotype persisted through subsequent generations. These results indicated that the functional T-DNA sequences in this line are inserted into a single locus and that this insertion created a mutation in a gene involved in flowering time determination.

T-DNA Insertion Mutant Is an *ld* Allele

The late-flowering mutant containing a T-DNA insert was crossed to 12 previously described late-flowering mutants; they are *fca*, *fd*, *fe*, *fha*, *fpa*, *ft*, *fve*, *fwa*, and *fy* (Koornneef et al., 1991) and *co*, *gi*, and *ld* (Rédei, 1962). In the F_1 generation, only the cross to *ld* failed to complement the late-flowering mutation (i.e., the F_1 plants of this cross were late flowering). To confirm that the insertion mutant was indeed an *ld* allele, the flowering time of F_2 progeny resulting from self-pollination of an F_1 plant from the cross to *ld* was examined. All F_2 plants were late flowering, which confirmed that the insertion mutant was a new *ld* allele. This T-DNA insertion allele was designated *ld-3*. The first *ld* allele was described by Rédei (1962) and is designated *ld-1*. During the analyses of other late-flowering mutants from T-DNA insertion lines, we isolated another *ld* allele, *ld-2*, which was not due to T-DNA insertion. The mutant *ld-1* was isolated in the Columbia genetic background, and *ld-2* and *ld-3* are in the Wassilewskija genetic background. To determine the degree of recessiveness, *ld-1* and *ld-3* were crossed to the Columbia and Wassilewskija wild types, respectively, and the flowering time of the F_1 progeny and the parental lines was compared (Table 1). The flowering time, measured by the number of rosette leaves formed prior to flowering, was the same in the F_1 and the wild type, indicating that these *ld* mutations are fully recessive.

Flowering Behavior of *ld* Mutant Plants

Rédei (1962) first reported that *ld-1* mutant plants are late flowering in both long and short days and that the delay of flowering is more extreme in short days. We have characterized the response of the *ld-3* mutant to photoperiod and vernalization (Figure 1 and Table 2). *ld-3* is likely to be a null mutant because no transcript was detectable and the T-DNA was inserted into the 5′ end of the coding region of the gene (see below). Similar to *ld-1*, we found that *ld-3* mutant plants were extremely late flowering in short days. In long days consisting of relatively high intensity light, flowering of the mutant was accelerated when compared to short days, but flowering of the mutant occurred much later than in the corresponding wild-type strain. The late-flowering phenotype of *ld-3* under long-day conditions of sunlight (~14.5 hr of daylight) was similar to that observed under high-intensity long-day conditions in the growth chamber (data not shown). However, if long days

Table 1. Comparison of Rosette Leaf Number for F_1 and Parental Plants

Line	Leaf Number ± SE
Columbia (wild type)	11.2 ± 0.8
ld-1	36.0 ± 5.1
Columbia × *ld-1*; F_1	10.0 ± 0.7
Wassilewskija (wild type)	5.7 ± 0.5
ld-3	26.5 ± 6.6
Wassilewskija × *ld-3*; F_1	6.1 ± 0.7

Each value represents the average of nine plants.

Figure 1. Mutant Phenotype of *ld*-3.

Wild-type and *ld* mutant plants at a similar stage: opening of the first flower. From left to right: Wassilewskija wild type, not vernalized; *ld-3* mutant, not vernalized; *ld-3* mutant, vernalized. All plants were grown in continuous light.

were provided by a low-intensity daylength extension, a condition which is sufficient to promote flowering in the wild-type Wassilewskija background, the mutant flowered equally late in long and short days (Table 2). The flowering of *ld-3* mutant plants was accelerated by vernalization in both long- and short-day conditions (Table 2). Thus, vernalization partially alleviated the block in a flowering pathway caused by the *ld* mutation.

Isolation of the *LD* Gene

DNA gel blot analyses indicated that there was a single T-DNA insert in *ld-3* consisting of a tandem array of three to four T-DNA copies (data not shown). Plant DNA flanking the site of insertion was isolated by plasmid rescue of the left border of the T-DNA (Yanofsky et al., 1990). When used as a probe in DNA gel blot analyses, this plant DNA fragment detected restriction fragment length polymorphisms (RFLPs) between DNAs from wild-type and *ld-3* mutant plants (Figure 2); this confirmed that the rescued plant DNA was indeed derived from sequences that flanked the site of a DNA insertion. The rescued plant DNA was then used to identify genomic clones of wild-type sequences in this region, and the physical map of an 18-kb region surrounding the site of insertion was constructed from two overlapping genomic clones (Figure 3A).

