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EARLY IN SHORT DAYS 1 (*ESD1*) encodes ACTIN-RELATED PROTEIN 6 (AtARP6), a putative component of chromatin remodelling complexes that positively regulates *FLC* accumulation in *Arabidopsis*

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We have characterized *Arabidopsis esd1* mutations, which cause early flowering independently of photoperiod, moderate increase of hypocotyl length, shortened inflorescence internodes, and altered leaf and flower development. Phenotypic analyses of double mutants with mutations at different loci of the flowering inductive pathways suggest that *esd1* abolishes the *FLC*-mediated late flowering phenotype of plants carrying active alleles of *FRI* and of mutants of the autonomous pathway. We found that *ESD1* is required for the expression of the *FLC* repressor to levels that inhibit flowering. However, the effect of *esd1* in a *flc-3* null genetic background and the downregulation of other members of the *FLC*-like/*MAF* gene family in *esd1* mutants suggest that flowering inhibition mediated by *ESD1* occurs through both *FLC*- and *FLC*-like gene-dependent pathways. The *ESD1* locus was identified through a map-based cloning approach. *ESD1* encodes ARP6, a homolog of the actin-related protein family that shares moderate sequence homology with conventional actins. Using chromatin immunoprecipitation (ChIP) experiments, we have determined that ARP6 is required for both histone acetylation and methylation of the *FLC* chromatin in *Arabidopsis*.

KEY WORDS: Flowering time, Floral repression, Chromatin remodelling, *Arabidopsis*

INTRODUCTION

The floral transition is highly regulated in many plant species to modulate flowering time in response to environmental and endogenous factors, and to ensure reproductive success. *Arabidopsis thaliana* is a facultative long-day (LD) species in which winter and summer annual accessions can be distinguished. In winter annual accessions, flowering time is regulated by the vernalization, photoperiod and gibberellin (GA) pathways (Boss et al., 2004; Komeda, 2004; Puterill et al., 2004; Amasino, 2005). Winter annuals require exposure to an extended period of cold (vernalization) to become flowering competent, thus preventing premature flowering in the fall (Michaels and Amasino, 2000; Henderson and Dean, 2004). This requirement is mainly conferred by dominant alleles at the *FRIGIDA* (*FRI*) (Johanson et al., 2000) and *FLOWERING LOCUS C* (*FLC*) loci (Michaels and Amasino, 1999; Sheldon et al., 1999), as well as by other *FLC*-related genes within the *MAF* clade (Scortecci et al., 2001; Ratcliffe et al., 2003; Werner et al., 2005). Active alleles of *FRI* increase *FLC* expression to levels that delay flowering (Michaels and Amasino, 1999; Sheldon et al., 1999). FLC is a MADS box transcription factor that acts to delay flowering, in part by suppressing the expression of the floral promoters *FT* and *SUPPRESSOR OF OVEREXPRESSION OF CO 1* (*SOC1*), which function as integrators of flowering signals (Kobayashi et al., 1999; Samach et al., 2000). Vernalization promotes flowering by overcoming the effect of *FRI* and repressing *FLC* expression; this repression is stably maintained after plants are returned to warm growth conditions, allowing plants to flower (Michaels and Amasino, 1999; Sheldon et al., 1999). The photoperiod pathway promotes flowering in response to LD through the activation of the floral integrators *FT* and *SOC1*. Mutations in photoperiod-pathway genes [e.g. *constans* (*co*), *fd*, *fe*, *fha*/*cryptochrome2* (*cry2*), *ft*, *fwa* and *gigantea* (*gi*)] delay flowering in LD but have little effect on flowering time under short days (SD) (Searle and Coupland, 2004). The GA pathway is required for flowering in non-inductive photoperiods, and mutants with reduced GA levels are extremely delayed in flowering time under SD (Wilson et al., 1992).

Many summer annual accessions of *Arabidopsis* lack an active *FRI* allele (Johanson et al., 2000; Gazani et al., 2003; Shindo et al., 2005). Under these circumstances, *FLC* expression is low and flowering occurs rapidly without vernalization. In these accessions, the reduction of *FLC* expression depends on the function of the autonomous pathway (Michaels and Amasino, 2001). In fact, mutations in autonomous pathway genes [*fca*, *flowering locus d* (*fld*), *fpa*, *fve*, *fy*, *flowering locus k* (*flk*) and *luminidependens* (*ld*)] cause a flowering delay under any photoperiod (Boss et al., 2004) that is associated with higher *FLC* expression, and can be rescued by vernalization (Michaels and Amasino, 1999; Sheldon et al., 1999; Michaels and Amasino, 2001).

Thus, transcriptional regulation of the *FLC* repressor is a central checkpoint in both winter and summer annual accessions of *Arabidopsis*. Recently, the involvement of chromatin modification in *FLC* regulation has been described (for a review, see He and Amasino, 2005). In non-vernalized winter annual plants, *FLC* chromatin is in an active conformation and is enriched in modifications, such as the acetylation of histones 3 (H3) and 4 (H4),

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and the trimethylation of lysine 4 of H3 (H3-K4), which are hallmarks of active genes (He et al., 2003; Ausin et al., 2004; He et al., 2004). Late-flowering autonomous pathway mutants also have increased levels of H3-K4 trimethylation and histone acetylation compared with the rapid-flowering parental line (He et al., 2003; Ausin et al., 2004; He et al., 2004; Kim et al., 2005). Many early flowering mutations suppressing the late flowering phenotype of *FRI*-containing lines have identified components that are required to maintain high levels of *FLC* expression. This is the case of mutants such as *early flowering in short days* (*efs*), *photoperiod independent early flowering 1* (*pie1*), *early flowering 5* (*elf5*), *vernalization independence3* (*vip3*) and *frigida-like1* (*frl-1*), and mutants in genes encoding components of the PAF1 complex (*ELF7*, *VIP4*, *VIP5* and *VIP6*/*ELF8*) (Zhang and Van Nocker, 2002; Noh and Amasino, 2003; Zhang et al., 2003; Noh et al., 2004; He et al., 2004; Michaels et al., 2004; Oh et al., 2004; Kim et al., 2005). Most of these mutations also appear to affect flowering in an *FLC*-independent manner.

After exposure to an extended winter and the completion of vernalization, the level of modifications associated with 'active' chromatin is reduced, and the histone tails of *FLC* chromatin are deacetylated and become enriched in methylation of lysine 9 (K9) and 27 (K27) of H3 (Bastow et al., 2004; Sung and Amasino, 2004), which are hallmarks of repressed genes (Orlando, 2003). Mutants that are unable to reduce *FLC* transcript levels by vernalization or to maintain the vernalised state have permitted the identification of some of the proteins participating in this process, such as the chromatin remodelling factors VERNALIZATION INSENSITIVE 3 (VIN3) and VERNALIZATION 2 (VRN2) (Gendall et al., 2001; Sung and Amasino, 2004), and a plant-specific DNA-binding protein, VRN1 (Levy et al., 2002).

In summer annual accessions, reduced expression of *FLC* depends on the autonomous pathway, and is associated with lower histone acetylation of *FLC* chromatin as a result of *FVE* and *FLD* function (He et al., 2003; Ausin et al., 2004). Mutations in both genes cause *FLC* chromatin to become more acetylated at H3 and H4 concomitantly with an increase in *FLC* expression (He et al., 2003; Ausin et al., 2004).

Here, we report the identification of *EARLY IN SHORT DAYS1* (*ESD1*), a gene that is required for the maintenance of *FLC* expression. The *esd1* mutation causes early flowering through the reduction of *FLC* expression, although the mutation also appears to affect flowering through other *FLC*-like repressors. Using a map-based approach, we have determined that *ESD1* encodes ARP6, a member of the actin-related protein family that share moderate sequence homology and basal structure with conventional actins. Recently, ARPs and actins have been discovered in the nucleus as integral components of several chromatin remodelling and histone acetyltransferase (HAT) complexes (Schafer and Schroer, 1999; Galarneau et al., 2000; Rando et al., 2000; Shen et al., 2000; Olave et al., 2002; Blessing et al., 2004). We present evidence that *ESD1* is needed to achieve the levels of both H3 acetylation and H3-K4 methylation required for high *FLC* expression.

