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early in short days 4, a mutation in *Arabidopsis* that causes early flowering and reduces the mRNA abundance of the floral repressor *FLC*

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SUMMARY

The plant shoot is derived from the apical meristem, a group of stem cells formed during embryogenesis. Lateral organs form on the shoot of an adult plant from primordia that arise on the flanks of the shoot apical meristem. Environmental stimuli such as light, temperature and nutrient availability often influence the shape and identity of the organs that develop from these primordia. In particular, the transition from forming vegetative lateral organs to producing flowers often occurs in response to environmental cues. This transition requires increased expression in primordia of genes that confer floral identity, such as the *Arabidopsis* gene *LEAFY*. We describe a novel mutant, *early in short days 4* (*esd4*), that dramatically accelerates the transition from vegetative growth to flowering in *Arabidopsis*. The effect of the mutation is strongest under short photoperiods, which delay flowering of *Arabidopsis*. The mutant has additional phenotypes, including premature termination of the shoot and an alteration of phyllotaxy along the stem, suggesting that *ESD4* has a broader role in plant development. Genetic analysis indicates that *ESD4* is most closely associated with the autonomous floral promotion pathway, one of the well-characterized pathways proposed to promote flowering of *Arabidopsis*. Furthermore, mRNA levels of a floral repressor (*FLC*), which acts within this pathway, are reduced by *esd4*, and the expression of flowering-time genes repressed by FLC is increased in the presence of the *esd4* mutation. Although the reduction in *FLC* mRNA abundance is likely to contribute to the *esd4* phenotype, our data suggest that *esd4* also promotes flowering independently of *FLC*. The role of *ESD4* in the regulation of flowering is discussed with reference to current models on the regulation of flowering in *Arabidopsis*.

Key words: Flowering, *Arabidopsis thaliana*, Photoperiod, Vernalization, *ESD4*

INTRODUCTION

The plant shoot is derived from stem cells within the shoot apical meristem (SAM). In *Arabidopsis*, the primordia that form on the flanks of the SAM soon after germination give rise to leaves, while those that form later in shoot development produce flowers. This change in the identity of the lateral organs formed on the shoot is regulated by environmental conditions, such as temperature and daylength, and by the age of the plants. Early in the development of floral primordia, the mRNA of the *LEAFY* gene accumulates and encodes a transcription factor that activates the expression of floral homeotic genes (Weigel et al., 1992; Parcy et al., 1998). *LEAFY* expression is increased by environmental conditions that promote flowering (Blázquez et al., 1998; Blázquez and Weigel, 2000), and this complex response is mediated by signals that act on the shoot meristem and probably directly on the developing primordium (Hempel and Feldman, 1994). Some of the signals are formed in the mature leaves and promote or repress flowering at the apex of the shoot, while others act within the apex and determine its competence to respond to these signals (reviewed by Aukerman and Amasino, 1998).

A systematic genetic approach to identifying genes that regulate flowering time has been taken in *Arabidopsis* (reviewed by Araki, 2001; Mouradov et al., 2002; Simpson and Dean, 2002). Many mutations have been identified that delay flowering, and genetic and physiological analysis has placed these mutations in at least three independent pathways that promote flowering (Koornneef et al., 1998). These are the long day pathway, the autonomous pathway and the gibberellic acid (GA)-dependent pathway. Mutations affecting the long day pathway (*co*, *fd*, *fe*, *fha*, *ft*, *fwa*, *gi* and *lhy*) delay flowering under long day conditions, whereas those affecting the autonomous pathway (*fca*, *fpa*, *fve*, *fy* and *ld*) delay flowering under all photoperiods. Mutations that strongly reduce GA biosynthesis delay flowering under long days, and almost abolish flowering under short days (Wilson et al., 1992). The existence of these pathways is supported by the phenotypes of double mutants (Koornneef et al., 1991; Koornneef et al., 1998). Partial redundancy between the long day, autonomous and GA pathways probably explains why no single mutation

has been identified that prevents flowering. However a triple mutant, in which all three flowering pathways are impaired does not flower under long or short days, indicating that these pathways are absolutely required for flowering under these conditions (Reeves and Coupland, 2001).

The cloning of several flowering-time genes, and analysis of their expression in wild-type and mutant backgrounds, have led to detailed models of the mechanisms underlying the flowering response (Lee et al., 1994; Putterill et al., 1995; Macknight et al., 1997; Schaffer et al., 1998; Michaels and Amasino, 1999a; Sheldon et al., 1999; Kardailsky et al., 1999; Kobayashi et al., 1999; Samach et al., 2000; Lee et al., 2000; Suárez-López et al., 2001; El-Assal et al., 2001; Gendall et al., 2002). These models are supported by the phenotypes of transgenic plants in which flowering-time genes are overexpressed (Kardailsky et al., 1999; Kobayashi et al., 1999; Onouchi et al., 2000; Lee et al., 2000). An endogenous circadian clock acts to control the expression patterns of genes within the long day pathway, enabling the promotion of flowering under appropriate day lengths (Schaffer et al., 1998; Fowler et al., 1999; Park et al., 1999; Suárez-López et al., 2001). The autonomous pathway appears to promote flowering by reducing the expression of the *FLC* gene that encodes a repressor of flowering (Michaels and Amasino, 1999a; Sheldon et al., 1999; Michaels and Amasino, 2001). More recently, it has become apparent that the long day, autonomous, and GA pathways converge on a common set of target genes to regulate flowering time and flower development. For example, all three pathways are involved in the regulation of expression of *LEAFY* (Simon et al., 1996; Blázquez et al., 1998; Nilsson et al., 1998; Blázquez and Weigel, 2000), *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS* (*SOC1*)/*AGAMOUS-LIKE 20* (*AGL20*) (Samach et al., 2000; Lee et al., 2000; Borner et al., 2000; Michaels and Amasino, 2001) and *FT* (Kardailsky et al., 1999; Kobayashi et al., 1999; Samach et al., 2000; Suárez-López et al., 2001; Ohto et al., 2001).

Additional flowering pathways promote flowering specifically in response to vernalization. The vernalization response shares common targets with the autonomous pathway, and acts to repress *FLC* mRNA abundance (Michaels and Amasino, 1999a; Sheldon et al., 1999). However, vernalization is not mediated by any of the three pathways described above, because the vernalization response is not abolished in mutants representative of each pathway (Koornneef et al., 1991; Chandler et al., 2000; Michaels and Amasino, 1999b), nor in a *co-2 fca-1 ga1-3* triple mutant in which all three pathways are impaired (Reeves and Coupland, 2001). The *FRI* gene confers a vernalization response on naturally occurring varieties of *Arabidopsis* (Johanson et al., 2000), and is involved in the promotion of *FLC* mRNA levels (Michaels and Amasino, 1999a; Sheldon et al., 1999; Johanson et al., 2000). Stable repression of *FLC* by vernalization requires VERNALIZATION 2, which was proposed to act within a protein complex similar to Polycomb group complexes of *Drosophila* (Gendall et al., 2001). Both the promotion of flowering by the autonomous pathway and the delay in flowering by strong *FRI* alleles are absolutely dependent on active *FLC*, although the vernalization response also has an *FLC* independent component (Michaels and Amasino, 2001).

A diverse group of mutations causes early flowering in *Arabidopsis* (Alvarez et al., 1992; Zagotta et al., 1996; Hicks et al., 1996; Goodrich et al., 1997; Telfer and Poethig, 1998; Somers et al., 1998; Soppe et al., 1999; Scott et al., 1999; Hartmann et al., 2000; Michaels and Amasino, 2001; Gomez-Mena et al., 2001). For example, the *phyB* mutation disrupts the gene encoding the red/far-red light receptor PHYTOCHROME B (PHYB) and causes early flowering under both long and short days (Reed et al., 1993; Koornneef et al., 1995). The *elf3* mutation causes early flowering under short days so that mutants flower at the same time irrespective of daylength (Hicks et al., 1996). Under continuous light, *elf3* also disrupts the rhythmic expression of the circadian clock-regulated gene *CAB2*, and the rhythmic movement of leaves. *ELF3* is proposed to act by gating light signalling to the circadian clock (McWatters et al., 2000). The early-flowering mutant, *hasty*, forms adult leaves earlier in vegetative development than wild type (Telfer and Poethig, 1998). The apical meristem of the *hasty* mutant can also respond more rapidly to expression of the floral meristem-identity gene *LEAFY*, suggesting that *HASTY* reduces the competence of the shoot to respond to flowering signals. *EMBRYONIC FLOWER* mutations cause extreme early flowering, probably by inactivating transcriptional repression complexes that in wild-type plants repress the expression of floral identity genes such as *APETALA1* (Chen et al., 1997; Kinoshita et al., 2001).