To determine whether the genomic region into which the T-DNA had inserted contained the *LD* gene, we subcloned a 9.6-kb region of wild-type DNA corresponding to sequences flanking the T-DNA insertion site into a binary vector (Figure 3B) and transferred this subclone into the genome of *ld-2* mutant plants via Agrobacterium-mediated transformation (Valvekens et al., 1988). The primary transformants exhibited a normal flowering time. Four of the primary transformants were further examined. The progeny resulting from self-pollination of these primary transformants contained both late- and early-flowering plants; this segregation behavior was expected because the inserted DNA should segregate in these populations. In one of these populations, the cosegregation of the introduced genomic fragment and complementation of the mutant phenotype were confirmed: all of the early-flowering (i.e., wild-type) plants contained the introduced subclone (based on kanamycin resistance conferred by the vector), whereas all of the late-flowering plants were kanamycin sensitive. These results demonstrated that the introduction of the 9.6-kb genomic fragment into *ld-2* mutant plants complements the mutation.

The plant DNA flanking the T-DNA insert was also used to screen a cDNA library, which resulted in the isolation of a clone containing an insert of 1.8 kb (data not shown). RNA gel blot analysis using this clone as a probe detected a single transcript of 3.2 kb in wild-type poly(A)$^+$ RNA (see below), indicating that the cDNA was not full length. The presence of a poly(A) segment in this clone indicated that the 5′ end of the cDNA was lacking, and an anchored polymerase chain reaction (PCR) technique (Loh et al., 1989) was used to obtain the remainder of the cDNA. Comparison of the cDNA to the genomic sequence revealed that the *LD* gene contains 12 introns, most of which are quite short and clustered at the 5′ end (Figure 3C).

Sequence Analysis of *LD*

The putative full-length cDNA contained a single, long open reading frame encoding a hydrophilic protein of 953 residues. Although the predicted amino acid sequence does not exhibit significant similarity to other genes in the data bases, it has

Table 2. Effect of Photoperiod and Vernalization on Rosette Leaf Number

	Without Vernalization			After Vernalization[a]	
	Short Day[b]	Long Day[c]	Long Day[d]	Short Day[b]	Long Day[c]
ld-3	63 ± 6	25 ± 2	62 ± 4	40 ± 1	9 ± 1
Wassilewskija	25 ± 3	6 ± 1	6 ± 1	22 ± 7	5 ± 1

[a] Imbibed seeds were incubated at 4°C for 30 days under short-day conditions.

[b] Eight hours at 100 μmol m^{-2} sec^{-1} of fluorescent (75%) and incandescent (25%) light; 16-hr dark.

[c] Twenty hours of light at the same intensity as short days; 4-hr dark.

[d] Same as short days with a 12-hr extension of 4 μmol m^{-2} sec^{-1} of cool-white fluorescent light.

The leaf numbers represent the mean value and the range of a minimum of six plants.

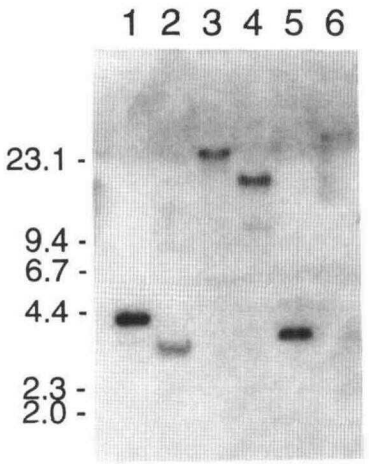

Figure 2. DNA Gel Blot Hybridization Analysis of Genomic DNA from the Wild Type and *ld-3*.

The plant DNA flanking left border of the T-DNA insertion was used as a probe for blots of genomic DNA (3 μg) from the wild type (lanes 1, 3, and 5) and *ld-3* (lanes 2, 4, and 6) that had been digested with EcoRI (lanes 1 and 2), SalI (lanes 3 and 4), or BglII (lanes 5 and 6). Molecular length markers at left are given in kilobases.

three interesting features, which are indicated in Figure 4. First, two consensus bipartite nuclear localization signals are present in the LD. This consensus sequence has been defined as two basic amino acids separated by a spacer region followed by three basic residues among the next five (Dingwall and Laskey, 1991; Raikhel, 1992). Second, LD contains a glutamine-rich domain at the carboxy terminus that resembles the glutamine-rich domains found in several transcription factors (Mitchell and Tjian, 1989). Third, the central region of the protein has a QPVNG sequence repeated five times with each repeat separated by a four–amino acid spacer.