MATERIALS AND METHODS

Genetic stocks and growth conditions

Mutant seed stocks used were in the Landsberg *erecta* (L*er*) and Columbia (Col) genetic backgrounds, and were obtained from the Arabidopsis Biological Resource Centre (ABRC) of Ohio State University (Columbus, USA), the Nottingham Arabidopsis Centre (NASC) in UK and personal donations. The monogenic *fve-1*, *fca-1*, *co-2* and *gi-3* mutants were described by Koornneef et al. (Koornneef et al., 1991); *flc-3* was described by Michaels and Amasino (Michaels and Amasino, 2001) and the Col *FRI-Sf2* lines were described by Lee et al. (Lee et al., 1995). GA-deficient *ga1-3* and *ga2-1* mutants were described by Koornneef and van der Veen (Koornneef and van der Veen, 1980) and *spy-5* by Jacobsen and Olszewski (Jacobsen and Olszewski, 1993). The origin of the *esd1-1* to *esd1-9* alleles is summarized in Table 1. The *esd1-10* allele in Col corresponds to the T-DNA line Wisc Ds-Lox 289_29L8, and was kindly provided by the ABRC. We confirmed that all *esd1* mutations were allelic by their failure to complement the early flowering phenotype in F1 plants derived from crosses between them. Plants were grown in plastic pots containing a mixture of substrate and vermiculite (3:1). Controlled environmental conditions were provided in growth chambers at 21°C and 80% relative humidity. Plants were illuminated with cool-white fluorescent lights (approximately 120 μE m^{-2} $second^{-1}$). LD conditions consisted of 16 hours of light followed by 8 hours of darkness; SD conditions consisted of 8 hours of light followed by 16 hours of darkness.

Phenotypic analyses

Total leaf number was scored as the number of main leaves in the rosette (excluding cotyledons) plus the number of leaves in the inflorescence at the time of opening of the first flower (Koornneef et al., 1991). Cauline, adult and juvenile leaves were scored independently. Rosette leaves lacking abaxial trichomes were considered as juvenile leaves (Telfer et al., 1997). Floral organs were analyzed by scanning electron microscopy as described (Ruiz-Garcia et al., 1997).

Genetic analysis

Double mutants were constructed by crossing the monogenic *esd1-2* mutant with lines carrying the mutations *co-2*, *gi-3*, *fve-1*, *fca-1*, *ga1-3*, *ga2-1* or *spy-5*. *esd1-3* was crossed with a line carrying the *flc-3* mutation in Col and with Col *FRI Sf-2* (Lee and Amasino, 1995). Double mutants were isolated from selfed F2 progeny that showed the *esd1* phenotype and that segregated for the second mutation.

Molecular characterization of the *esd1* alleles and map-based cloning

The *esd1-2* mutation was initially mapped to chromosome 3 between markers GAPab and nga6, using the cleaved-amplified polymorphic sequence (CAPS) and the simple sequence length polymorphism (SSLP) molecular markers indicated in Table S1 in the supplementary material. Additional analysis of 925 *esd1*-like F2 plants allowed us to locate *ESD1* to a pericentromeric region of 1.4 cM, between the T8N9 and ATA1 markers (see Table S2 in the supplementary material). To fine map the *esd1* mutation within the interval deleted in the *esd1-1* and *esd1-6* mutant plants, which is located between the 5F21A14 and 1T14A11 markers, we designed specific PCR molecular markers (see Table S3 in the supplementary material) that were used to amplify the genomic DNA of each *esd1* mutant allele, in order to score the presence or absence of the amplified product. Southern blot hybridizations with genomic DNA were performed to confirm the PCR results (data not shown).

Plant transformation

Four overlapping binary TAC clones (JAtY74I04, JAtY64M05, JAtY54G02, JAtY49O18) spanning the minimum deleted region in the *esd1* alleles were obtained from the Genomic Arabidopsis Resource Network (GARNET) and introduced into the *esd1-3* allele by *Agrobacterium tumefaciens*-mediated transformation using the floral-dip method (Clough and Bent, 1998). The *Agrobacterium* strain used was C58C1. Transformant plants were selected on soil by spraying seedlings with BASTA.

Only *esd1*-3 mutant plants transformed with the JAtY T74I04 TAC clone that spans from position 28823 bp of T4P3 BAC clone to 78776 bp of T14A11 BAC clone, showed complementation of the early flowering phenotype. To check whether the integration of the TAC clone was complete in transformed *esd1-3* plants, we used a set of specific molecular markers (see Table S3 in the supplementary material) contained in the deleted region. We chose markers that amplify PCR products over genomic DNA extracted from wild-type plants, but not from *esd1-3* mutant plants. In this way, we demonstrated that the genomic region of the JAtYT74I04 TAC clone integrated in the complementing transgenic plants contained only two ORFs

predicted to encode proteins, *At3g33520* and *At3g33530*. The rest of the ORFs present in this region correspond to pseudogenes and retrotransposon elements.

Expression analysis

Total RNA was isolated using TRIzol (Invitrogen-Gibco), electrophoresed and transferred onto Hybond N^+ membranes (Amersham), following described protocols. For the *FLC* probe, we used a 700 bp *Eco*RI/*Sph*I fragment from pFLC lacking the MADS-box domain (Michaels and Amasino, 1999). As loading controls, we used a 305-bp *Eco*RI fragment of the cauliflower 18S ribosomal DNA gene. *ARP6* transcript levels were assayed by RT-PCR. cDNA was prepared by reverse transcription of total RNA from *Arabidopsis* roots, stems, cauline leaves, floral buds and flowers, according to described procedures (Piñeiro et al., 2003). *ARP6* gene-specific primers, 5′-GAGCTTCGACCACTTGTCCCAGAT-3′ and 5′-GCATTACAATATACGACAAATAATGTG-3′, were designed to amplify the C-terminal end of the coding region, including the last intron and a portion of the 3′ untranslated region. For low abundance mRNAs, such as the *MAF*, *FT* and *SOC1* genes, we also performed reverse transcriptase-mediated PCR, according to described procedures (Scortecci et al., 2001; Piñeiro et al., 2003; Ratcliffe et al., 2003). *UBIQUITIN 10* (*UBQ10*) was used as control in these experiments.

Histochemical β-glucuronidase assays

esd1-2 fca-1 FLC:GUS plants were obtained by crossing *esd1-2* with *fca-1* plants carrying a 6 kb *FLC:GUS* translational fusion construct (Sheldon et al., 2002). GUS activity in *fca-1* and *esd1-2 fca-1 FLC:GUS* plants was revealed by incubation in 100 mM $NaPO_4$ (pH 7.2), 2.5 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide, 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6$ and 0.25% Triton X-100. Plant tissue was incubated at 37°C for 20 hours. After staining, chlorophyll was cleared from the samples by dehydration through an ethanol series.

ChIP assays and PCR

ChIP assays were carried out as described (Ausin et al., 2004). Chromatin proteins and DNA were cross linked in 10-day-old Col, *esd1*-3, *FRI*, *esd1-3FRI*, Ler, *esd1-2*, *fca-1*, *esd1-2 fca-1*, *fve-1* and *esd1-2 fve-1* seedlings by formaldehyde fixation. After chromatin isolation, the H3 acetylated and methylated fractions were immunoprecipitated using specific antibodies to acetylated K9 and K14, and trimethylated K4, residues (06-599 and 07-473 from Upstate Biotechnology, respectively). Cross-links were reversed by incubations at 65°C for 2 hours, and DNA was purified with QIAquick spin columns (QIAGEN) and eluted in 40 μl of TE (pH 8.0). Semiquantitative PCR was used to amplify six different fragments of the *FLC* gene (Michaels and Amasino, 1999) (details and primer sequences are available on request). All PCR reactions and quantification of the amplified DNA were done as described previously (Ausin et al., 2004). We carried out three independent experiments and data provided in Fig. 7 are from one representative. *UBQ10* served as an internal control for the ChIP analysis. To calculate the fold enrichment in H3 acetylation or methylation, *FLC* was first normalized to *UBQ10* in each sample, and, subsequently, these values were normalized against their respective wild-type controls.