Combining mutations causing early or late flowering can provide information on how the functions of the affected genes are inter-related (Yang et al., 1995; Koornneef et al., 1995; Koornneef et al., 1998a; Weller et al., 1997; Soppe et al., 1999; Michaels and Amasino, 2001). For example, in *Arabidopsis*, the *efs* gene was suggested to act within the autonomous pathway on the basis of double mutant analysis (Soppe et al., 1999). However, in general, little information is available on how mutations causing early or late flowering interact in *Arabidopsis* and the molecular basis for such interactions is even less clear.

Here we describe a novel mutation, *early in short days 4* (*esd4*), that causes an extreme early-flowering phenotype in *Arabidopsis*. The analysis demonstrates genetic and molecular relationships between *ESD4* and genes in the autonomous flowering pathway.

MATERIALS AND METHODS

Plant material

The *early in short days* mutants were isolated in the *Arabidopsis thaliana* ecotype Landsberg *erecta* (L*er*). L*er* seeds were subjected to 90 kRad gamma irradiation by Dr Mary Anderson, University of Nottingham. Around 40,000 M_2 plants derived from approximately 2,200 M_1 parents were screened under short-day conditions and three early-flowering mutants were recovered. Together with two mutants isolated independently by Maarten Koornneef (University of Wagningen, Netherlands), these were named *early in short days 1* to *early in short days 5* (*esd1* to *esd5*). The two mutants provided by M. Koornneef were *esd2* and *esd3*.

Mutant seed stocks were all in L*er* and were provided by the following individuals: *fca-1, fve-1, co-2, fwa-1, ft-1* M. Koornneef (University of Wageningen), *ag-3* J. Goodrich (University of Edinburgh), *gai-1, ga1-3,* (Nottingham Stock Centre).

Growth conditions and measurement of flowering time

Flowering time was measured under controlled conditions as described previously (Reeves and Coupland, 2001). Short days consisted of a photoperiod of 10 hours light, whereas long days

Table 1. Flowering time and shoot determinancy of wild-type and *esd4* mutant plants

	Long days			Short days		
Genotype	Rosette leaves	Cauline leaves	Flower number	Rosette leaves	Cauline leaves	Flower number
L*er*	5.4±0.5	3.1±0.2	37.2±9.2	38.4±2.0	10.3± 0.9	58.5±9.5
esd4	2.2±0.4	2.5±0.6	19.1±3.0	6.1±0.6	3.4±0.5	34.4±3.9
esd4/+	5.0±0.5	3.1±0.7	ND	38.3±2.2	9.8±1.3	ND

ND, not determined. Flowering times are shown as mean leaf number ± standard deviation of the mean.

consisted of 10 hours light with a 6 hour daylength extension supplied by incandescent bulbs. At least 15 plants were used to examine the flowering time of each genotype. For analysis of *FT*, *SOC1* and *CO* mRNA expression patterns, plants were grown in cabinets under true long days of 16 hours light with no daylength extension.

Mapping

The map position of *ESD4* was defined using the CAPS markers 326 (5′-GTGACGTACTCGGTGAAG, 5′-CTCTACTACACACCACAC; *StyI*) and SC5 (5′-TCGACGACTCTCAAGAACCC, 5′-CACAAGC-TATACGATGCTCACC; *AccI*).

Cryo-scanning electron microscopy

The sample was mounted on an aluminium stub which was then plunged into liquid nitrogen slush and transferred onto the cryostage of a CT1000 Hexland cryo-transfer system at –85°C (Oxford Instruments, Oxford) fitted to a CamScan Mark IV scanning electron microscope (Gresham-CamScan, Cambridge). The sample was transferred to the pre-chamber, placed on a stage at –195°C and sputter coated with gold for 6 minutes at 2 mA. It was returned to the main cryo-stage of the microscope and viewed at 16 kV.

Construction and analysis of double mutants

Information on the construction of double mutants can be obtained from the authors.

To examine the flowering times of *esd4 ga1-3* and *ga1-3* plants seeds were germinated without applying exogenous GA as described previously (Reeves and Coupland, 2001).

Northern analysis of mRNA abundance

RNA extraction, northern blotting and hybridisation was as described by Suárez-López et al. (Suárez-López et al., 2001). The *FLC* probe was provided by A. Gendall and C. Dean (JIC, Norwich), and consisted of a 403 bp PCR fragment corresponding to nucleotides 298-700 of the *FLC* cDNA sequence. The *FT* probe was described previously (Samach et al., 2000). The *UBQ10* probe was as described by Wang et al. (Wang et al., 1997). The *SOC1* probe was a 459 bp PCR fragment, amplified from the *SOC1* cDNA with the primers 5′-AATATGCAAGATACCATAGATCG-3′ and 5′-TCTTGAAGAACA-AGGTAACCCAAT-3′. The strength of hybridisation signals was assessed using a Phosphorimager (Molecular Dynamics). The ratio of signal intensity compared to the *UBQ10* control was calculated for each sample using ImageQuant software (Molecular Dynamics).

Analysis of CO mRNA abundance

RT-PCR analysis of CO mRNA abundance was as described by Suárez-López et al. (Suárez-López et al., 2001).

RESULTS

Isolation and mapping of the *early in short days 4* (*esd4*) mutation

Early-flowering mutants were identified under short-day conditions after mutagenesis with gamma rays (see Materials and Methods). To exclude the possibility of recovering previously analysed *hy* or *elf3* mutants (Reed et al., 1993; Hicks et al., 1996), only early-flowering individuals that did not show an elongated hypocotyl were selected. The *early in short days 4* (*esd4*) mutant was the most extreme. None of the other mutations recovered were alleles of *esd4*, because F_1 plants derived from crossing each of them to *esd4* flowered at the same time as wild type.

The *esd4* mutant was back-crossed to L*er* three times before further phenotypic analysis, and crossed to Columbia to enable its position to be determined relative to RFLP markers. For the mapping, DNA was extracted from 200 F_2 plants that were homozygous for *esd4* and analysed with several CAPS markers. Linkage was detected to markers on the lower arm of chromosome 4. For example, *esd4* was located approximately 1.8 cM distal to marker 326, and 0.5 cM proximal to SC5. Relative to phenotypic markers, *esd4* is therefore located around 2.3 cM distal to *cop9* and 0.5 cM proximal to *fca*. The map position of *esd4* excluded the possibility that it was an allele of any previously described mutation causing early flowering.

esd4 causes early flowering and has pleiotropic effects on shoot development

Plants homozygous for *esd4* were grown under long and short days, and their flowering time compared with that of L*er* (Table 1; Fig. 1). The early-flowering phenotype was most dramatic under short days where wild-type plants flowered after forming around 49 rosette and cauline leaves compared to only 10 for *esd4*. The mutants also flowered slightly earlier than wild-type plants under long days, forming only 5 leaves compared to 9 for L*er*. The *esd4* mutation therefore causes early flowering under long and short days, and the flowering time of the mutant is influenced by daylength, although less strongly than that of wild-type plants (Table 1).

Arabidopsis plants exhibit heteroblasty, forming juvenile rosette leaves that have trichomes only on their adaxial (upper) side; adult rosette leaves that develop trichomes on their adaxial and abaxial (lower) sides, and cauline leaves that form on the stem above the rosette and have trichomes on both surfaces (Chien and Sussex, 1996; Telfer et al., 1997). The early-flowering *hasty* mutant forms fewer juvenile rosette leaves than wild type but approximately the same numbers of adult rosette and cauline leaves (Telfer and Poethig, 1998). As shown in Fig. 2, *esd4* showed a reduction in all types of leaf. However, the most dramatic effect was on adult rosette leaves, which were absent from *esd4* mutants grown under long days and dramatically reduced in number under short days.

