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Rapid Communication

Ecotype-Specific Expression of a Flowering Mutant Phenotype in *Arabidopsis thaliana*[1]

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The majority of mutations that delay flowering in *Arabidopsis thaliana* have been identified in studies of the Landsberg *erecta* (L*er*) ecotype. In this report we describe a gene (referred to as *FLD*) that, when mutated, delays flowering in the Columbia ecotype but has a minimal phenotype in the L*er* genetic background. The late-flowering phenotype of *fld* mutants requires a non-L*er* allele of another gene involved in the control of flowering time, *Flowering Locus C*. *fld* mutants retain a photoperiod response, and the flowering time of *fld* mutants can be reduced by cold treatment and low red/far-red light ratios.

The initiation of flowering is a complex process that, in many plant species, is regulated by a combination of developmental programs and responses to environmental signals, such as temperature and daylength (Lang, 1965; Vince-Prue, 1975). The physiology and genetics of flowering have been extensively studied in *Arabidopsis thaliana*. Flowering in Arabidopsis is promoted by long days, and in many genotypes, exposure to low temperatures also promotes flowering (vernalization). The response to low temperature and long days is quantitative; in the absence of these environmental signals most genotypes of Arabidopsis will eventually flower (Napp-Zinn, 1985).

Genes involved in the initiation of flowering have been identified by two approaches. One approach is to cross Arabidopsis ecotypes that exhibit different flowering behavior. These analyses have revealed that the major difference in flowering time among many ecotypes resides at the *FRI* locus (Napp-Zinn, 1985; Burn et al., 1993; Lee et al., 1993; Clarke and Dean, 1994). Plants containing *FRI* flower later under short-day than long-day photoperiods, and the late-flowering phenotype is reduced by cold treatment. Another approach is to isolate mutants in which the initiation of flowering is either delayed (late-flowering mutants) or accelerated (early-flowering mutants) (for review, see Martinez-Zapater et al., 1994). The late-flowering mutants can be further classified into at least two groups, depending on their response to photoperiod and low temperature (Martinez-Zapater et al., 1994). One group retains a photoperiod and vernalization response, i.e. flowering is more delayed in short days than in long days and the late-flowering phenotype can be reduced by low temperature. The other group does not exhibit a large difference in flowering time in short days compared to long days, and the vernalization response is reduced.

The majority of late-flowering mutations have been identified and characterized in the L*er* ecotype (Koornneef et al., 1991). Redei (1962) identified three late-flowering mutants in the Col ecotype. One of the mutations, which identified the *LD* locus, has not been found among the late-flowering mutants generated in L*er* (Koornneef et al., 1991), because L*er* has been shown to contain an allele of the *FLC* gene that suppresses the late-flowering phenotype of *FRI* and *ld* mutations (Koornneef et al., 1994; Lee et al., 1994). The *FLC* alleles of most ecotypes, such as Col, appear to act as flowering inhibitors, whereas the L*er* allele of *FLC* does not mediate this inhibition (Lee et al., 1994). The role of *LD* may be to counteract this *FLC*-mediated inhibition of flowering, and thus *ld* mutations in L*er* have little effect on flowering time.

In this report, a novel late-flowering mutation is described, which, similar to *ld*, requires a non-L*er* allele of *FLC* to cause a late-flowering phenotype. These results indicate that genetic studies in the L*er* ecotype do not reveal the full range of genes that regulate flowering in most ecotypes of Arabidopsis.

MATERIALS AND METHODS

Plant Lines

The late-flowering *fld* mutant was selected from ethyl methanesulfonate-mutagenized plants of the Col ecotype. The original *fld* mutant was backcrossed to Col, and several lines derived from late-flowering plants in the F_2 generation were selected for study. *FLC*-Col introgressed into L*er* (the *FLC*-Col line) was described by Lee et al. (1994; table I, line 11) and was provided by M. Koornneef (Department of Genetics, Wageningen Agricultural University, Wageningen, The Netherlands).

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Abbreviations: Col, Columbia; FLC, *Flowering Locus C*; FLD, *Flowering Locus D*; *FRI*, *FRIGIDA*; L*er*, Landsberg *erecta*; *LD*, *LUMINIDEPENDENS*.

Growth Conditions

Unless otherwise stated, plants were grown as described by Lee et al. (1993) with continuous illumination of approximately 100 μmol m^{-2} s^{-1} of cool-white fluorescent light at 22 to 23°C. Temperature and light conditions for the photoperiod and vernalization treatments were as described by Lee and Amasino (1995). The flowering response was measured as the number of leaves in the rosette when the flowering stalk reached 1 cm in height as previously described (Lee et al., 1994).