RESULTS

esd1 mutants are early flowering and display a pleiotropic vegetative and reproductive phenotype

Mutants at the *ESD1* locus were independently identified in screens for L*er* mutations conferring early flowering under SD (*esd1-1* and *esd1-2*) or for Col mutations that accelerate developmental phase transitions (*esd1-3* to *esd1-9*) (Table 1). All of the selected alleles produced a similar array of phenotypes, independently of their genetic background. Plants homozygous for *esd1* mutations were early flowering under both LD and SD photoperiods (Table 1), showing a more extreme phenotype under SD (Table 1, Fig. 1A,B). The fact that *esd1* mutants flower earlier under inductive photoperiods indicates that the mutations do not abolish the photoperiod responses. Earliness was associated with a reduction in the length of all developmental phases of the plant (Fig. 1C), based on leaf shape and leaf trichome distribution (Telfer et al., 1997). This reduction was more dramatic in the case of adult rosette leaves, which were almost absent from *esd1* mutants grown under LD and highly reduced in *esd1* mutant plants under SD (Fig. 1C). This behaviour is similar to that exhibited by other early flowering

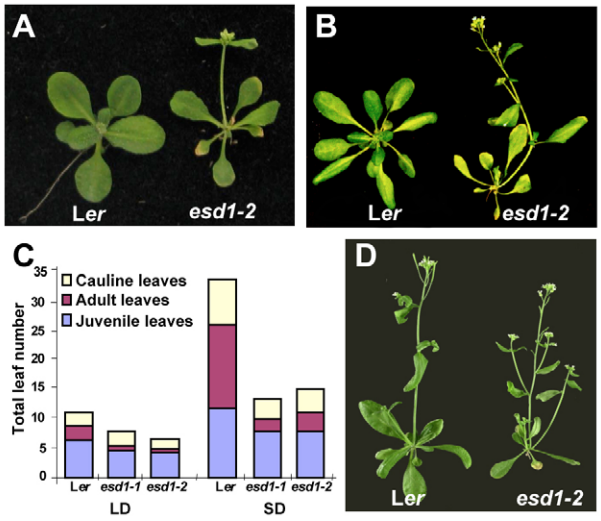

Fig. 1. The flowering phenotype of *esd1* mutants. (**A**) Wild-type L*er* and *esd1-2* 2-week-old plants grown under LD. (**B**) L*er* and *esd1-2* 4-week-old plants grown under SD. (**C**) Histogram comparing the number of juvenile, adult and cauline leaves in L*er* and *esd1* mutants. Plants were grown under both LD and SD. (**D**) L*er* and *esd1-2* 3.5-week-old plants grown under LD.

Table 1. Flowering time of wild-type and *esd1* mutant plants, indicating the nature of each allele

Genotype	Long days	Short days	Mutagen	Origin
L*er*	9.87±0.81	32.91±1.57		
esd1-1	6.81±0.48	14.10±1.40	Gamma rays	Coupland (1995)
esd1-2	7.03±0.57	15.10±1.84	Fast neutron	This work
Col	14.13±0.96	66.00±2.12		
esd1-3	9.47±0.64	28.09±1.95	Fast neutron	This work
esd1-4	9.13±0.62	29.10±1.79	Fast neutron	This work
esd1-5	9.73±0.53	31.20±1.97	Ionizing carbon	This work
esd1-6	9.71±0.64	26.81±1.75	Fast neutron	This work
esd1-7	9.67±0.61	29.96±1.94	Fast neutron	This work
esd1-8	9.48±0.51	30.83±1.90	Fast neutron	This work
esd1-9	9.47±0.51	31.05±1.81	Fast neutron	This work

Flowering times are shown as mean leaf number±s.d. of the mean.

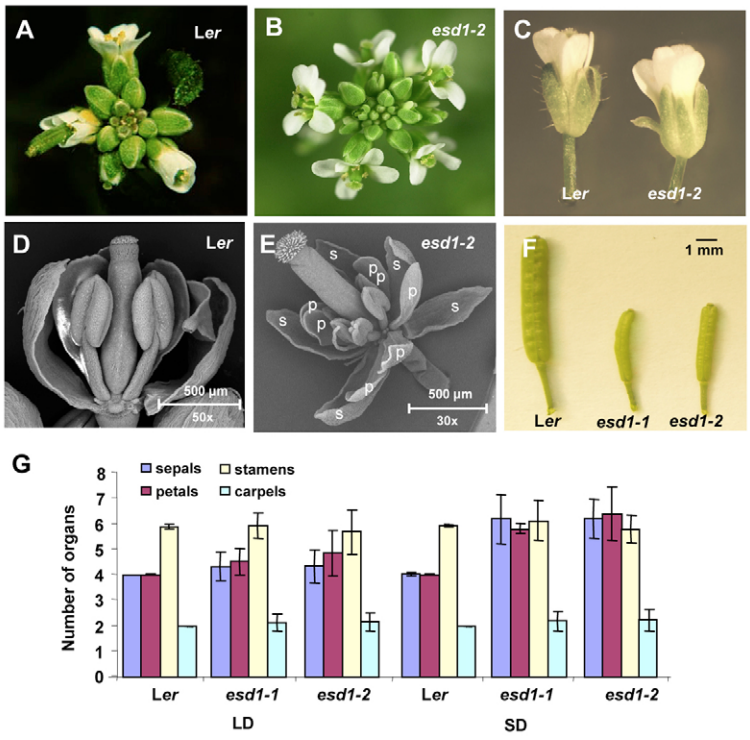

Fig. 2. The inflorescence phenotype of *esd1* mutants. (**A**) Apex of 3-week-old L*er* plants. (**B**) Apex of 2.5-week-old *esd1* mutant plants, showing open flowers with extra sepals and petals. (**C**) Detached flowers showing the increased number of sepals and petals in *esd1* mutant flowers. (**D**,**E**) Scanning electron micrographs of L*er* (D) and *esd1* (E) flowers of plants grown under SD. s, sepal; p, petal. (**F**) A comparison of silique shape and length in L*er*, *esd1-1* and *esd1-2* plants. (**G**) Number of sepals, petals, stamens and carpels in L*er* and *esd1* mutants. Plants were grown under both LD and SD. Bars represent the standard error.

mutants such as *esd4* and *ebs*, which also show a major reduction of the adult vegetative phase (Gomez-Mena et al., 2001; Reeves et al., 2002).

Apart from their flowering time phenotype, *esd1* mutants also displayed a complex pleiotropic vegetative and reproductive phenotype. *esd1* mutants show a moderate increase in hypocotyl length but have shortened inflorescence internodes. Furthermore, *esd1* leaves are smaller and more curled than wild-type leaves, and usually have serrated margins (Fig. 1D). *esd1* flowers are smaller than wild-type ones (Fig. 2C) and frequently bear extra perianth organs. This phenotype was more extreme under SD, where *esd1* flowers contained an average of two extra sepals and two extra petals per flower (Fig. 2B,E,G). Mutant flowers showed a reduced fertility that was associated with a reduction in the amount of pollen and approximately a 50% reduction in seed set when compared with wild-type plants. Mutant carpels were much smaller and the filaments of the stamens of mutant flowers were shorter than those of wild-type plants (Fig. 2E). Concomitantly, siliques were approximately 50% shorter in *esd1* mutants than in wild-type plants (Fig. 2F). Thus, mutations at the *ESD1* locus directly or indirectly alter multiple aspects of plant development.

Genetic interactions between *esd1* and mutations affecting flowering time regulatory pathways

The early flowering phenotype of *esd1* mutants suggested that *ESD1* could negatively interact with a flowering promoting pathway in *Arabidopsis*. To test this hypothesis, we analyzed the phenotype of double mutants carrying *esd1* and mutations causing a delay in flowering time. We chose representative mutations for each of the photoperiod, GA and autonomous pathways. Within the photoperiod pathway, mutations at the *CO* and *GI* loci delay flowering mainly under LD (Koornneef et al., 1998) (Table 2). *esd1-2 co-2* and *esd1-2 gi-3* double mutants flowered later than *esd1-2* mutants, and earlier than *co-2* and *gi-3* plants, and thus displayed an additive phenotype (Table 2). Similar to *co-2* and *gi-3* single mutants, *esd1-2 co-2* and *esd1-2 gi-3* double mutants lack the capacity to respond to inductive photoperiods, and flowered with a similar number of leaves under both LD and SD photoperiods (Table 2).

Mutations affecting GA synthesis, such as *ga1-3* (Sun and Kamiya, 1994) and *ga2-1* (Yamaguchi et al., 1998), delay flowering in both LD and SD (Wilson et al., 1992). By contrast, mutations in *SPINDLY* (*SPY*) cause constitutive GA signalling and accelerated flowering time (Jacobsen and Olszewski, 1993). To determine whether the GA synthesis and response pathways are required for the early flowering phenotype of *esd1*, we analyzed the phenotype of *esd1-2 ga1-3*, *esd1-2 ga2-1* and *esd1-2 spy-5* double mutants. Under LD, the *esd1-2 ga1-3* and *esd1-2 ga2-1* double mutants showed an additive flowering time phenotype, in that they flowered earlier than their late parent and later than their early parent (Table 2). Under SD conditions, *esd1-2 ga2-1* also showed an intermediate flowering time phenotype; however, the *esd1-2 ga1-3* double mutant was unable to flower under SD. This is similar to the phenotype of the *ga1-3*

Table 2. Flowering time of *esd1* double mutants

Genotype	Long days	Short days
L*er*	8.18±0.72	32.91±1.57
esd1-2	6.11±0.42	15.10±1.84
gi-3	24.10±1.97	39.36±2.16
esd1-2 gi-3	14.52±0.79	15.68±1.49
co-2	23.15±1.28	35.63±1.75
esd1-2 co-2	16.17±0.87	16.44±1.53
ga1-3	17.8±2.2	>65
esd1-2 ga1-3	11.00±1.10	>65
ga2-1	22.01±2.81	56.31±2.83
esd1-2 ga2-1	12.81±1.56	39.30±2.31
spy-5	6.41±0.50	14.60±1.55
esd1-2 spy-5	5.20±0.51	6.60±0.90
fve-1	20.23±0.81	61.93±1.87
esd1-2 fve-1	7.32±0.61	15.96±2.18
fca-1	27.80±1.07	61.83±2.32
esd1-2 fca-1	8.84±0.80	20.56±1.73

mutant, and indicates that the early flowering of *esd1* mutants requires GA biosynthesis under SD. In agreement with these results, *esd1-2 spy-5* double mutants also display an additive early flowering phenotype that is more readily observed under SD (Table 2).