The main inflorescence of *esd4* mutants showed several abnormalities. Internodes between the cauline leaves and solitary flowers are shorter, and the leaves are smaller than in wild-type plants (Fig. 1). Also at the nodes at which the last leaf or the first solitary flower form there are often alterations

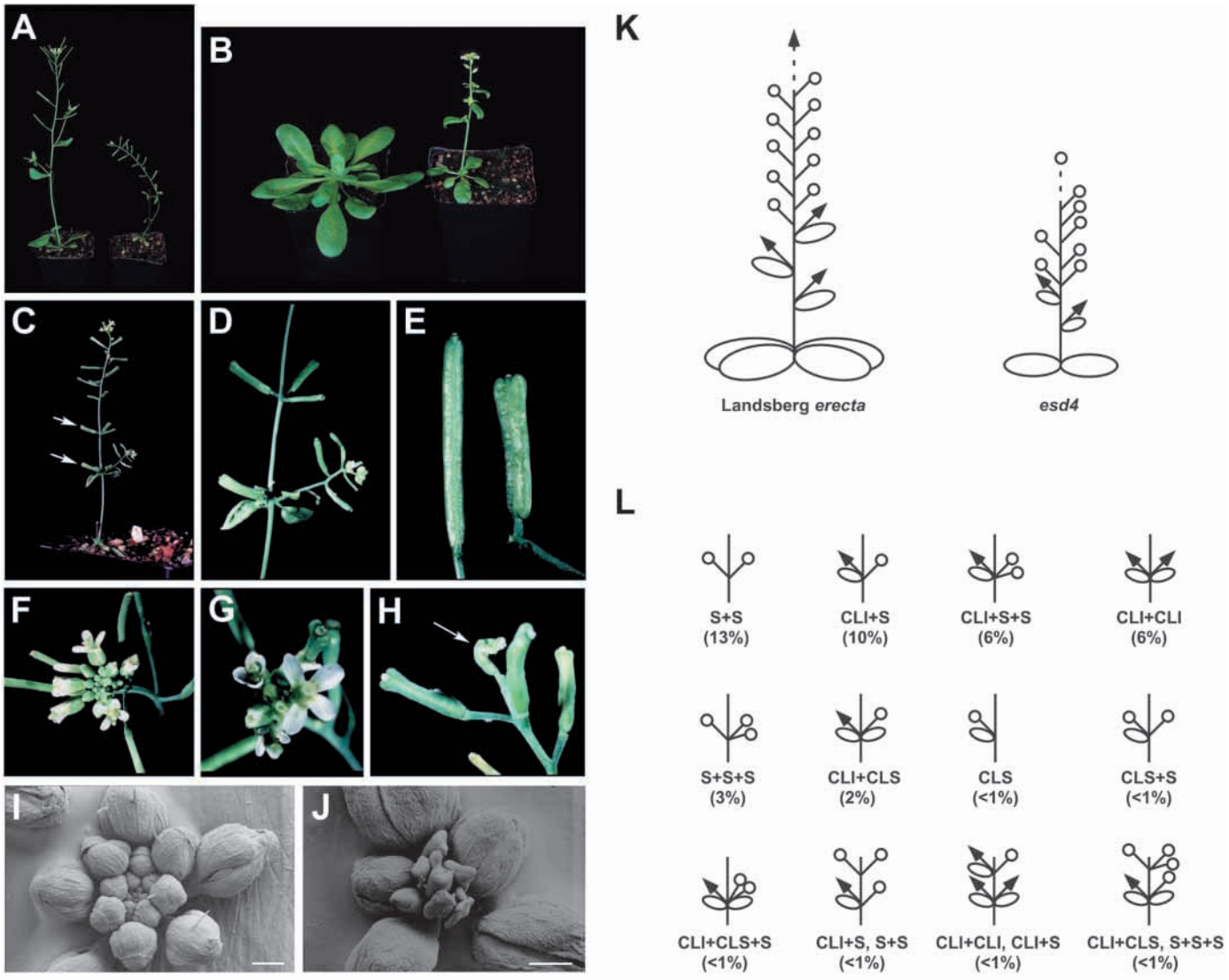


Fig. 1. Phenotype of the *esd4* mutant**.** (A) A wild-type plant (left) and an *esd4* mutant (right) grown under long days. Both plants are 5 weeks old. (B) A wild-type plant (left) and an *esd4* mutant (right) grown under short days. Both are 6 weeks old. (C) An *esd4* mutant grown under long days. The arrows indicate siliques that have developed in unexpected positions. (D) The section of the stem of *esd4* mutants shown in C at higher magnification. (E) A comparison of silique shape in wild-type (left) and *esd4* (right) plants. (F-H) The apex of 4-week-old plants: (F) wild-type; (G) *esd4* mutant; (H) *esd4* mutant showing the pistil-like structure (arrow) that terminates the shoot. (I,J) Scanning electron micrographs of the apex of the shoot 4-week-old wild-type (I) and *esd4* mutant (J) plants grown under long days. The scale bars are 300 μm. (K) Schematic diagram illustrating the structure of wild-type and *esd4* plants. The arrows represent indeterminate shoots, the circles flowers, and rosette and cauline leaves are shown as ovals. (L) The frequency with which various abnormalities were recorded at the node containing the last cauline leaf or the first flower. CL, cauline leaf; S, solitary flower; I, inflorescence. Percentages illustrate the proportion of plants that showed each abnormality. Fewer than 1% of wild-type plants showed any of these abnormalities.

to phyllotaxy compared to wild-type plants (Fig. 1). For example, in approximately 13% of *esd4* mutants, two solitary flowers develop at the first node after the last cauline leaf, while in another 10% of mutants the last cauline leaf to develop and the first solitary flower form at the same node on opposite sides of the stem. More complex abnormalities also occur at this point of transition from cauline leaves to solitary flowers (Fig. 1).

In addition, *esd4* mutants formed fewer flowers than wild-type plants (Table 1). The main inflorescence of wild-type plants contained approximately 37 solitary flowers under long days, and around 59 under short days. However, the main inflorescence of *esd4* mutants formed many fewer solitary flowers: approximately 19 and 34 under long and short days, respectively. This reduction in flower number was in part associated with the conversion of the shoot apical meristem into a carpelloid, pistil-like structure (Fig. 1) that does not occur in wild-type plants (Fig. 1). Under long days, the main inflorescence of approximately 80% of *esd4* mutants terminated with this structure. Under short days, this phenotype was less severe, and was only observed in around 25% of plants.

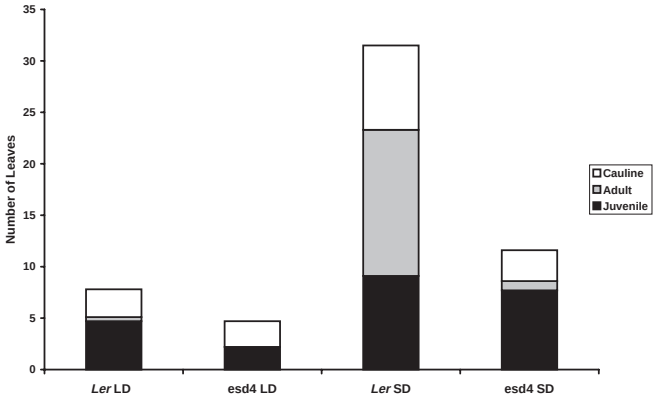


Fig. 2. Histogram comparing the number of juvenile, adult and cauline leaves in wild-type (L*er*) and *esd4* mutants. Plants were grown under both long days (LD) and short days (SD).

After fertilisation, the gynoecium of *Arabidopsis* plants elongates to form a silique that contains the developing seeds (Bowman, 1993). The siliques of *esd4* mutants are shorter than those of wild type, and are broader at the tip (Fig. 1). The siliques of *esd4* mutants contain 2 valves, as do those of wild-type plants (Bowman, 1993).

The *esd4* mutation is recessive and behaves as a single genetic locus

Plants heterozygous for *esd4* were generated by back-crossing to L*er*. The shoot of the heterozygotes showed a wild-type phenotype, and the heterozygous plants flowered at the same time as wild-type under long and short days (Table 1). The *esd4* mutation is therefore recessive with respect to all aspects of the mutant phenotype.

To determine whether the *esd4* phenotype was due to a mutation at a single locus, 78 F_2 plants derived from a back-cross of *esd4* to wild-type plants were examined. Nineteen plants flowered at a similar time to *esd4* and showed all of the shoot phenotypes previously described for the *esd4* mutant, while 59 plants flowered at a similar time to the L*er* wild-type, and showed a wild-type shoot phenotype. The ratio of *esd4*-like plants to wild-type like plants was approximately 3:1, suggesting that the *esd4* phenotype is caused by a mutation at a single genetic locus.