The predicted amino acid sequence initiates from the third start codon (ATG) of the *LD* cDNA. The first and second ATGs give rise to putative polypeptides of only 13 and 47 amino acids (data not shown) and are not in a favorable sequence context for translational initiation in plants (Joshi, 1987; Lutke et al., 1987), whereas the third ATG is in a favorable context. Furthermore, all three reading frames above the third ATG are interrupted by stop codons. Thus, it is highly likely that this ATG represents the initiation codon of the LD polypeptide. There are several examples of translational initiation from internal initiation codons documented in eukaryotes, including plants (Lohmer et al., 1993). To confirm that the 3.2-kb cDNA encodes the LD gene product, we sequenced the corresponding cDNA from *ld-2* and determined that the lesion in this allele is an in-frame deletion in the coding region resulting in the loss of amino acids 266 to 268.

Chromosomal Location of *LD*

The approximate chromosomal location of *LD* was determined using the *LD* genomic clone as a probe for RFLP mapping in populations resulting from crosses of Landsberg *erecta* with the Columbia and Niederzenz ecotypes as described previously (Chang et al., 1988). This analysis placed *LD* distal to

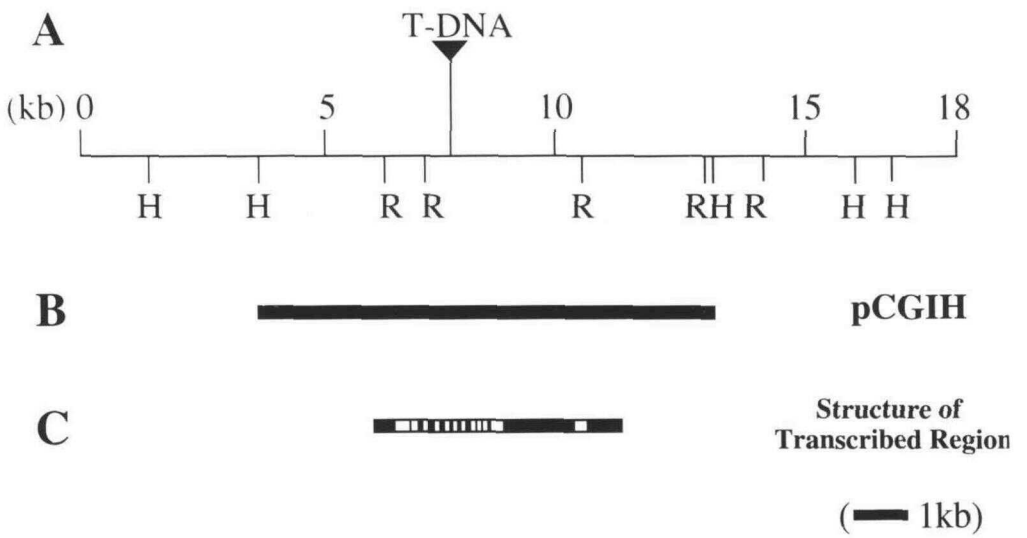

Figure 3. Map of the *LD* Region.

(A) EcoRI (R) and HindIII (H) restriction sites are shown for an 18-kb region flanking the site of T-DNA insertion in *ld-3*.
(B) The 9.6-kb region of the genomic DNA that complements the *ld* mutation.
(C) Transcribed region of the *LD* gene. Exons are black and introns are white.