Molecular Techniques

The map position of *FLD* and the allele at *FLC* were determined by microsatellite analysis according to the method of Bell and Ecker (1994), except that 20 μL of PCR product were prepared and 35 PCR cycles were used. *FLC* is closely linked to the nga249 locus (Lee et al., 1994). At least one marker on each chromosome arm (except for the top arm of chromosome 2) was tested for segregation with the late-flowering phenotype to determine the map location of *FLD*. PCR products were visualized by staining with ethidium bromide after electrophoresis in a 7% nondenaturing acrylamide gel.

RESULTS

Screening for New Flowering-Time Mutations

The M_2 progeny of ethyl methanesulfonate-mutagenized plants of the Col ecotype were screened for plants that produced a greater number of rosette leaves than Col before initiating flowering. One of the late-flowering mutations isolated from this screen, which appeared to identify a novel flowering locus based on its map location, was chosen for further study (Fig. 1). This locus was designated *FLD*.

Genetic Characterization and Mapping of *fld*

When the *fld* mutant was crossed to wild-type Col, the resulting F_1 plants were slightly later flowering than wild-type Col (Table I). Self-pollinated F_1 plants produced F_2 populations that segregated for early- and late-flowering plants in a ratio of 3:1 (Table II). Thus, the *fld* mutation behaves as a single, moderately recessive trait.

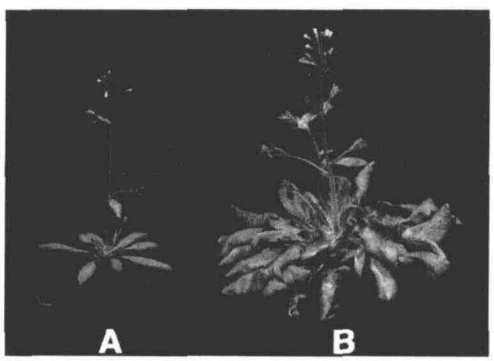

Figure 1. The phenotype of the *fld* mutation in the Col background. A, Col wild type. B, *fld* mutant line (after two backcrosses to Col).

Table I. *Rosette leaf number and days to flowering of the fld mutant in the Col background*

The averages and SDs of the values from 10 plants of each genotype are presented. Plants were grown in continuous light. The *fld* mutant plants used in this study had been backcrossed once to wild-type Col.

Genotype	Leaf No.	Days to Flowering
Col	8.9 ± 1.2	25.1 ± 2.0
Col × *fld* F_1	13.3 ± 2.4	27.6 ± 1.7
fld	49.1 ± 12.8	59.3 ± 10.8

Microsatellite analysis (Bell and Ecker, 1994) of plants from the F_2 progeny of crosses between *fld* (in Col) and L*er* were used to obtain the genetic map location of *fld*. The late-flowering phenotype co-segregated with Col DNA at two chromosomal locations, one near the top of chromosome 5 and the other on the upper arm of chromosome 3. The chromosome 5 locus was closely linked to *FLC* (see below). The chromosome 3 locus did not map near any known flowering-time gene. This locus was required in a homozygous state to cause late flowering and was designated *FLD*. The relationship of *FLD* to three microsatellite markers is shown in Figure 2.

The Late-Flowering Phenotype of *fld* Requires the Col Allele of FLC

To determine whether the *fld* mutant phenotype was dependent on the Col allele of *FLC* (*FLC*-Col), the F_2 population resulting from the cross of the *fld* mutant to L*er* was analyzed. In this population, three-sixteenths of the progeny were late flowering (Table II; Fig. 3). The genotype at *FLC* was assessed by microsatellite analysis of the tightly linked marker nga249 (Lee et al., 1994). All late-flowering plants in this population contained at least one copy of the Col allele of *FLC*. The late-flowering plants that were heterozygous for *FLC*-Col flowered before producing 40 leaves, whereas the majority of the late-flowering plants homozygous for *FLC*-Col flowered after producing more than 40 leaves (Fig. 3). The *fld* mutation, therefore, requires *FLC*-Col to produce a late-flowering phenotype, and the effect of *FLC*-Col is dosage dependent.