AGAMOUS is not required for *esd4* to cause early flowering

Ectopic expression of the floral-organ identity gene *AGAMOUS* was previously shown to cause early flowering (Mizukami and Ma, 1992; Goodrich et al., 1997). To test whether *AG* was required for the early flowering of *esd4*, double mutants carrying both *esd4* and *ag-3* were made. These plants flowered at the same time as *esd4* under long and short days. *AG* is therefore not required for the early flowering of *esd4* plants. However, ectopic expression of other genes that encode MADS box containing proteins can also cause early flowering. We therefore constructed the *esd4 ap3*, *esd4 pi* and *esd4 ap1* double mutants and they all flowered at the same time as *esd4*, indicating that AP3, PI and AP1 are also not required for early flowering of *esd4*.

The interaction of *esd4* with mutations affecting the long day promotion pathway

To test the relationship between *ESD4* and genes that promote flowering, double mutants were made containing *esd4* and mutations causing late flowering. The *co*, *ft* and *fwa* mutations were proposed to affect the long-day pathway (Koornneef et al., 1998). The double mutants *esd4 co-2*, *esd4 ft-1* and *esd4 fwa-1* were constructed to test the effects of the mutations on the *esd4* phenotype.

Under long days, all three double mutants flowered later than *esd4* (Table 2; Fig. 3). The flowering times of the *esd4 co-2*, *esd4 fwa-1* and *esd4 ft-1* double mutants were similar to that of wild type. Under short days, the *co-2*, *ft-1* and *fwa-1* mutants do not show late flowering compared to wild type. However, these mutations delayed flowering of the *esd4* mutant under these conditions (Table 2, Fig. 4). The *ft-1* and *fwa-1* mutations caused a more severe delay in the flowering time of *esd4* than *co-2*, and under short days the *esd4 ft-1* and *esd4 fwa-1* plants flowered almost as late as wild-type and the late flowering parent. The latest flowering genotype was *esd4 fwa-1* that formed around 23 rosette leaves under short days compared to approximately 9 for *esd4* (Table 2).

The pleiotropic effects of *esd4* were also reduced in severity in the *esd4 co-2*, *esd4 ft-1* and *esd4 fwa-1* plants. More flowers were formed on the primary inflorescences of the double mutants, and the proportion of plants in which the primary inflorescence was determinate was reduced. For example, in long days *esd4 co-2*, *esd4 ft-1* and *esd4 fwa-1* produced over 35, 72 and 61 flowers, respectively, compared to the 20 flowers produced by *esd4*. Only around 37% of *esd4 co-2* mutants showed a determinate main inflorescence, whereas no *esd4 ft-1* or *esd4 fwa-1* plants showed this phenotype. The frequency with which abnormalities in floral phyllotaxy were observed at the node representing the transition from cauline leaves to flowers (Fig. 1) was also reduced. These were visible in fewer than 8% of *esd4 co-2*, and 3% of *esd4 ft-1* mutants. No *esd4 fwa-1* mutants showed these abnormalities. The double

Table 2. Flowering time of *esd4* double mutants

	Long days		Short days	
Genotype	Rosette leaves	Cauline leaves	Rosette leaves	Cauline leaves
L*er*	5.1±0.4	2.8±0.5	24.6±1.3	10.6±1.2
esd4	2.2±0.4	2.5±0.5	8.6±0.9	2.9±0.6
co-2	16.3±1.4	7.8±1.0	17.9±1.3	5.6±1.7
esd4 co-2	6.0±0.7	3.9±0.6	10.6±0.9	4.2±0.7
ft-1	10.3±0.7	5.5±0.5	26.4±1.3	11.9±1.5
esd4 ft-1	4.5±0.6	3.5±0.6	21.1±2.4	7.8±1.0
fwa-1	9.8±1.0	5.5±0.6	24.1±0.9	8.7±0.7
esd4 fwa-1	5.2±0.4	3.3±0.6	23.2±0.8	8.3±0.9
fca-1	16.5±2.7	6.3±1.5	36.5±4.8	12.9±1.0
esd4 fca-1	3.7±0.5	2.5±0.5	14.9±1.4	3.8±0.9
fve-1	12.1±2.0	3.8±0.7	41.8±4.6	11.0±0.8
esd4 fve-1	2.8±0.5	2.4±0.5	12.1±1.3	3.2±0.8
*ga1-3**	7.8±1.0		80.6±6.4	
*esd4 ga1-3**	8.8±1.2		15.3±1.3	
*gai**	11.9±1.1		55.2±3.9	
*esd4 gai**	6.0±0.5		11.3±0.8	
*co-2 fca-1 ga1-3**	>90		ND	
*esd4 co-2 fca-1 ga1-3**	28.1±2.0		ND	

*In plants carrying *ga1-3* or *gai*, rosette and cauline leaves could not be distinguished. ND, not determined. Flowering times are shown as mean leaf number ± standard deviation of the mean.

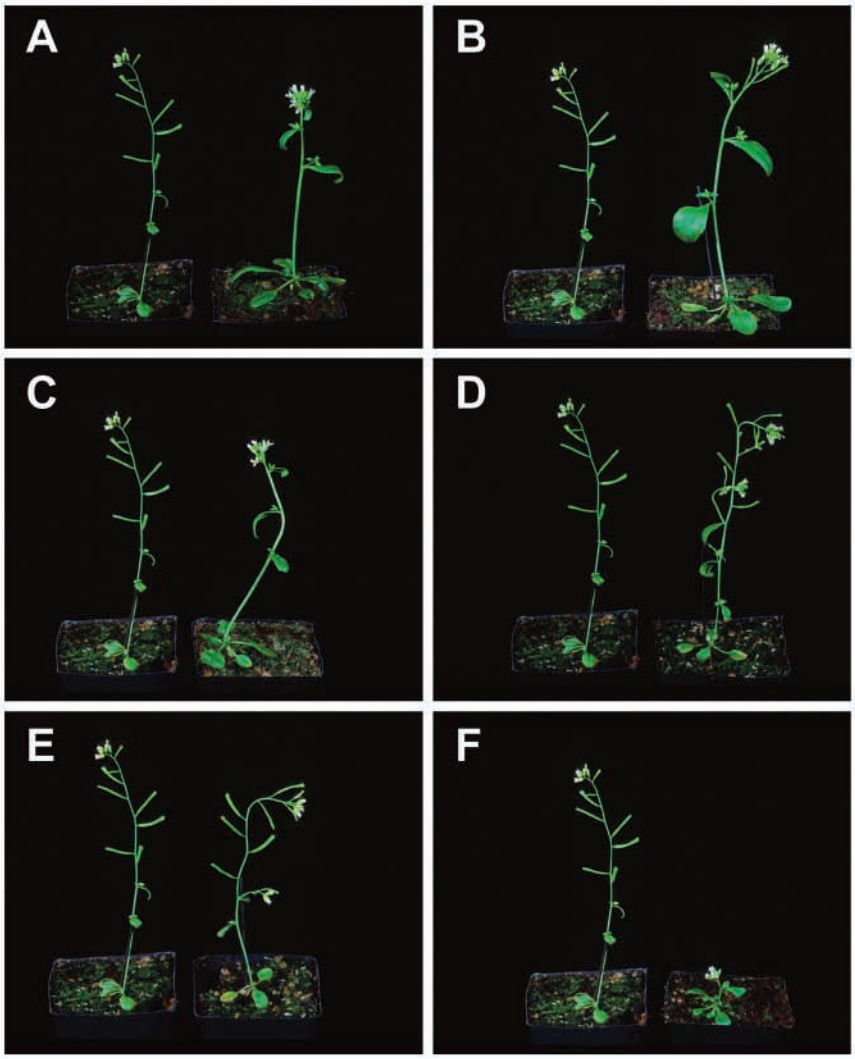


Fig. 3. Photographs illustrating the phenotypes of double mutants carrying *esd4* grown under long days. In each panel an *esd4* mutant is shown on the left and the following double mutants on the right. (A) *esd4 co-2*; (B) *esd4 ft-1*; (C) *esd4 fwa-1*; (D) *esd4 fca-1*; (E) *esd4 fve-1*; (F) *esd4 gai*. All plants are 4 weeks old.

mutants retained the altered silique shape of *esd4*, and remained slightly dwarfed with respect to wild type, with the exception of *esd4 ft-1*, which was taller than wild type.

The *FCA* and *FVE* genes are not required for *esd4* to cause early flowering

The *fca* and *fve* mutations affect the autonomous flowering-time pathway (Koornneef et al., 1998). The double mutants *esd4 fve-1* and *esd4 fca-1* were made and their flowering times scored under long and short days (Table 2; Figs 4 and 5).