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MDAFKEEIEIGSSVESLMELLDSQK   25
VLFHSQIDQLQDVVVAQCKLTGVNP   50
LAQEMAAGALSIKIGKRPRDLLNPK   75
AVKYLQAVFAIKDAISKRESREISA  100
LFGITVAQVREFFVTQKTRVRKQVR  125
LSREKVVMSNTHALQDDGVPENNNA  150
TNHVEPVPLNSIHPEACSISWGEGE  175
TVALIPPEDIPPDISDSDKYFVENI  200
FSLLRKEETFSGQVKLMEWIMQIQD  225
ASVLIWFLSKGGVLILTTWLSQAAS  250
EEQTSVLLLILKVLCHLPLHKASPE  275
NMSAILQSVNGLRFYRISDISNRAK  300
GLLSRWTKLFAKIQAMKKQNRNSSQ  325
IDSQSQLLLKQSIAEIMGDSSNPED  350
ILSLSNGKSENVRRIESSQGPKLLL  375
TSADDSTKKHMLGSNPSYNKERRKV  400
QMVEQPGQKAAGKSPQTVRIGTSGR  425
SRPMSADDIQKAKMRALYMQSKNSK  450
KDPLPSAIGDSKIVAPEKPLALHSA  475
KDSPPIQNNEAKTEDTPVLSTVQPV  500
NGFSTIQPVNGPSAVQPVNGPLAVQ  525
PVNGPSALQPVNGPSAVIVPVQADE  550
IKKPSTPPKSISSKVGVMMKMSSQT  575
ILKNCKRKQIDWHVPPGMELDELWR  600
VAAGGNSKEADVQRNRNRRERETTY  625
QSLQTIPLNPKEPWDREMDYDDSLS  650
PEIPSQQPPEESLTEPQDSLDERRI  675
AAGAATTSSSLSSPEPDLELLAALL  700
KNPDLVYALTSGKPSNLAGQDMVKL  725
LDVIKTGAPNSSSSSNKQVEERVEV  750
SLPSPTPSTNPGMSGWGQEGIRNPF  775
SRQNQVGTAVARSGTQLRVGSMQWH  800
QTNEQSIPRHAPSAYSNSITLAHTE  825
REQQQYMQPKLHHNLHFQQQQQQPI  850
STTSYAVREPVGQMGTGTSSSWRSQ  875
QSQNSYYSHQENEIASASQVTSYQG  900
NSQYMSSNPGYESWSPDNSPSRNQL  925
NMRGQQQQASRKHDSSTHPYWNQNK  950
RWR                        953
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Figure 4. Predicted Amino Acid Sequence of LD.

The two putative bipartite nuclear localization signals and the QPVNG repeat sequence are underlined. The glutamine residues in the glutamine-rich domain at the carboxy terminus are in italic, boldface type. Amino acid and nucleotide sequence data have been submitted to GenBank as accession number U03456.

RFLP marker 506 on chromosome 4. We previously characterized a naturally occurring, late-flowering gene, *FLA*, in this region of chromosome 4 by RFLP mapping of F_2 populations from the cross between the ecotypes San feliu-2 and Columbia (Lee et al., 1993). Therefore, we were able to use the same populations to refine the chromosomal location of *LD* to the position between *FLA* and m506, as shown in Figure 5.

LD is a Unique Sequence

To determine if there are other genes related to *LD* in the Arabidopsis genome, we probed blots of genomic DNA at reduced stringencies with the *LD* cDNA. Under high- and reduced-stringency conditions, only the expected cross-hybridizing bands from the *LD* genomic region that we had characterized could be detected in Arabidopsis DNA (Figures 6A and 6B), indicating that other *LD*-related sequences are not likely to be present in the Arabidopsis genome. However, sequences related to *LD* could be detected in other plant species, such as cauliflower and *Nicotiana sylvestris*, under reduced-stringency conditions (Figure 6C); thus, *LD* homologs are likely to be present in other plant species.

LD Transcript Analysis

We examined *LD* mRNA levels in shoots of wild-type and mutant plants by RNA gel blotting. As shown in Figure 7A, transcripts of identical size were detected in all lines, except for the T-DNA insertion mutant *ld-3* in which no mRNA was detectable. The *LD* transcript could only be detected when highly poly(A)$^+$ enriched samples were analyzed, indicating that this message is present in very low abundance or that expression is restricted to a small subset of cells of the shoot. We also compared the *LD* mRNA levels of the wild-type plants grown under different photoperiodic conditions. There was no difference in *LD* transcript levels in plants grown in long and short days (Figure 7B).

