**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, fp, 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

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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
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; Ohito 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 esd4 mutation disrupts the expression of the floral identity genes such as APETALA1 (Chen et al., 1997; Kinoshita et al., 2001).

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 (Ler), Ler seeds were subjected to 90 kRad gamma irradiation by Dr Mary Anderson, University of Nottingham. Around 40,000 M2 plants derived from approximately 2.200 M1 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 Wageningen, 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 Ler and were provided by the following individuals: fca-1, fve-1, co-2, fwa-1, ft-1 J. M. Koornneef (University of Wageningen), ag-3 J. Goodrich (University of Edinburgh), gai-1, gai-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

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Long days</th>
<th>Short days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rosette leaves</td>
<td>Cauline leaves</td>
</tr>
<tr>
<td>Ler</td>
<td>5.4±0.5</td>
<td>3.1±0.2</td>
</tr>
<tr>
<td>esd4</td>
<td>2.2±0.4</td>
<td>2.5±0.6</td>
</tr>
<tr>
<td>esd4+</td>
<td>5.0±0.5</td>
<td>3.1±0.7</td>
</tr>
</tbody>
</table>

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

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 F1 plants derived from crossing each of them to *esd4* flowered at the same time as wild type.

The *esd4* mutant was back-crossed to *Ler* 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 F2 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 *Ler* (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 *Ler*. 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...
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.
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 esd4 mutation is recessive and behaves as a single genetic locus

Plants heterozygous for esd4 were generated by back-crossing to Ler. 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 F2 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 Ler 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.

Table 2. Flowering time of esd4 double mutants

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Rosette leaves</th>
<th>Caulline leaves</th>
<th>Rosette leaves</th>
<th>Caulline leaves</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Long days</td>
<td>Short days</td>
<td>Long days</td>
<td>Short days</td>
</tr>
<tr>
<td>fer</td>
<td>2.8±0.5</td>
<td>2.4±0.5</td>
<td>12.1±1.3</td>
<td>3.2±0.4</td>
</tr>
<tr>
<td>fca-1</td>
<td>2.6±1.1</td>
<td>2.4±0.6</td>
<td>13.2±1.0</td>
<td>4.3±0.6</td>
</tr>
<tr>
<td>fve-1</td>
<td>2.8±0.5</td>
<td>2.3±0.5</td>
<td>11.8±1.4</td>
<td>3.6±0.4</td>
</tr>
<tr>
<td>esd4 co-2</td>
<td>2.6±0.5</td>
<td>2.4±0.6</td>
<td>12.1±1.3</td>
<td>3.2±0.4</td>
</tr>
<tr>
<td>gai*</td>
<td>8.8±1.2</td>
<td>15.3±1.3</td>
<td>80.6±6.4</td>
<td>28.1±2.0</td>
</tr>
</tbody>
</table>

*In plants carrying gal-3 or gal, rosette and caulline leaves could not be distinguished. ND, not determined. Flowering times are shown as mean leaf number ± standard deviation of the mean.
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 Ler 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 $gal-3$ 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 $esd4$ gal-1-3 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 $esd4$ gal-1-3 plants showed a flowering time intermediate between the $esd4$ and gal-1-3 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.

**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 Ler 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 mutants, 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 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 Ler, esd4, fca-1, esd4 fca-1, fve-1, and esd4 fve-1 plants. The panel on the right is a longer exposure of the Ler and esd4 samples. (B) Northern blot comparing FT mRNA levels in 7-day-old Ler, 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 Ler, 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 Ler, 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.

**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 mutations and the autonomous pathway that affect 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 fts (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 gal 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 fts 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 ftc 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. P. H. Reeves and others
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 fca 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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