Under long days, *esd4 fca-1* and *esd4 fve-1* double mutants flowered earlier than wild type, and were earlier flowering than the *esd4 co-2*, *esd4 ft-1* and *esd4 fwa-1* plants described above. Some of the double mutant plants were indistinguishable from *esd4*, although on average *esd4 fve-1* and *esd4 fca-1* formed 1 or 2 rosette leaves more than *esd4* (Table 2). *esd4 fve-1* double mutants flowered slightly earlier than *esd4 fca-1* double mutants, probably because *fve-1* causes a weaker phenotype than *fca-1* (Table 2) (Koornneef et al., 1991).

Under short days, *esd4 fca-1* and *esd4 fve-1* mutants again flowered earlier than wild-type L*er* and the late-flowering parent (Table 2). The double mutant plants flowered later than the *esd4* mutant, with *esd4 fca-1* plants and *esd4 fve-1* plants forming a total of 19 and 15 leaves, compared to 12 in the *esd4* mutant. Furthermore, although the *fca-1* and *fve-1* mutants flowered much later than any of the long-day pathway mutants described in the previous section (Table 2; see above), the relative severity of their phenotypes was in general reversed in the presence of the *esd4* mutation: the *esd4 fca-1* and *esd4 fve-1* double mutants flowered much earlier under short days than *esd4 fwa-1* and *esd4 ft-1*.

Under both long and short days, the double mutants still showed the pleiotropic effects caused by the *esd4* mutation; the siliques retained their club-like appearance, the shoot terminated in a carpelloid structure, and the phyllotaxy of flowers on the shoot was disrupted. The frequency with which an abnormality occurs at the node representing the transition from cauline leaves to flowers was reduced from 45% in *esd4* to 19% in *esd4 fca-1* and 15% in *esd4 fve-1*. The number of flowers formed on the main inflorescence was also affected: under long days, 29 flowers were formed by *esd4 fca-1* plants and 21 by *esd4 fve-1* plants compared to 20 for *esd4*. However, the *fca-1* and *fve-1* mutations reduced the severity of the pleiotropic effects of *esd4* to a much lesser extent than the long-day pathway mutations did (see previous section).

The effect of mutations affecting synthesis or response to the growth regulator gibberellin on the flowering time of *esd4* mutants

Mutations that affect GA synthesis or signal transduction delay flowering weakly under long days, and severely under short days (Wilson et al., 1992). The severe mutation *ga1-3* disrupts an early step in GA biosynthesis, and prevents flowering under short days (Sun and Kamiya, 1994; Wilson et al., 1992). The *gai* mutation affects GA signalling (Peng et al., 1997). To determine whether GA synthesis and response pathways are required for the early flowering caused by *esd4*, plants carrying *esd4 ga1-3* and *esd4 gai* were constructed.

Under both long and short days the *esd4 gai* double mutants flowered earlier than wild type, particularly under short days, and at approximately the same time as *esd4* mutants (Table 2). The *gai* mutation therefore has almost no effect on the early-flowering phenotype caused by *esd4*. The *esd4 ga1-3* plants showed a flowering time intermediate between the *esd4* and *ga1-3* parents, indicating that GA synthesis is required for the extreme early-flowering phenotype caused by *esd4*. However,

esd4 still has a dramatic effect on the flowering time of *ga1-3* mutants, particularly under short days where *ga1-3* flowered after forming approximately 80 leaves, while the *esd4 ga1-3* double mutants formed approximately 15 (Table 2; Fig. 4).

esd4 can promote flowering in a *co-2 fca-1 ga1-3* background

A *co-2 fca-1 ga1-3* triple mutant, in which all three flowering time pathways are impaired, does not flower under long days (Reeves and Coupland, 2001). To determine whether *esd4* promotes flowering in this triple mutant background, *esd4 co-2 fca-1 ga1-3* quadruple mutants were constructed and their flowering time examined under long days. As previously shown, the *co-2 fca-1 ga1-3* control plants did not flower under long-day conditions (Table 2) (Reeves and Coupland, 2001). However, the *esd4 co-2 fca-1 ga1-3* quadruple mutant flowered after the production of 28 leaves.

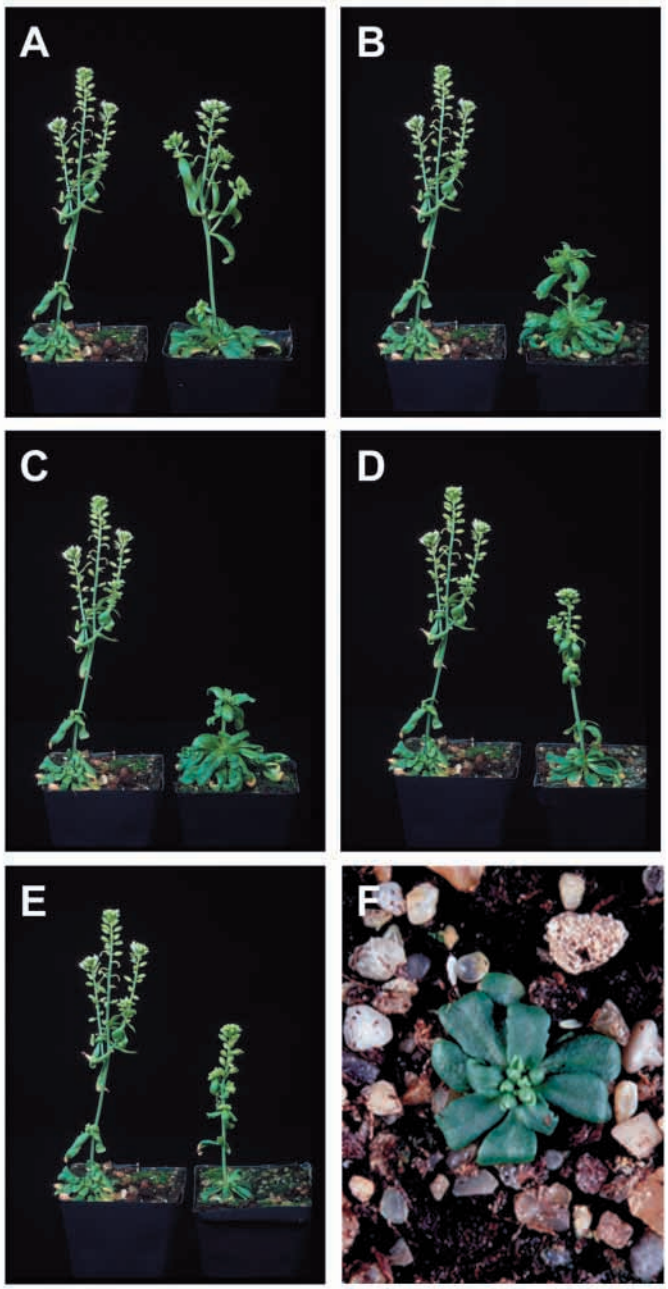


Fig. 4. Photographs illustrating the phenotypes of double mutants carrying *esd4* grown under short days. In A-E an *esd4* mutant is shown on the left and the following double mutants on the right. (A) *esd4 co-2*; (B) *esd4 ft-1*; (C) *esd4 fwa-1*; (D) *esd4 fca-1*; (E) *esd4 fve-1*. (F) An *esd4 ga1-3* double mutant. All plants are 6 weeks old.

The level of *FLC* mRNA is reduced in *esd4* mutants

The *esd4* mutation most effectively suppressed the late-flowering phenotype of mutations that impair the autonomous pathway (Fig. 3; Table 2). Therefore, *esd4* might cause early flowering by increasing the activity of the autonomous pathway downstream of *FCA* and *FVE*, or by bypassing the requirement for the autonomous pathway. *FLC*, which encodes a repressor of flowering, is a downstream target of both the autonomous and the vernalization-dependent floral promotion pathways (Michaels and Amasino, 1999a; Michaels and Amasino, 2001; Sheldon et al., 1999; Sheldon et al., 2000). *FLC* mRNA abundance is increased in mutants impaired in the autonomous pathway and this increase is responsible for their late-flowering phenotype. Therefore, *esd4* may suppress the effect of autonomous pathway mutations by reducing *FLC* mRNA levels.