DISCUSSION

We have isolated and characterized a gene, *LD*, that is involved in the regulation of flowering time in Arabidopsis. The function of the *LD* gene product is presumably to promote flowering

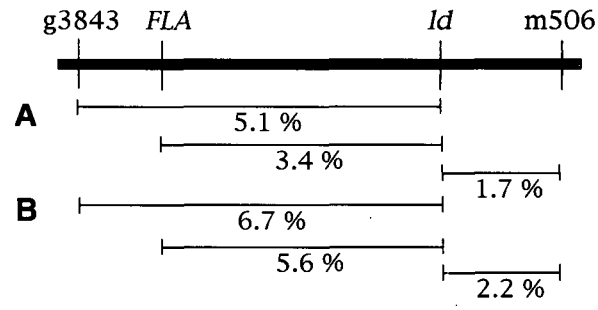

Figure 5. Map Position of the *LD* Locus.

The position of *LD* was determined by RFLP mapping using the *LD* genomic DNA fragment as a probe for blots containing DNA from crosses of Sf-2 and Columbia (see Lee et al., 1993, for details of these F_2 populations).

(A) Fifty-eight F_2 plants from the cross of Sf-2 and Columbia.

(B) Ninety F_2 plants from the second backcross generation.

For each population, the recombination percentages between the indicated markers are shown.

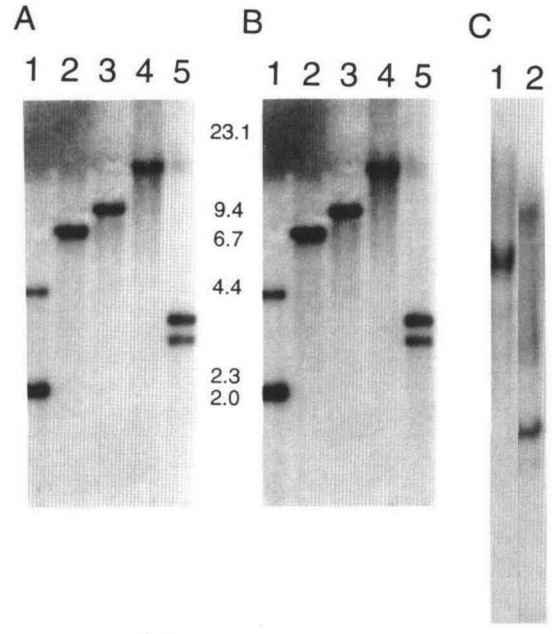

Figure 6. Reduced-Stringency DNA Blot Hybridization to Detect *LD*-Related Sequences in Arabidopsis, *Brassica oleracea* (Cauliflower), and *N. sylvestris*.

Shown are autoradiograms of filters hybridized with *LD* cDNA probe.
(A) Arabidopsis (Wassilewskija strain) genomic DNA (3 μg) was digested with PstI (lane 1), XbaI (lane 2), HindIII (lane 3), SalI (lane 4), and EcoRI (lane 5). Hybridization was done under high-stringency conditions (0.25 M Na^+, 65°C) as described by Reid et al. (1988).
(B) As described in **(A)** except that hybridization was performed at 50°C.
(C) Cauliflower (lane 1) and *N. sylvestris* (lane 2) genomic DNAs (3 μg) were digested with EcoRI. Hybridization conditions were as described in **(A)** except that hybridization was at 42°C with 20% formamide.
Between **(A)** and **(B)**, the size in kilobases and the position of migration of molecular weight markers are given.

because flowering is delayed by *ld* mutations. The effects of a mutation in *LD* were first described by Rédei (1962). Plants containing *ld* mutations flower later than the wild type in both inductive long-day and noninductive short-day photoperiods, but the delay of flowering time is more severe in short days when long- and short-day photoperiods consist of full-intensity growth chamber light (Table 2). Thus, under high light intensities, *ld* mutant plants retained photoperiod responsiveness. However, mutant plants were insensitive to inductive photoperiods consisting of low-intensity daylength extensions provided by cool-white fluorescent bulbs which emit light with a high red/far-red ratio. Although incandescent bulbs, which emit light with a lower red/far-red ratio, are often used for daylength extension studies, the conditions employed were sufficient to fully induce flowering in wild-type plants of the Wassilewskija ecotype (Table 2). Therefore, the *LD* gene may be involved in the perception of light levels or light quality. Vernalization of mutant seedlings for 30 days significantly accelerated the flowering time of mutant plants grown in both long and short days, although a phenotypic effect of the *ld* mutation was still apparent after this length of cold treatment. It remains to be determined whether longer periods of vernalization can further reduce the delay in flowering time caused by this mutation.