To test the interaction between *ESD1* and autonomous pathway genes, we analyzed the flowering phenotype of *esd1-2 fve-1* and *esd1-2 fca-1* double mutants (Table 2; Fig. 3A). Under LD, some of the *esd1-2 fve-1* and *esd1-2 fca-1* double mutants were indistinguishable from *esd1*, although, on average, *esd1-2 fve-1* and *esd1-2 fca-1* produced one and two leaves more than *esd1*, respectively (Table 2; Fig. 3A). Under SD, *esd1-2 fve-1* and *esd1-2 fca-1* mutants were also very similar to *esd1-2* single mutants (Table 2), producing a few more leaves (16 and 21 leaves, respectively) than the early flowering parental plants (15 leaves); this result indicates that the late flowering phenotype of autonomous pathway mutations requires *ESD1*. In summary, these results suggest that *ESD1* does not interact with the photoperiod and GA floral induction pathways, but shows an almost epistatic interaction with genes in the autonomous pathway.

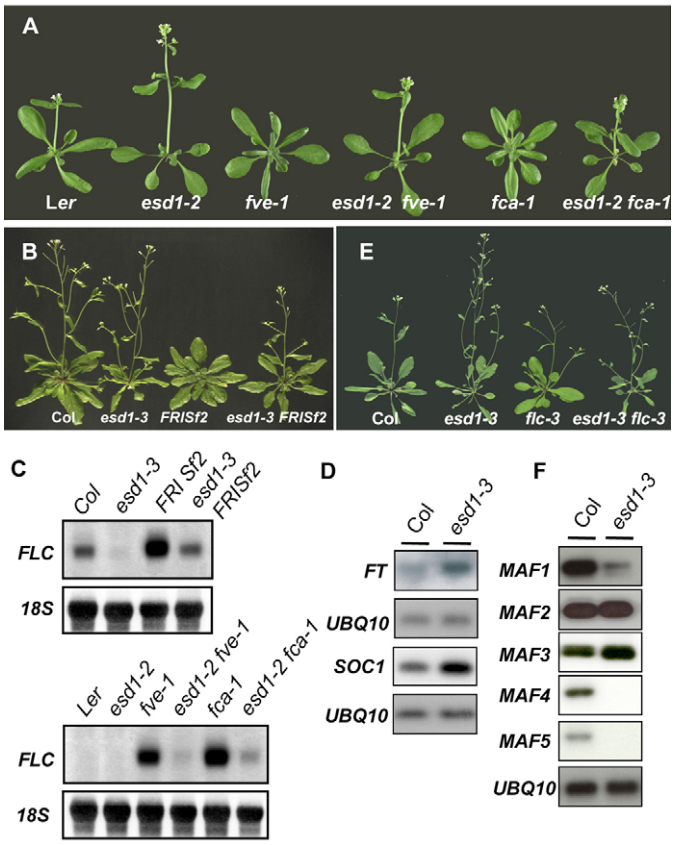

Fig. 3. Suppression of *FLC*-dependent late flowering by *esd1* mutations. (**A**) Photograph illustrating the flowering phenotype of double mutant *esd1 fve* and *esd1 fca* plants grown under LD. (**B**) Flowering phenotype of lines where an active allele of *FRI* is combined with *esd1* grown under LD. (**C**) Analysis of the expression of *FLC* in the late-flowering genotypes *FRI*, *fve* and *fca* combined with *esd1*. RNA blot hybridizations were performed using total mRNA from 9-day-old Col, *esd1-3*, *FRI*, *esd1-3 FRI*, L*er*, *esd1-2*, *fve-1*, *esd1-2 fve-1*, *fca-1* and *esd1-2 fca-1* plants grown under LD. (**D**) Analysis of the expression of *FT* and *SOC1* genes in *esd1* mutants. RT-PCR assays comparing *FT* and *SOC1* expression in 9 day-old Col and *esd1*-3 plants. The samples were taken at the time of the day with the maximum expression; for *FT* expression analysis, before dusk, and for *SOC1* analysis, 8 hours after dawn. (**E**) Flowering phenotype of *esd1 flc* double mutant plants grown under LD. (**F**) Analysis of the expression of *MAF* genes in *esd1* mutant plants. Total RNA was extracted from pools of 50 9-day-old seedlings grown under LD conditions. Expression was monitored by RT-PCR over 32 cycles for *MAF1*, 28 cycles for *MAF2*, and 35 cycles for *MAF3*, *MAF4* and *MAF5*. For the *UBQ10* control, we amplified during 22 cycles. RT- PCR products were blotted and hybridized with specific probes for each *MAF* gene.

The epistatic interaction with mutations in the autonomous pathway suggest that *esd1* might cause early flowering either by increasing the activity of the autonomous pathway downstream of *FCA* and *FVE*, or by bypassing the requirement for the autonomous pathway by reducing *FLC* expression. Because other flowering pathways, besides the autonomous pathway, converge on the regulation of *FLC* expression, it was of interest to evaluate the interaction of *esd1* with *FRI*, a dominant-positive regulator of *FLC* (Lee and Amasino, 1995; Michaels and Amasino, 1999; Johanson et al., 2000), and to study *FLC* expression in the different mutant backgrounds. When the *FRI* allele introgressed from the San Feliu-2 ecotype (*FRI*-Col) (Lee and Amasino, 1995) was combined with the *esd1-3* mutation in a Col genetic background, plants showed an additive phenotype in which the *FRI* late-flowering phenotype was only partially suppressed by *esd1-3* (Table 3, Fig. 3B).

In order to check if *esd1* suppresses the effect of the autonomous pathway mutations and *FRI* by reducing *FLC* mRNA levels, we compared the abundance of the *FLC* mRNA in wild-type, *esd1-2*, *fca-1*, *esd1-2 fca-1*, *fve-1*, *esd1-2 fve-1*, *FRI* and *esd1-3 FRI* seedlings (Fig. 3C). *FLC* mRNA was present at higher levels in both *fca-1* and *fve-1* mutants and in *FRI*-containing lines than in wild-type plants, as has been previously shown (Michaels and Amasino, 1999; Sheldon et al., 1999). *FLC* transcript levels in *FRI* and in *fca-1* and *fve-1* mutants were decreased by the *esd1* lesion (Fig. 3C). *FLC* mRNA levels were also compared between wild-type plants and *esd1* mutants. Because *FLC* is expressed at a low level in L*er* wild-type plants, we could not observe a clear reduction in its expression in the *esd1* alleles isolated in L*er* background. However, we were able to detect a reduction in the *FLC* expression in the *esd1* alleles isolated in Col background, which bears an *FLC* allele expressed at higher levels (Fig. 3C). In summary, *ESD1* is required to maintain high *FLC* expression levels, either as promoted by *FRI* or by mutations that impair the autonomous pathway, and, consistent with the genetic analysis, *esd1* mutations suppress the increase in *FLC* expression caused by autonomous pathway mutations more effectively than that caused by active *FRI* alleles. In agreement with this scenario, the expression of the floral integrator genes *FT* and *SOC1*, normally repressed by *FLC* (Moon et al., 2003), was upregulated in the *esd1* mutants (Fig. 3D).

Although the effects of *esd1* mutations on flowering time are more readily observed in the late-flowering *FRI* and autonomous pathway mutant backgrounds, as discussed above, the fact that *esd1* mutants also flower earlier than the rapid-flowering wild-type strains L*er* and Col (Fig. 1 and Table 1) suggests that, in addition to regulating *FLC* expression, *ESD1* plays other roles in the control of flowering time.