The abundance of the *FLC* mRNA was compared in wild-type, *esd4*, *fca-1*, *esd4 fca-1*, *fve-1* and *esd4 fve-1* seedlings that were 7 days old and had been grown in long days (Fig. 5A). *FLC* mRNA abundance was higher in both *fca-1* and *fve-1* mutants than in wild-type plants, as previously shown (Sheldon et al., 1999; Michaels and Amasino, 1999a). However, in *esd4 fca-1* and *esd4 fve-1* double mutants, the level of *FLC* mRNA was reduced compared to the late flowering *fca-1* and *fve-1* parents. Nevertheless, *FLC* mRNA abundance was still higher than in wild-type plants, which flower later than *esd4 fca-1* and *esd4 fve-1* mutants. *FLC* mRNA levels were also compared between wild-type plants and *esd4* mutants. Although *FLC* is expressed at a low level in L*er* wild-type plants, this was further reduced in *esd4* mutant plants. Similar reductions in the level of *FLC* mRNA were also observed under short days (data not shown).

esd4 can partially suppress the effect of *fca-1* on *FT* and *SOC1* mRNA levels

The reduction in *FLC* mRNA levels in *esd4* may contribute to the early-flowering phenotype of the mutant. The repression of flowering by FLC is probably caused in part by reduced expression of the flowering-time genes *SOC1* and *FT* (P.Suárez-López and G.Coupland, unpublished results) (Michaels and Amasino, 2001; Ohto et al., 2001). Therefore, whether the early flowering and reduced expression of *FLC* in genotypes containing *esd4* was also associated with increased expression of *SOC1* and *FT* was tested. Over a 24-hour long day cycle, *FT* and *SOC1* mRNA abundance was compared between wild-type and *esd4* plants, and between *fca-1* and *esd4 fca-1* plants.

In wild-type plants, *FT* showed the expected pattern of expression, with the main peak in mRNA abundance occurring between 12 and 16 hours after dawn (Fig. 5B) (Suárez-López et al., 2001). In *esd4* single mutants, *FT* mRNA abundance was slightly higher than in wild-type plants (Fig. 5B). The *fca-1* mutation caused a reduction in the level of *FT* mRNA, so that

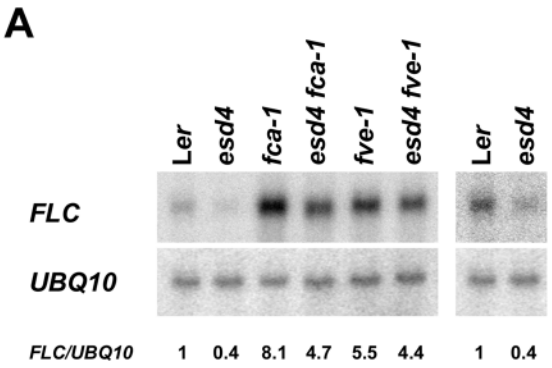
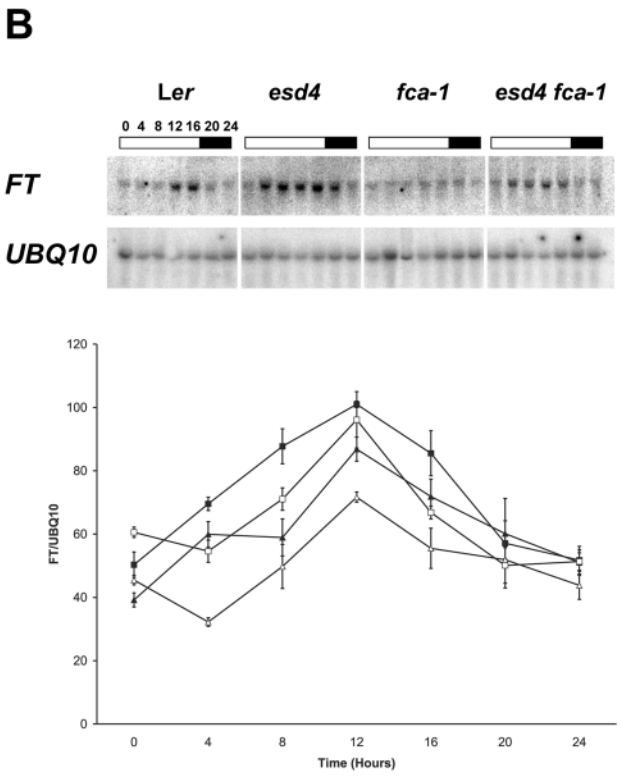
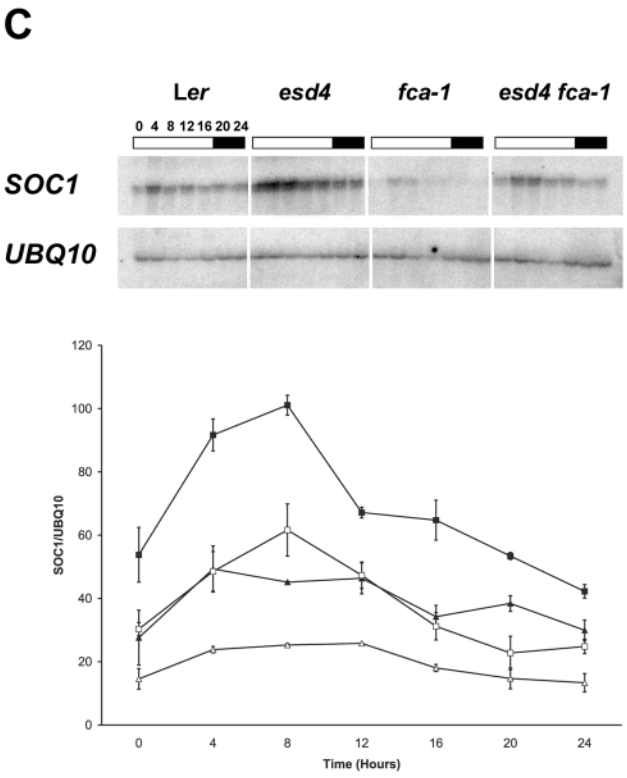
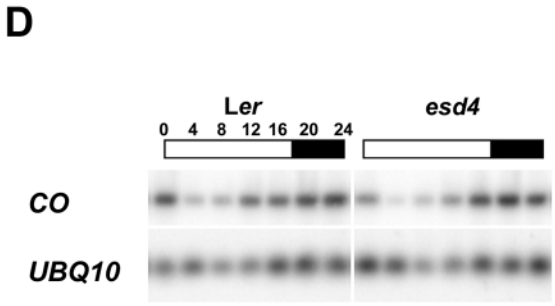
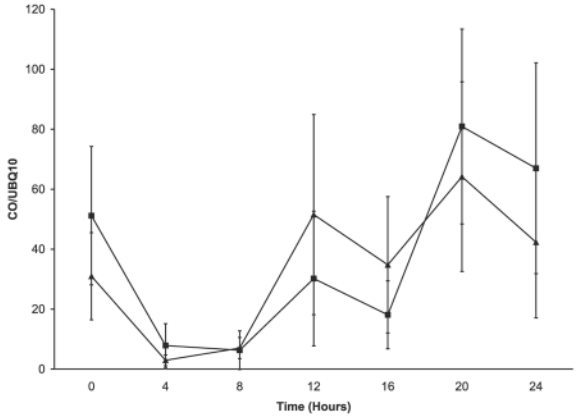


Fig. 5. Analysis of the of the expression of the flowering time genes *FLC*, *FT*, *SOC1* and *CO* in wild-type and *esd4* mutant plants. (A) Northern blot comparing *FLC* mRNA levels in 7-day-old L*er*, *esd4*, *fca-1*, *esd4 fca-1*, *fve-1*, and *esd4 fve-1* plants. The panel on the right is a longer exposure of the L*er* and *esd4* samples. (B) Northern blot comparing *FT* mRNA levels in 7-day-old L*er*, *esd4*, *fca-1* and *esd4 fca-1* plants. The graph shows the mean level of *FT* gene expression relative to the *UBQ10* control. In all graphs, black triangles represent L*er*, black squares *esd4*, white triangles *fca-1* and white squares *esd4 fca-1*; error bars indicate the standard error of the mean. (C) Northern blot comparing *SOC1* mRNA levels in 7-day-old L*er*, *esd4*, *fca-1* and *esd4 fca-1* plants. The graph shows the mean level of *SOC1* gene expression relative to the *UBQ10* control. Error bars indicate the standard error of the mean. (D) RT-PCR analysis of *CO* expression in 7-day-old wild-type and *esd4* plants. The graph shows the level of *CO* gene expression relative to the *UBQ10* control. Error bars indicate the standard error of the mean. (A-D) All experiments were performed three times using RNA from independently grown plant material.









a lower level peak was detectable at the same time as in wild-type plants. This reduction in *FT* expression is probably caused by increased *FLC* expression in *fca-1* mutants (Michaels and Amasino, 2001), and was partially suppressed by the *esd4* mutation so that *FT* mRNA levels were increased in the *esd4 fca-1* double mutant compared to *fca-1*, and similar to those of wild-type plants (Fig. 5B).