Koornneef et al. (1991) have placed the late-flowering mutants of Arabidopsis into three categories based upon flowering behavior in short days and the response to vernalization. The phenotype of *ld* mutant plants is similar to that of plants with mutations at the *FCA*, *FPA*, *FVE*, and *FY* loci, indicating that *LD* is likely to act in the flowering pathway proposed for these genes (Koornneef et al., 1991).

The phenotype of *ld-3* is likely to be that of the null mutant because in this allele, the T-DNA insert interrupts the coding sequence close to the amino terminus of the LD protein. The phenotypic effects on flowering of the three *ld* alleles were similar, indicating that all alleles may result in a loss of *LD* function. The effect on flowering of the *ld-1* and *ld-3* alleles appeared to be fully recessive when plants were grown in continuous light (Table 1). This behavior is in contrast to the majority of the late-flowering mutations at other loci that cause some delay in flowering time in the heterozygous state (Koornneef et al., 1991).

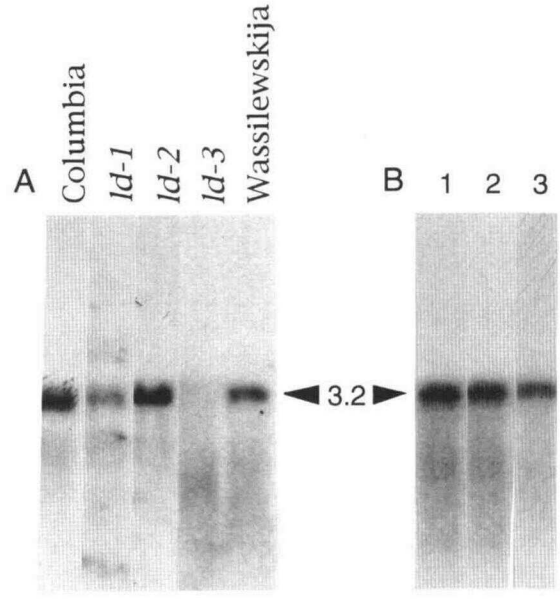

Figure 7. RNA Gel Blot Analysis of *LD* Transcript Levels.

Autoradiogram of RNA gel blots hybridized with ^{32}P-labeled *LD* cDNA are shown. Three micrograms of poly(A)$^+$ RNA derived from whole shoots was loaded for each lane.
(A) Comparison of Columbia and Wassilewskija wild types and *ld* mutants.
(B) Expression of *LD* mRNA in the leaves of 25-day-old Wassilewskija wild-type plants grown under long days (lane 1) and short days (lane 2) and 11-day-old seedlings (lane 3) grown under long days. The *LD* transcript of 3.2 kb is indicated by arrowheads.

The predicted amino acid sequence of the LD protein does not exhibit significant relatedness to other sequences in the data bases. However, the sequence does contain two putative motifs that may provide clues regarding LD function. First, the protein contains two regions that fit the consensus pattern for bipartite nuclear localization signals (Dingwall and Laskey, 1991; Raikhel, 1992). Second, LD has an additional characteristic of a class of nuclear proteins: a glutamine-rich domain that is present in certain transcription factors. The glutamine-rich domains of the mammalian Sp1 and the Drosophila Antennapedia transcription factors have been shown to function in transcriptional activation (Courey and Tjian, 1988; Mitchell and Tjian, 1989). The length and glutamine content of a region in the carboxy terminus of LD, 23 glutamines in 106 residues, are similar to those found in Sp1 and Antennapedia (Mitchell and Tjian, 1989). The isolation of the *LD* gene will provide the opportunity to test whether these putative motifs in LD actually function in nuclear localization and transcriptional regulation and to explore the specific role of *LD* in the regulation of flowering time.

METHODS

Arabidopsis Strains and Growth Conditions

The *ld-1* mutation is in the Columbia ecotype, and *ld-2* and *ld-3* are in the Wassilewskija ecotype. Seeds were sown on 0.8% agar containing one-fourth of the recommended level of minerals in Murashige–Skoog medium (Murashige and Skoog, 1962) and incubated at 4°C under short-day conditions (8-hr light/16-hr dark) for 24 hr to break dormancy or for 30 days for vernalization studies and then cultured at 25°C for an additional 5 to 7 days prior to transplanting. Seedlings were transferred to a potting mixture (2:1:1 peat/perlite/vermiculite [Fafard Superfine Mix; Fafard Inc., Springfield, MA]) that had been soaked with a nutrient solution as described previously (Somerville and Ogren, 1982). Plants were grown at 23 ± 2°C, 60 ± 10% relative humidity under ~100 μmol m^{-2} sec^{-1} of continuous fluorescent (75%) and incandescent (25%) light unless otherwise indicated.