The *FLC* dependence of the mutant phenotype was also examined by crossing the *fld* mutant in Col to a line of L*er* containing *FLC*-Col (*FLC*-Col line; Lee et al., 1994). The F_2 population resulting from this cross produced early- and late-flowering plants segregating in a ratio of 3:1 as expected if the late-flowering trait in this cross

Table II. *Segregation data and χ^2 analysis of the F_2 generations of fld mutants crossed to Col, Ler, and a Ler line containing the FLC allele from Col (FLC-Col).*

Cross	Early Flowering	Late Flowering	Expected Ratio	χ^2
fld × Col F_2	57	26	3:1	1.8
fld × Ler F_2	85	18	13:3	0.11
fld × *FLC*-Col F_2	122	38	3:1	0.13

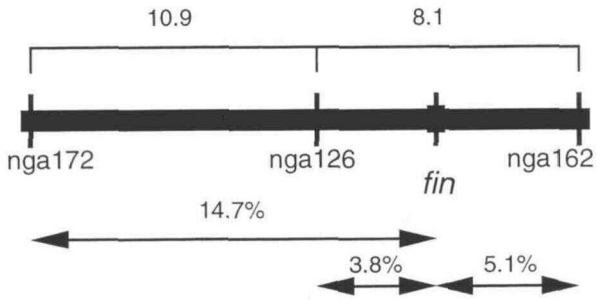

Figure 2. Map location of *fld* on chromosome 3. Distances shown with brackets were derived from the recombinant inbred lines (Lister and Dean, 1995). Distances shown with arrows indicate the recombination percentages between the microsatellite markers and late flowering. From a total of 146 plants, the numbers of crossing over events detected for the markers nga126, nga162, and nga172 were 11, 15, and 43, respectively. *fin*, *FLD*.

were due to a single locus (Table II), confirming that the *fld* mutation requires *FLC*-Col to create a strong, late-flowering phenotype.

To analyze the effect of the *fld* mutation in the L*er* genetic background, four plants homozygous for both *fld* and *FLC*-L*er* were identified based on flanking microsatellite markers in an F_2 population resulting from the second backcross of *fld* to L*er*. The genotypes of these plants were confirmed by test crosses. The self-pollinated progeny of these lines flowered only slightly later than L*er* (Table III; Fig. 4). Thus,

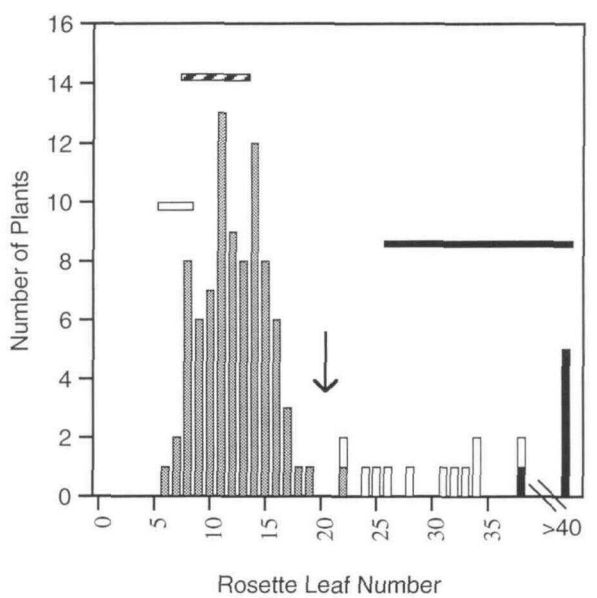

Figure 3. Frequency distribution of rosette leaf number in a segregating population from a cross between *fld* (Col background) and L*er*. The arrow marks the division between early- and late-flowering plants. The *FLC* genotypes of the early-flowering plants were not determined (dotted columns). Among the late-flowering plants, those that were homozygous for the *FLC* allele from Col are shown as filled columns, and *FLC* heterozygotes (*FLC*-Col/*FLC*-L*er*) are shown as open columns. The horizontal bars represents the leaf number distributions of the parental lines *fld* (filled bar) and L*er* (open bar) and the F_1 (striped bar).

Table III. *Rosette leaf number and days to flowering of the fld mutation in the Ler background*

The averages and SDs of the values from eight plants are presented. Plants were grown in continuous light. The *fld* mutant plants used in this study had been backcrossed once to wild-type *Ler*.

Genotype	Leaf No.	Days to Flowering
L*er*	8.4 ± 0.5	24.8 ± 0.9
fld in L*er*	12.9 ± 2.0	27.8 ± 2.9

the *fld* mutation in L*er* produces a minimal alteration of flowering time.

Effect of Light Quality and Vernalization on the *fld* Mutant Phenotype

The flowering response of *fld* to photoperiod, light quality, and vernalization was examined (Fig. 5). The *fld* mutant flowered later than did wild type under long- and short-day photoperiods. However, *fld* mutant plants were still photoperiod responsive; the mutants flowered considerably later under short-day conditions than under long-day conditions. Cold treatment greatly reduced time to flowering in *fld* mutants under both photoperiods. Far-red light supplementation also reduced the number of leaves produced before flowering in *fld* mutants, and the reduction in flowering time was proportional to the amount of far-red light.