Similar observations were made with *SOC1*. In wild-type plants *SOC1* mRNA peaked around 8 hours after dawn, although the peak was of lower amplitude than that of *FT* (Fig. 5C) (Samach et al., 2000). In *esd4* single mutants, *SOC1* expression was increased, and the *fca-1* mutation caused a severe reduction in the level of *SOC1* mRNA, although a low level peak was still detectable at the same time as in wild-type plants (Fig. 5C). As for *FT*, the *esd4* mutation partially suppressed the effect of *fca-1* on *SOC1* expression (Fig. 5C), so that the level of *SOC1* mRNA in the double mutant was similar to that of wild-type plants.

The increases in *FT* and *SOC1* expression in *esd4 fca-1* plants compared to *fca-1* mutants are consistent with the proposal that *esd4* causes earlier flowering by reducing *FLC* expression and thereby increasing the expression of genes that are repressed by FLC.

esd4 does not affect the expression pattern of *CONSTANS*

To test at the molecular level whether *esd4* influenced the activity of the long-day pathway, *CO* mRNA abundance was compared in wild-type and *esd4* plants. *CO* acts downstream of many of the other long-day pathway genes and is not repressed by FLC, although its expression is increased in several early-flowering mutants or transgenic plants that affect the long-day pathway (Onouchi et al., 2000; Suárez-López et al., 2001). *CO* shows a diurnal pattern of expression, with the main peak of mRNA abundance occurring around 16 hours after dawn in long-day grown plants (Suárez-López et al., 2001). In wild-type plants, *CO* mRNA levels showed the expected pattern (Fig. 5D) (Suárez-López et al., 2001). No significant difference in either the diurnal expression pattern or the amplitude of expression of *CO* was observed in the *esd4* mutant.

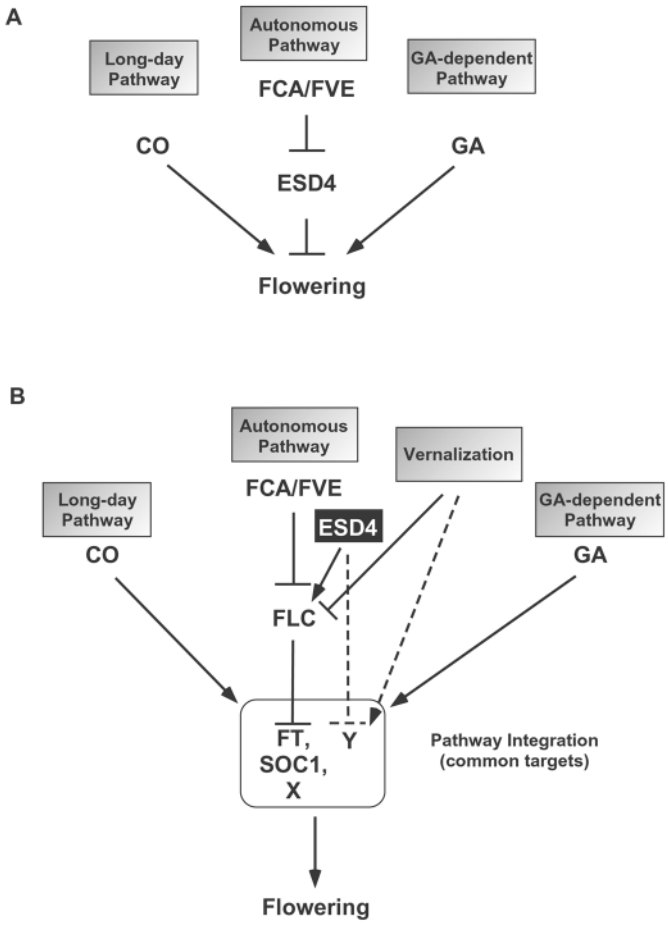


Fig. 6. Models for the role of *ESD4* in the regulation of flowering time. (A) ESD4 acts within the autonomous pathway to repress flowering and ESD4 activity is repressed by the activity of FCA/FVE. (B) ESD4 acts to repress flowering, in part by promoting high levels of the floral repressor FLC. Genes repressed by FLC include FT and SOC1, but it is likely that other genes (X) are also regulated by FLC. Both the autonomous and vernalization pathways act to regulate *FLC* mRNA levels (Sheldon et al., 1999; Sheldon et al., 2000; Michaels and Amasino, 1999; Michaels and Amasino, 2001), and ESD4 may interact with genes in these pathways. Genetic and molecular evidence suggests that ESD4 is likely to repress flowering independently of FLC (represented by dotted line), as does the promotion of flowering by vernalization (Michaels and Amasino, 2001). The genes regulated in an FLC-independent manner by ESD4 are represented by Y and probably include *FT* and *SOC1*, as the level of expression of *FT* and *SOC1* is similar in wild type and *esd4 fca-1* mutants, despite higher levels of *FLC* mRNA in *esd4 fca-1* plants. Mutations in the long day and gibberellin pathways delay the flowering of *esd4* mutants by affecting the activity of genes that are common targets of several floral promotion pathways.

DISCUSSION

The location of *esd4* on chromosome 4 indicates that it is not an allele of a previously described mutation causing early flowering. The *esd4* mutation is recessive and segregates as a single locus, suggesting that the role of the *ESD4* gene is to delay flowering. However, the pleiotropic effects of the mutation suggest that *ESD4* also has a broader role in plant development.

Pleiotropy of the *esd4* mutation suggests ESD4 plays a broad role in plant development

The *esd4* mutation has pleiotropic effects on the architecture of the shoot and on silique shape (Fig. 1). These pleiotropic effects of *esd4* may be related to the early flowering of the mutant, perhaps due to ectopic expression of flowering time or floral meristem identity genes. Some of the previously described early flowering mutants show pleiotropic effects (Goodrich et al., 1997; Soppe et al., 1999; Gomez-Mena et al., 2001) whereas others do not (Scott et al., 1999; Michaels and Amasino, 2001). Moreover, transgenes causing ectopic expression of flowering time genes can also result in developmental defects (Kardailsky et al., 1999; Kobayashi et al., 1999; Onouchi et al., 2000). Some of the mutations causing late flowering largely suppressed the effects of *esd4* on flowering time, shoot determinacy and floral phyllotaxy, but

none of these mutations abolished the effect of *esd4* on plant height or silique shape. It is therefore unlikely that all the pleiotropic effects of *esd4* can be explained by altered expression of flowering time genes, and therefore *ESD4* probably plays a broader role in the regulation of plant development.

Genetic interactions between *esd4* and mutations causing late flowering

Models for the genetic control of flowering time in *Arabidopsis* propose that three independent genetic pathways promote flowering under long photoperiods (see Introduction) (reviewed by Araki, 2001; Mouradov et al., 2002; Simpson and Dean, 2002). Double mutants carrying *esd4* and mutations previously shown to affect each of these three pathways were constructed to determine whether *esd4* promotes early flowering by acting through one or more of these pathways. No true epistatic relationships were identified between *esd4* and mutations causing late flowering, although epistatic relationships have previously been reported between *Arabidopsis* mutations causing early and late flowering (Yang et al., 1995; Soppe et al., 1999). However, mutations that affect the autonomous pathway, such as *fca* and *fve*, had only weak effects on the *esd4* phenotype under long days. One interpretation of these results is that *ESD4* acts within the autonomous pathway downstream of *FCA* and *FVE* (Fig. 6A). In this model FCA and FVE promote flowering by repressing the activity of ESD4, as was proposed previously for *efs* (Soppe et al., 1999).