Table 3. Flowering time of *esd1 flc* double mutant and *FRI* combined with *esd1*

Genotype	Long days	Short days
Col	14.13±0.96	66.00±2.12
esd1-3	9.47±0.64	28.09±1.95
FRI	61.70±9.60	83.57±5.22
esd1-3 FRI	13.50±1.10	62.30±2.30
flc-3	11.70±0.91	55.20±1.75
esd1-3 flc-3	8.54±0.64	32.93±2.15

To determine the fraction of the *esd1* early-flowering phenotype that is independent of the effect of the *esd1* mutation on *FLC* expression, we analyzed the phenotypic effect of the *esd1-3* mutation in an *flc* null (*flc-3*) genetic background (Michaels and Amasino, 1999) under both LD and SD. When combined with *flc-3*, the *esd1* mutation significantly reduces the number of leaves produced by *flc-3* under both photoperiods (Fig. 3E and Table 3), confirming that *esd1* mutations have an *FLC*-independent effect on flowering time. Indeed, loss of function of *ESD1* also resulted in downregulation of some other members of the *FLC/MAF* gene family, particularly *MAF1*, *MAF4* and *MAF5* (Fig. 3F). RT-PCR analysis indicated a modest but reproducible decrease in *MAF1* gene expression and a marked silencing of the *MAF4* and *MAF5* genes, suggesting that these *MAF* gene family members represent additional regulatory targets of *ESD1*.

ESD1 encodes ACTIN-RELATED PROTEIN 6 (ARP6)

To understand the molecular function of *ESD1*, we decided to identify the gene responsible for the observed phenotypes in the mutant by a map-based cloning approach. For this, 925 *esd1* F2 plants derived from the cross between *esd1-2* and Col were screened with the markers shown in Table S1 in the supplementary material. This allowed us to locate *ESD1* south of the T8N9 marker and north of the ATA1 marker, close to the pericentromeric region of chromosome 3 (see markers used in Table S2 in the supplementary material). Owing to the severe suppression of recombination in the vicinity of the centromere and because this chromosomal region is almost completely sequenced, we designed an alternative strategy to complete the identification of the *ESD1* gene, based on the observation that all of the isolated alleles harbour a deletion in the pericentromeric region of chromosome 3. We identified the shorter overlapping genomic region that was deleted in all of the *esd1* alleles by using PCR molecular markers to amplify specific genomic DNA fragments from all of the *esd1* alleles, and looked for the presence or absence of an amplified product (see Table S3 in the supplementary material). In this way, we delimited the *ESD1* locus to a deleted genomic region between the 5F21A14 and 1T14A11 markers. This region spans three overlapping BAC clones, F21A14, T4P3 and T14A11 (Fig. 4A), and is enriched in retrotransposon and transposase elements, pseudogenes and highly repeated sequences.

Subsequently, different overlapping binary TAC clones spanning the deleted region were identified and introduced into the *esd1-3* allele by *Agrobacterium*-mediated transformation, in order to identify those that complemented the mutant phenotype. Only certain transgenic *esd1-3* mutant plants transformed with JAtYT74I04 TAC flowered at a similar time as wild-type plants, and had lost the pleiotropic phenotype of *esd1* mutant plants (Fig. 4B). The analysis of these transgenic plants showed that TAC clone integration had been incomplete (see Materials and methods), and that only in those lines that contained the genomic region harbouring open reading frames *At3g33520* y *At3g33530* had the mutant phenotype been complemented, suggesting that one of those ORFs represent *ESD1*. To determine which one of them corresponded to *ESD1*, we searched for T-DNA insertions within the *At3g33520* and *At3g33530* ORFs and identified the Wisc Ds-Lox 289_29L8 line for *At3g33520* and the SALK_003098 line for *At3g33530* (Alonso et al., 2003). We obtained seeds of these lines and identified plants homozygous for the T-DNA insertions. Only the plants that harbour a T-DNA insertion in *At3g33520* flowered early under both LD and SD (producing around nine and 29 leaves, respectively; wild-type plants produce 14 leaves in LD and 66 in SD), and showed a pleiotropic phenotype similar to that of *esd1* mutants regarding

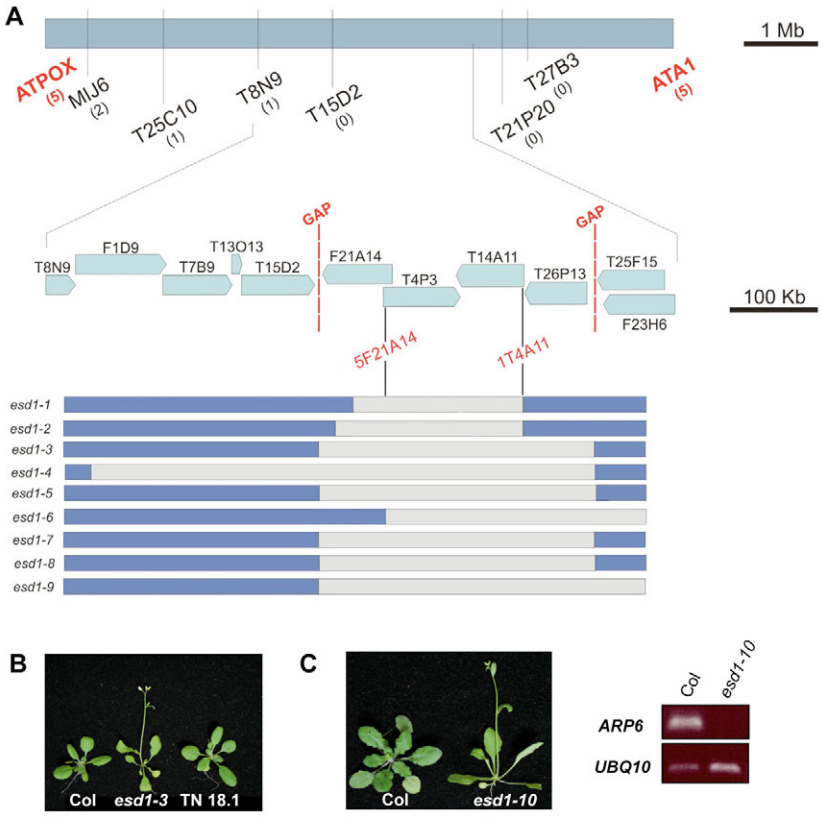

Fig. 4. Identification of *ESD1*. (**A**) Map-based cloning of *ESD1*. The genetic interval, molecular markers and BAC clones in the *ESD1* region are shown. The number of recombinant events between molecular markers is given in parentheses. The centromere is located between the T15D2 and T25F15 markers (http://www.arabidopsis.org/info/agicomplete.jsp). GAP indicates the existence of genomic regions of unknown size, where it was not possible to get overlapping BAC clones. Gray bars correspond to the deleted region in each *esd1* allele. The *ESD1* locus was delimited to a deleted overlapping genomic region between the 5F21A14 and 1T14A11 molecular markers. Mb, megabases. (**B**) Complementation of the *esd1* mutant. Col, *esd1-3* and TN 18.1, a transgenic *esd1-3* plant containing the genomic region harbouring open reading frames *At3g33520* y *At3g33530*, shown at the time of bolting initiation. (**C**) Flowering phenotype of *esd1-10*, a T-DNA insertion allele. Left, Col plant; right, a homozygous plant for the T-DNA insertion within the *At3g33520* gene (Wisc Ds-Lox 289 line), showing an early flowering phenotype. RT-PCR analyses of the expression of *At3g33520 in esd1-10* show no expression of this gene in the T-DNA mutant, indicating that it is a loss-of-function allele.

leaf shape, extra perianth organs and small siliques (Fig. 4C). Reverse-transcription (RT-PCR) analyses showed no expression of the *At3g33520* mRNA in these insertional mutant plants (Fig. 4C), indicating that the T-DNA insertion causes a loss-of-function allele. Complementation tests confirmed that this T-DNA mutation was allelic to *esd1*. Thus, we refer to the Wisc Ds-Lox 289_29L8 line as the *esd1-10* allele, and conclude that the *ESD1* locus corresponds to the *At3g33520* gene.

To determine the genomic structure of *ESD1*, a cDNA was identified and sequenced. The *ESD1* gene possesses six exons and five introns, and encodes a protein of 421 amino acids (Fig. 5A). This protein corresponds to ARP6, a member of the actin-related protein family that shares moderate sequence homology and basal structure with conventional actins, but it has two peptide insertions that seemingly provide divergent surface features from actins (Fig. 5B). ARPs are normally grouped into several classes or subfamilies that are highly conserved in a wide range of eukaryotes, from yeast to plants and humans (Goodson and Hawse, 2002). Database searches with the AtARP6 protein sequence identified eight potential ARP proteins in *Arabidopsis* (ARP2-ARP9) (McKinney et al., 2002). In particular, AtARP6 is a likely ortholog of a group of less-characterized ARPs, including ARP6s from yeast, *C. elegans*, fruit fly and humans (Fig. 5B). RT-PCR analysis revealed that *ARP6* mRNA is detected in most plant organs, with the highest levels found in roots and floral buds (Fig. 5C). Lower levels were detected in cauline leaves, stems and flowers. These results indicate that ARP6 is expressed ubiquitously.