DISCUSSION

In this paper we report a novel flowering time gene, *FLD*, located on chromosome 3. Mutations in *fld* cause a strong late-flowering phenotype in the Col ecotype. The *fld* mutation joins an environmental response class that includes *FRI* and mutations in *ld*, *fca*, *fy*, *fve*, and *fpa* (Koornneef et al., 1991; Bagnall, 1993; Martinez-Zapater et al., 1994). This class is late flowering under long-day photoperiods and even later flowering under short-day photoperiods. Thus, mutants in this class exhibit a photoperiod response. As is the case for other mutants in this class (Bagnall, 1993), flowering time in *fld* mutant plants is reduced by cold treatment and low red/far-red light ratios. Another characteristic of this response class is that the late-flowering

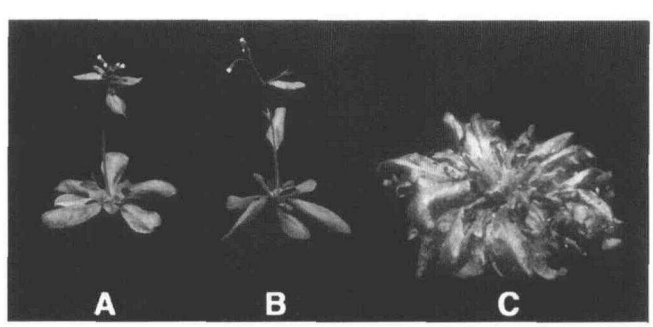

Figure 4. Phenotype of *fld* in the L*er* background. A, L*er* wild type. B, *fld* mutant line with the L*er* allele of *FLC* after two backcrosses to L*er*. C, *fld* mutant line with the Col allele of *FLC* after two backcrosses to L*er*.

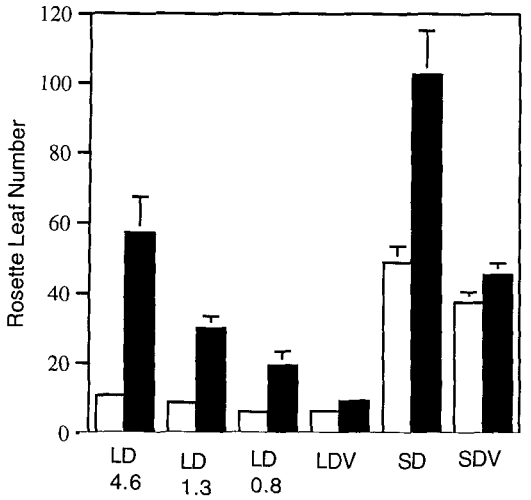

Figure 5. Average rosette leaf number of *fld* mutant plants under different environmental conditions. Columbia (open columns) and *fld* mutants (filled columns) were grown under long-day (LD) photoperiods with varying ratios of red/far-red light, with short-day (SD) photoperiods, and after cold treatment. Long-day photoperiods consisted of 20 h of light with a red/far-red ratio of 4.6, 1.3, and 0.8 (Lee and Amasino, 1995). The short-day photoperiod consisted of 8 h of light with a red/far-red ratio of 1.3. Plants were cold treated for 40 d in short days as described by Lee et al. (1994) and then grown under long-day (LDV) or short-day (SDV) photoperiods with a red/far-red light ratio of 1.3. The *fld* mutant plants were derived from one backcross of the original mutant to Col.

phenotype is strongly enhanced when these mutants are combined with a non-L*er* *FLC* allele such as *FLC* from Col (Sanda and Amasino, 1996). The *fld* mutation also exhibits this interaction with *FLC*. Similar to *FRI* and *ld* (Lee et al., 1994), the late-flowering phenotype of *fld* is greatly increased by the presence of *FLC*-Col. This effect of *FLC* on the *fld* phenotype is dosage dependent; maximum late flowering of *fld* mutants requires *FLC*-Col in a homozygous state, although a single copy of *FLC*-Col causes a substantial delay of flowering in *fld* mutants.

Mutations in the *LD* and *FLD* genes have not been identified in genetic analyses of flowering time in L*er* because the late-flowering phenotype of these mutations is dependent on a non-L*er* allele of *FLC*. The function of *FLD*, as suggested for *LD* (Lee et al., 1994), may be to counteract the inhibition of *FLC* alleles, which act as flowering inhibitors. Because the L*er* allele of *FLC* does not act to inhibit flowering, *fld* mutations in the L*er* background have little effect. We are screening for additional genes involved in the promotion of flowering for which the late-flowering mutant phenotype depends on a non-L*er* allele of *FLC*.

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