Construction of double mutants demonstrated that under both long and short days mutations in the long-day pathway (*co*, *ft* and *fwa*) caused a severe delay in flowering of *esd4* mutants in comparison to the effect of autonomous pathway mutations. A similar effect was observed for GA pathway mutations under long days. ESD4 could therefore act in the long-day pathway before the *CO*, *FT* and *FWA* genes to reduce their expression, or within the GA pathway to reduce GA signalling or synthesis. This is consistent with previous observations that overexpression of the long-day pathway gene *CO* from the 35S promoter causes early flowering and largely suppresses the late-flowering caused by *fca* (Onouchi et al., 2000), and that mutations in suppressors of GA signalling cause early flowering (Jacobsen and Olszewski, 1993). However, no increase in *CO* mRNA abundance was detected in an *esd4* mutant, and these mutant plants are not resistant to the GA biosynthesis inhibitor paclobutrazol (data not shown) nor do they show elongated internodes, which are effects characteristic of mutations such as *spindly* that cause GA-signal transduction independently of GA (Jacobsen and Olszewski, 1993).

An alternative explanation for the partial suppression of mutations in the long-day and GA pathways by *esd4* is that *ESD4* acts predominantly in pathways related to the autonomous pathway (Fig. 6), and that increased activity of these pathways in *esd4* mutants can partially suppress the effect of mutations in the long-day or GA pathways. The *esd4* mutation would then be proposed to activate the autonomous pathway downstream of *FCA* and *FVE*, because of the early flowering of *esd4 fca* and *fve esd4* double mutants, or would somehow bypass the effect of these mutations on the autonomous pathway. The autonomous pathway appears to facilitate the action of the long day and GA pathways, but on its own to have only a weak floral promotion activity (Nilsson et al., 1998; Reeves and Coupland, 2001). Thus, if *esd4* acts predominantly through the autonomous pathway (Fig. 6A), then mutations within other floral promotion pathways would be expected to reduce the severity of the *esd4* phenotype. This reduction in severity of *esd4* by mutations in the long-day and GA pathways did occur, but not for *ga1* under short days, suggesting that under these conditions there may be a closer relationship between *esd4* and the GA pathway. Nevertheless, we propose that the effect of ESD4 on flowering time is most closely associated with the autonomous pathway.

Response of *esd4* mutants to daylength

The *esd4* mutant flowers later under short than long days, and is therefore still responsive to daylength. In wild-type plants the daylength response is conferred by the long-day pathway. Therefore, *esd4* mutants that are mainly affected in the autonomous pathway would be expected to retain a response to daylength. Such an argument can also explain why *esd4* suppresses mutations that impair the autonomous pathway much less effectively under short-day conditions, because the activity of the long-day pathway would be reduced under these conditions and this would further delay flowering of *esd4 fca-1* plants. The *esd4 co-2* double mutant is only slightly later flowering under short days compared to long days, consistent with the long-day pathway not having a strongly promotive effect on flowering under short days even in an *esd4* mutant. The *co* mutation had a similarly weak effect on the flowering time of the *efs* mutant under short days (Soppe et al., 1999).

The role of *ESD4* in the regulation of *FLC* and its downstream targets

Mutations within the autonomous pathway cause an increase in the expression of the floral repressor *FLC* (Michaels and Amasino, 1999; Sheldon et al., 1999). To test the genetic model of ESD4 function, the effect of *esd4* on the regulation of *FLC* mRNA was examined. *FLC* mRNA abundance was reduced in *esd4* compared to wild-type plants, and in *esd4 fca-1* and *esd4 fve-1* double mutants compared to the late flowering mutants. This suggests that *ESD4* may act to delay flowering by increasing the abundance of *FLC* mRNA. However, *FLC* mRNA levels are still higher in *esd4 fca-1* double mutants than in wild-type plants, although the *esd4 fca-1* plants flowered earlier than wild type. This indicates that *ESD4* is unlikely to act solely through *FLC*. However, we cannot rule out the possibility that *esd4* may reduce *FLC* mRNA abundance to a very low level in a small subset of cells that are critical for the regulation of flowering or that ESD4 may have an additional post-transcriptional effect on FLC protein. Nevertheless, the observation that *flc* null mutants do not flower as early as *esd4* mutants under short days (Michaels and Amasino, 2001), supports our view that the regulation of *FLC* is not the only role for *ESD4* in the regulation of flowering time.

Although the reduction in *FLC* mRNA levels is not the only cause of the *esd4* phenotype, it is likely to contribute to the early flowering of *esd4* mutants and the suppression of the late flowering phenotype of autonomous pathway mutations. Therefore we examined the effect of *esd4* on *FT* and *SOC1*, two flowering time genes whose expression levels are repressed by high levels of *FLC* (P. Suárez-López and G.

Coupland, unpublished results) (Michaels and Amasino, 2001; Ohto et al., 2001). In *esd4* plants, an increase in *FT* and *SOC1* mRNA levels relative to wild-type was observed, suggesting that these genes may contribute to the early flowering of *esd4*. *esd4* partially suppressed the reduction in *FT* and *SOC1* mRNA caused by the *fca-1* mutation, consistent with the partial reduction in *FLC* mRNA levels observed in the *esd4 fca-1* genotype. In *esd4 fca-1* plants the levels of *FT* and *SOC1* were similar to those of wild-type plants. This suggests that it is unlikely that the increase in *FT* and *SOC1* expression alone explains the effect of *esd4* on flowering time, as *esd4 fca-1* plants flower earlier than wild-type despite having similar levels of *FT* and *SOC1*. Furthermore, it is unlikely that the effects on *FT* and *SOC1* are mediated solely through *FLC* as *esd4 fca-1* have higher levels of *FLC* mRNA compared to wild type. Thus, the genetic and molecular data indicate that *ESD4* has a role in increasing *FLC*, and consequently decreasing *FT* and *SOC1* expression, but also that it has additional functions in the control of flowering.

Other genes have previously been shown to increase the level of *FLC*. For example, the *FRIGIDA* gene, which is responsible for the vernalization requirement of many naturally occurring winter varieties of *Arabidopsis* promotes *FLC* expression (Michaels and Amasino, 1999a; Sheldon et al., 1999; Johanson et al., 2000). *ESD4* may therefore act together with *FRI* to promote *FLC*. However, *esd4* was isolated in the Landsberg *erecta* ecotype, which lacks an active *FRI* allele (Johanson et al., 2000). Therefore, it is unlikely that the early flowering of *esd4* is due to effects on *FRI*. The vernalization response also acts through *FLC* and mutations that impair vernalization cause increased *FLC* mRNA levels (Chandler et al., 1996; Sheldon et al., 1999). Moreover, vernalization does not act solely through *FLC* (Michaels and Amasino, 2001). *ESD4* may therefore act to antagonize the activity of genes that are required to promote flowering in response to vernalization, such as the *VRN* genes (Chandler et al., 1996; Gendall et al., 2001). In this case loss of *esd4* would result in upregulation of their activity, in effect resulting in *esd4* behaving as a constitutively vernalized mutant as has been proposed for the early flowering *hos1* mutant (Lee et al., 2001). However, vernalization appears more effective in repressing *FLC* than loss of *ESD4* function (Michaels and Amasino, 1999a; Sheldon et al., 1999). The identification and cloning of additional genes that control the vernalization response should enable the interaction of *ESD4* with the vernalization pathway to be examined in more detail.

A model for *ESD4* in the control of flowering time

We propose that *ESD4* is closely associated with the regulation of *FLC* within the autonomous pathway. Mutations in *ESD4* promote flowering at least partly by decreasing *FLC* mRNA levels and thereby increasing expression of downstream flowering-time genes such as *FT* and *SOC1*. Increases in *FT* and *SOC1* expression would be expected to lead to earlier expression of floral meristem identity genes, such as *LEAFY* and *APETALA1* (Ruiz-Garcia et al., 1997; Kardailsky et al., 1999; Kobayashi et al., 1999). However, the late flowering phenotype of autonomous pathway mutations such as *fca* and *fve* is completely dependent on functional *FLC*: *fca* and *fve* mutants do not flower any later than wild type in a *flc* null mutant background (Michaels and Amasino, 2001), whereas the early flowering of *esd4* cannot be explained solely by its effects on *FLC*. We therefore propose that ESD4 has an additional role in a pathway that by-passes the requirement for the autonomous pathway (Fig. 6B). In this model, *ESD4* has two roles. The first is to ensure high *FLC* expression that leads to repression of flowering through repressing *FT* and *SOC1*, and probably other genes (represented by X in Fig. 6B). The second is to regulate flowering-time genes independently of FLC (represented by Y in Fig. 6B). This model is consistent with the analysis of gene expression in *esd4* mutants as well as with the single and double mutant phenotypes under long and short days.

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