DNA Extraction and DNA Gel Blot Analysis

DNA extraction and DNA gel blot analysis were performed as described by Lee et al. (1993). The restriction enzymes used for restriction fragment length polymorphism (RFLP) mapping of *LUMINIDEPENDENS* (*LD*) were EcoRI for the cross of Landsberg *erecta* and Columbia and HindIII for the cross of San feliu-2 and Columbia.

Molecular Cloning of *LD*

Plant DNA flanking the left border of the T-DNA insertion was isolated by plasmid rescue as described previously (Yanofsky et al., 1990). Briefly, genomic DNA from the *ld-3* mutant was cleaved with the restriction enzyme SalI, and the DNA was ligated and introduced into *Escherichia coli* by electroporation; the resulting colonies were screened for ampicillin resistance and kanamycin sensitivity. The rescued plasmids were used to probe DNA gel blots of wild-type and *ld-3* mutant DNAs to determine which plasmids contained plant DNA that detected polymorphisms between the wild type and the mutant and was therefore likely to represent plant DNA flanking the T-DNA insert. One such plasmid, pSAL48, was used to probe an Arabidopsis genomic library in λGEM11 (a gift of J. Esch and D. Marks, University of Nebraska, Lincoln). Two overlapping genomic clones were isolated and restriction mapped as described by Sambrook et al. (1989).

pSAL48 was also used to screen a cDNA library prepared from a mixed population of plants at all stages of development (a gift of S. Elledge and R. Davis, Stanford University, Palo Alto, CA), resulting in the isolation of a partial-length 1.8-kb cDNA. An anchored polymerase chain reaction (PCR) technique was used to isolate a 1.4-kb DNA fragment corresponding to the 5′ end of the *LD* cDNA as described previously (Loh et al., 1989). Briefly, the reverse transcription reaction was performed with poly(A)$^+$ RNA isolated from Arabidopsis leaves with random hexamer oligonucleotides as primers. A poly(G) extension was added to the first-strand cDNAs by incubating the cDNA with terminal deoxynucleotidyl transferase. *LD* cDNAs were then amplified using primers AN1 and AN2 (Loh et al., 1989) and an *LD*-specific primer. The PCR products were cloned into pBluescriptII KS+ (Stratagene), and positive clones were identified by colony hybridization.

DNA Sequencing Analysis

cDNA and genomic clones were subcloned into pBluescriptII KS+ (Stratagene), and sequential deletions were generated using the Erase-a-Base system (Promega). Double-stranded plasmid DNA was sequenced by the dideoxy chain termination method (Sambrook et al., 1989) using the Sequenase system (U. S. Biochemicals). All regions were sequenced on both strands. The nucleotide sequence of a region of *ld*-2 that was shown to be polymorphic by single-strand conformational polymorphism (Orita et al., 1989) was also determined. The predicted LD protein sequence was assembled from the cDNA using the GCG package of computer programs from the University of Wisconsin Genetics Computer Group (Devereux et al., 1984). To search the data bases for amino acid sequences similar to the predicted sequence of the LD protein, we used the FASTA program (Pearson and Lipman, 1988) included in the GCG package.

RNA Gel Blot Analysis

Total RNA was extracted as described previously (Puissant and Houdebine, 1990), and poly(A)$^+$ RNA was isolated by oligo(dT)-cellulose affinity columns. For RNA gel blot analysis, 3 μg of poly(A)$^+$ RNA was fractionated on 1% formaldehyde agarose gels, blotted onto membranes (Biotrace HP; Gelman, Ann Arbor, MI), and probed with ^{32}P-labeled *LD* cDNA as described previously (John and Amasino, 1988).

Molecular Complementation of *ld*

A 9.6-kb DNA fragment containing the *LD* gene was inserted into the binary vector pCGN1547 (a gift of Calgene, Inc., Davis, CA). This construct was transferred to *Agrobacterium tumefaciens* LB4404 (Hoekema et al., 1983). *ld*-2 mutant plants were transformed via a modification of the procedure of Valvekens et al. (1992) in which timentin (100 μg/mL) was substituted for vancomycin.

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