ESD1 is required to activate *FLC* transcription through both histone acetylation and methylation mechanisms

FLC gene expression integrates signals coming from different pathways involved in the regulation of the floral transition (Michaels and Amasino, 1999; Sheldon et al., 2000; Amasino, 2005). Recent work has demonstrated the role of histone modification in the regulation of *FLC* expression through *FRI*, the autonomous and the vernalization pathways (He et al., 2003; Ausin et al., 2004; Bastow et al., 2004; He et al., 2004; Sung et al., 2004; Kim et al., 2005). These results have also identified the first intron of *FLC* as a relevant region for histone modification (He et al., 2003; Ausin et al., 2004; Bastow et al., 2004; He et al., 2004; Sung et al., 2004) and transcriptional regulation (Gendall et al., 2001; Sheldon et al., 2002).

We first analyzed the effect of *esd1* mutations on a *FLC:GUS* translational fusion containing all the *FLC* promoter and intron elements required for proper regulation (Sheldon et al., 2002). For this purpose, we introduced the *FLC:GUS* construct into the *fca-1 esd1-2* background and analyzed five independent lines for *GUS* expression. In contrast to the pattern of *GUS* expression in the *fca-1* background, all of the *fca-1 esd1-2 FLC:GUS* lines we examined showed undetectable *FLC:GUS* expression in the shoot apical meristem (SAM) and in the root apical meristem (RAM) (Fig. 6). These results indicate that *ARP6* is required for the high level of *FLC* expression in the SAM and the RAM.

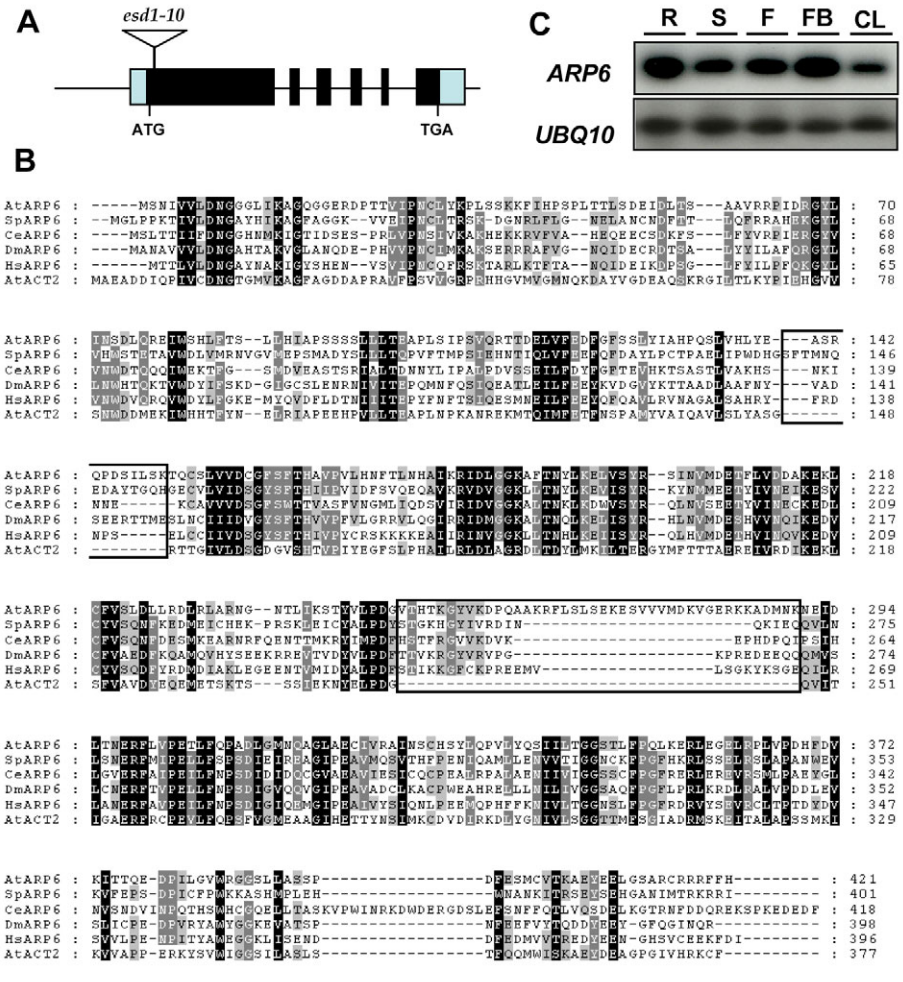

Fig. 5. *ESD1* encodes ARP6. (**A**) Scheme of the *ARP6* gene showing the position of the T-DNA insertion in the *esd1-10* mutant. Exons are shown as black boxes. The position of the start and stop codon are indicated. (**B**) Sequence comparison of AtARP6 with yeast (Sp), *C. elegans* (Ce), *Drosophila* (Dm) and human (Hs) ARP6s, and *Arabidopsis* Actin2. Amino acid residues in black are functionally similar in all sequences and those in gray are similar in at least four of them. Boxed regions indicate the two peptide insertions in ARP6s, which do not disrupt the conserved actin fold structure. GenBank Accession numbers are NP_566861 for AtARP6, AAF4849 for Dm ARP6, AAK14934 for Hs ARP6, CAA19116 for Sp ARP6, NP_495681 for Ce ARP6, and BAB01806 for AtACT2. (**C**) *APR6* expression in different organs of Col plants. RT-PCR assays were performed with RNA prepared from different tissues. R, roots; S, main stems; F, flowers; FB, flower buds; CL, cauline leaves. RT-PCR products were blotted and hybridized with a specific probe for *ARP6*. *UBQ10* was used as a loading control.

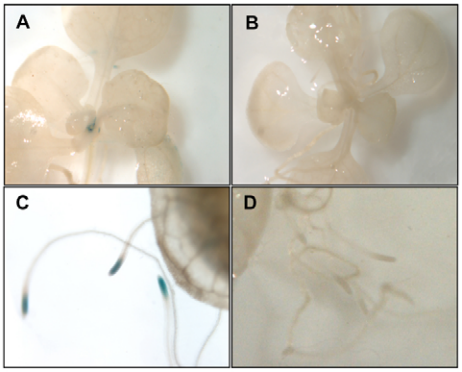

Fig. 6. Histochemical β-glucuronidase assays in *fca-1 FLC:GUS* and *esd1-2 fca-1 FLC:GUS* plants. (**A-D**) Gus staining is shown in the shoot apical meristem region (A,B) and the root tip (C,D) of representative *fca-1 FLC:GUS* (A,C) and *esd1-2 fca-1 FLC:GUS* (B,D) seedlings grown for 10 days under LD conditions.

Because *esd1* suppresses the late-flowering phenotype of autonomous pathway mutants such as *fve* and *fca*, and *FVE* represses *FLC* transcription through a histone deacetylation mechanism, we initially speculated that *ARP6* could be required for the acetylation of histones necessary to activate *FLC* expression. In fact, as mentioned above, there is considerable evidence implicating nuclear ARPs in chromatin remodelling complexes (Cairns et al., 1998; Boyer and Peterson, 2000; Olave et al., 2002).

To determine whether ARP6 promotes histone acetylation of the *FLC* chromatin, we used chromatin immunoprecipitation (ChIP) assays (Fig. 7B). High levels of expression of *FLC* in the *fve* mutant were correlated with H3 and H4 hyperacetylation at the *FLC* locus (He et al., 2003; Ausin et al., 2004). Chromatin of *esd1-2*, *fve-1* and *fve-1 esd1-2* plants was immunoprecipitated by using antibodies against acetylated H3, and PCR was used to amplify six DNA fragments spanning the promoter, the first exon and the first intron of *FLC* from the precipitated chromatin. For five out of the six probes assayed, *FLC* amplified sequences were consistently less abundant in DNA from precipitated chromatin of *fve-1esd1-2* double mutants than from chromatin of the *fve-1* mutant plants (Fig. 7B). Thus, in *fve-1 esd1-2* plants, *FLC* chromatin shows a reduction in acetylated H3 in comparison to the *fve-1* mutant, indicating that ARP6 affects the levels of H3 acetylation of *FLC*. We concluded that ARP6 is required to activate *FLC* expression through a mechanism involving the histone acetylation of *FLC* chromatin. We extended this assay to other genetic backgrounds with high levels of *FLC* expression, such as *fca-1* and *FRI*. For this, we focused our analysis on the *FLC V* and *FLC IX* probes, because they were among those that consistently showed the biggest effect of *esd1* on the histone acetylation of *FLC* chromatin in a *fve* background. In agreement

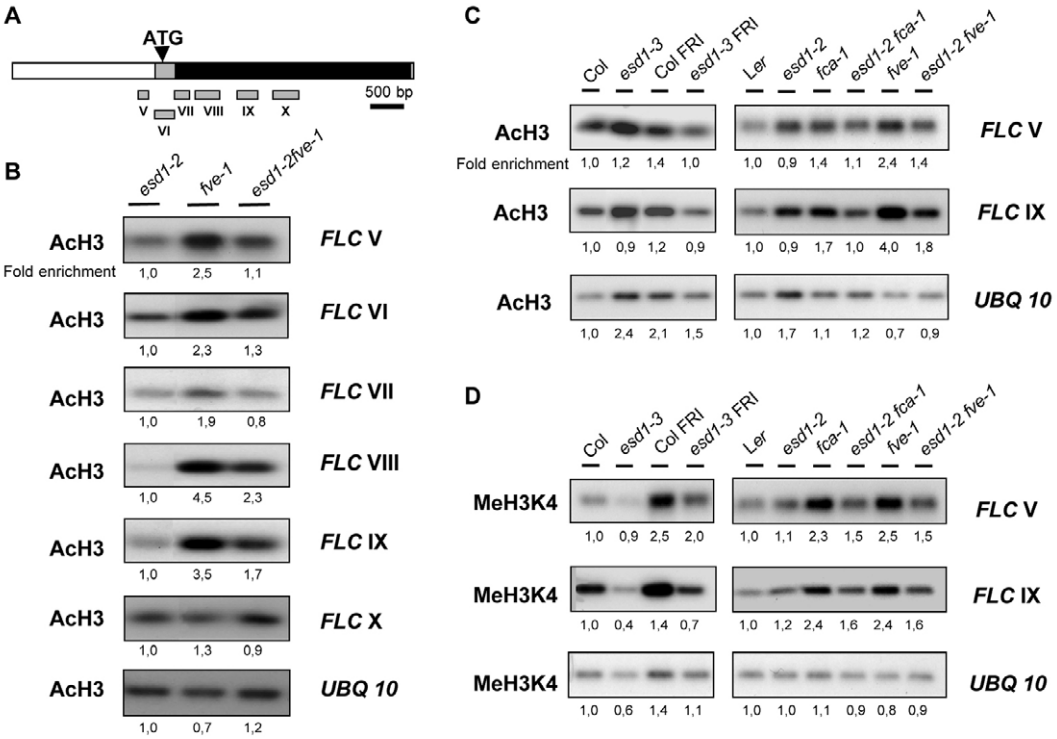

Fig. 7. Effect of *esd1* mutation on histone H3 acetylation and methylation in the *FLC* genomic region by ChIP analysis. (**A**) *FLC* genomic region analyzed by ChIP. The white box corresponds to the promoter *FLC* region, gray boxes to exons and the black box to the first intron. The six *FLC* fragments analyzed by semi-quantitative PCR are depicted and numbered. (**B**) PCR products after 25 cycles of *esd1-2*, *fve-1* and *esd1-2 fve-1* mutants, using as a template DNA purified from chromatin inmunoprecipitated with antibodies against acetylated H3 (AcH3). *UBQ10* was amplified during 22 cycles and used as control for DNA quantification. Fold enrichment in H3 acetylation of *fve-1* over *esd1-2* and *esd1-2 fve-1* double mutant is shown. (**C**) PCR products after 25 cycles of Col, *esd1-3*, *FRI*, *esd1-3FRI*, Ler, *esd1-2*, *fca-1*, *esd1-2 fca-1*, *fve-1*, and *esd1-2 fve-1* plants, using as a template DNA purified from chromatin inmunoprecipitated with antibodies against acetylated H3 (AcH3). *UBQ10* was amplified during 22 cycles and used as control for DNA quantification. Fold enrichment in H3 acetylation of mutants over wild-type ecotypes is shown. (**D**) PCR products as in C, but using a as template DNA purified from chromatin inmunoprecipitated with antibodies against trimethylated H3-K4 (MeH3-K4). Fold enrichment in H3-K4 methylation of mutants over wild-type ecotypes is shown.

with previous data, we only detected very small changes in acetylated H3 in *fca-1* and *FRI* backgrounds, when compared with those observed in the *fve-1* mutant (He et al., 2003; Ausin et al., 2004). These differences were suppressed to a certain degree when *fca-1* or *FRI* was combined with *esd1* (Fig. 7C).

Because *esd1* mutations reduced *FLC* expression in the *fca* and *FRI* background as shown, we hypothesized that ARP6 might be required for other chromatin modifications, in addition to histone acetylation, that are involved in the regulation of *FLC* expression. To further explore this hypothesis, we examined if ARP6 has an effect on histone methylation at the *FLC* locus. It has been shown recently that H3-K4 hypertrimethylation is associated with actively transcribed *FLC* chromatin (He et al., 2004), being elevated in *FRI*-containing winter annuals and autonomous pathway mutants. Given the fact that *esd1* mutations reduce *FLC* expression in these backgrounds, we wondered whether *ESD1* was required for the elevated trimethylation of H3-K4 in *FLC* chromatin. Compared with wild-type plants, the trimethylated H3-K4 levels were elevated in a *FRI*-containing line and in autonomous pathway mutants, as reported previously (He et al., 2004; Kim et al., 2005) (Fig. 7D). Introduction of *esd1* into *FRI*, *fca* and *fve* consistently eliminated the H3-K4 trimethylation increase in *FLC* chromatin associated with *FRI* and the autonomous pathway mutations (Fig. 7D). These data indicate that ESD1 is also required for the hypertrimethylation of H3-K4 in *FLC* chromatin.

DISCUSSION

esd1 mutants were selected from multiple screens for early flowering and accelerated phase change plants in *Arabidopsis*. Phenotypical analyses of these mutants revealed a complex pleiotropic phenotype affecting vegetative and reproductive development, together with a reduction in flowering time and phase length. The results of our genetic analyses revealed that the early flowering phenotype of *esd1* mutants is almost completely epistatic over the flowering time delay caused by mutations in the autonomous pathway, and that *esd1* partially suppresses the late flowering phenotype conferred by active *FRI* alleles (Fig. 3, Tables 2, 3). These epistatic effects correlate at the molecular level with a decrease in the steady state levels of *FLC* mRNA in lines carrying *esd1* mutant alleles. Together, these results indicate that *ESD1* is required for the expression of *FLC*. Thus, mutations of *ESD1* behave like mutations at the *EFS*, *ESD4*, *PIE1*, *ELF5*, *VIP3*, *ELF7*, *VIP4*, *VIP5* and *VIP6*/*ELF8* loci (Soppe et al., 1999; Reeves et al., 2002; Zhang and Van Nocker, 2002; Noh and Amasino, 2003; Zhang et al., 2003; Noh et al., 2004; He et al., 2004; Oh et al., 2004; Kim et al., 2005), all of which are also required for high *FLC* expression and flowering repression.

Additionally, the residual early flowering phenotype observed in *esd1-3 flc-3* double mutants, especially under SD, indicates an additional role of *ESD1* in the repression of flowering time that is independent of *FLC*. The most conservative hypothesis is that *ESD1* is also required for the expression of *FLC*-related repressors, such as some of the *MAF* genes, which is consistent with our results showing a decreased expression of *MAF1*, *MAF4* and *MAF5*, previously shown to play a role in flowering repression in *Arabidopsis* under certain environmental conditions (Scortecci et al., 2001; Ratclife et al., 2003). Finally, until triple and quadruple mutants carrying lesions at *FLC*, *ESD1* and these *MAF* genes are analyzed, we cannot discard possible additional effects of *ESD1* on flowering time through additional genes. The pleiotropic phenotype of *esd1* mutants together with the broad expression pattern detected for this gene suggest that its function could be required in other developmental processes apart from flowering time.

Positional identification of the genomic region deleted in *esd1* alleles and the complementation of the *esd1* phenotype by a genomic clone containing both *At3g33520* and *At3g33530* ORFs, together with the lack of genetic complementation between a T-DNA insertion line in *At3g33520* and *esd1-3*, identified *ESD1* as encoding ARP6. Recently, two publications have also described the characterization of early flowering mutants affected in the *ARP6* gene, proposing its role in the maintenance of *FLC* expression and repression of flowering in *Arabidopsis* (Choi et al., 2005; Deal et al., 2005). ARP6 belongs to the actin-related protein family that shares moderate sequence homology and basal structure with actins. In *Arabidopsis* and rice, four divergent ARP classes (ARP4, ARP5, ARP6 and ARP9) are sequence homologs of ARPs, which are nuclear located in animals and fungi (McKinney et al., 2002; Kandasamy et al., 2004). Most of the nuclear ARPs are essential components of large multiprotein chromatin-modifying complexes (Blessing et al., 2004). The fruit fly ARP6-related protein ARP13E is associated with heterochromatin and may also play a role in chromatin structure (Frankel and Mooseker, 1996; Kato et al., 2001). ARP13E colocalizes with heterochromatin protein 1 (HP1) (Frankel et al., 1997), which is also linked to heterochromatin-mediated gene silencing and chromatin structure (Eissenberg and Elgin, 2000). Moreover, in nuclei expressing mutant forms of HP1, the localization patterns of HP1 and dARP6 are altered in a parallel fashion (Frankel et al., 1997), implying that dARP6 interacts with HP1 directly or indirectly, and that they play a role in the organization of heterochromatin together. Mutants with a defect in an *Arabidopsis HP1* ortholog, *LIKE-HETEROCHROMATIN PROTEIN 1* (*LHP1*), also show an early flowering phenotype (Gaudin et al., 2001), raising the possibility that both proteins might be involved in the same chromatin-remodelling complexes in *Arabidopsis*.

Covalent modification of chromatin histones constitutes a code for maintaining states of gene activation and repression, and is a major component in the transcriptional regulation of *FLC* (Gendall et al., 2001; He et al., 2003; Ausin et al., 2004; Bastow et al., 2004; He et al., 2004; Sung et al., 2004). High levels of expression of *FLC* in autonomous pathway mutants are correlated with H3 and H4 hyperacetylation and trimethylation of H3-K4 at the *FLC* locus (He et al., 2003; Ausin et al., 2004; He et al., 2004; Kim et al., 2005). Furthermore, ARP6-like proteins have been found in other organisms as part of large protein complexes involved in chromatin remodelling (Krogan et al., 2003; Kobor et al., 2004; Mizuguchi et al., 2004). Because *esd1* mutations suppress the late-flowering phenotype of *fve* mutants, and *FVE* represses *FLC* transcription through a histone deacetylation mechanism, we initially hypothesized that *ESD1* could be required to activate *FLC* expression to levels that inhibit flowering, participating in chromatin remodelling complexes involved in histone acetylation of *FLC* chromatin. The lack of expression of *GUS* in *esd1 fca* plants expressing the *FLC:GUS* translational fusion, already suggested that if *ESD1* was required for active expression of *FLC*, this had to take place at the *FLC* sequences present in the construct used (promoter, first exon and first intron) (Sheldon et al., 2002). In fact, the results of ChIP experiments directed to that chromosomal region of *FLC* demonstrated that it is hypoacetylated in the *esd1 fve* mutant compared with the *fve* mutant (Fig. 7B). Thus, we conclude that ESD1 is required for histone acetylation at *FLC*, probably through its participation in HAT complexes. However, *esd1* mutations also reduce both the late-flowering phenotype and *FLC* expression in *FRI*-containing lines and *fca* mutants, despite the fact that in these backgrounds the levels of acetylated H3 of *FLC* chromatin did not

show significant changes in comparison to *five* (Fig. 7C). This raised the possibility that ARP6 would participate in other mechanisms besides histone acetylation; our results indicate that the hypermethylation of H3-K4 in *FLC* chromatin is one of these mechanisms (Fig. 7D). It remains to be determined whether the effect of *esd1* on the expression of other *MAF* genes takes place through similar mechanisms.

Our observation that ARP6 regulates the activation of *FLC* expression by promoting both histone acetylation and methylation is consistent with a role for plant ARPs in chromatin-mediated transcriptional regulation. ARP4 is also likely to be involved in transcriptional regulation via chromatin remodelling, as it is a component of the human SWI/SNF and yeast INO80 complexes that are involved in chromatin remodelling, transcriptional regulation and DNA damage repair (Zhao et al., 1998; Shen et al., 2003). Other ARP4-containing complexes, such as yeast NuA4 and human TIP60, are suggested to have roles in chromatin-mediated epigenetic control of transcription through modifications of core histones (Galarneau et al., 2000; Ikura et al., 2000). Yeast Arp4 interacts with all four core histones (Harata et al., 1999), and recent findings have shown that Arp4 and Arp6 are also part of the Swr1 chromatin-remodelling complex, which catalyzes the exchange of conventional histone H2A for the histone H2A.Z variant in nucleosome arrays (Krogan et al., 2003; Kobor et al., 2004; Mizuguchi et al., 2004). These histone variants are involved in the regulation of gene expression and the establishment of a buffer to the spread of silent heterochromatin (Meneghini et al., 2003). Indeed, a human H2A.Z complex, equivalent to the yeast Swr1 complex has histone acetyl transferase activity, which might help to understand the role of *ESD1* in histone acetylation (Owen-Hughes and Bruno, 2004). In the same way, the fact that components of the Swr1 complex were found to interact genetically with the PAF1 complex might explain the role of *ESD1* in the trimethylation of H3-K4 in *FLC* chromatin (Krogan et al., 2002; Mueller and Jaehning, 2002; Squazzo et al., 2002; Krogan et al., 2003; Krogan et al., 2004). Like the yeast PAF1 complex, the PAF1-like complex in *Arabidopsis* may also recruit an H3-K4 methyl transferase to *FLC* to regulate its expression (Kim et al., 2005). Indeed, mutations in *Arabidopsis* homologs of the components of the PAF1 complex cause a decrease in the trimethylation of H3-K4 in *FLC* chromatin, and provoke early flowering and small leaves, similar to the *esd1* mutation (He et al., 2004), raising the possibility that all of these genes are in the same pathway and regulate similar targets.

In agreement with the pleiotropic phenotype of *esd1* mutants, the general pattern of expression of *ESD1/ARP6* suggests that this gene is required in additional vegetative and reproductive developmental processes in which protein complexes harbouring ARP6 might play a relevant regulatory role. Given the molecular identity of *ESD1*, it seems reasonable to propose that loss-of-function alleles will cause a great effect on transcription, interfering with the expression of genes controlling various developmental pathways and thereby provoking changes in the morphology of different organs throughout the development of *Arabidopsis*. Among them, organ number in the perianth, which increases in *esd1* mutants, is affected in a similar way in *pie1* mutants. *PIE1* encodes a protein similar to the ATP-dependent, chromatin remodelling proteins of the ISWI and SWI/SNF2 family, and it is a close homolog to the Swr1 ATPase, the core subunit of the yeast Swr1 complex that harbours Arp6 (Mizuguchi et al., 2004). Loss of function of the *PIE1* gene causes strikingly similar phenotypes to those of the *esd1* mutant (Noh and Amasino, 2003), apart from the development of extra petals. In addition, *pie1* mutations also cause early flowering and suppress *FLC*-mediated delay of flowering as a result of the presence of *FRI* or of mutations in autonomous pathway genes, suggesting that PIE1 and ARP6 may act in the same genetic pathways and might be part of the same protein complexes. However, in contrast to *esd1* mutations, which suppress *FLC* expression in both SAM and RAM (Fig. 6), the effect of *pie1* lesions is restricted to the shoot apex (Noh and Amasino, 2003), suggesting that the root tip expression of *FLC* requires *ARP6* and probably other root-expressed relatives of *PIE1*, and that the level of *FLC* expression in the shoot apex, but not in the root apex, influences flowering behaviour.

Recent analyses of knockdown *AtARP4* expression in *Arabidopsis* have also revealed dramatic pleiotropic phenotypes, both similar to and entirely different from those of *esd1/arp6* (Kandasamy et al., 2005a). For example, silencing of the expression of *ARP4* or loss of function of *ARP6* caused early flowering; however, silencing of the expression of *ARP4* but not *ARP6* induced specific phenotypes, such as the altered organization of plant organs, delayed flower senescence and high levels of sterility (Kandasamy et al., 2005a), suggesting that both of these proteins may also be involved in the same and in different chromatin modifying complexes in *Arabidopsis*. Another ARP member, AtARP7 is required for normal embryogenesis, plant architecture, root growth and floral organ abscission (Kandasamy et al., 2005b), and may be also involved in chromatin-remodelling complexes.

In summary, our results demonstrate that *ESD1/ARP6* is required for both *FLC* and *FLC*-like gene expression in the shoot and the root apex, and for the activity of a floral repressor pathway. The role of *ESD1* in *FLC* regulation is to ensure competence for a high level of expression of this gene. We propose that ARP6 is required to activate *FLC* transcription through mechanisms involving both histone acetylation and methylation. We have determined that *FLC*, and maybe the *FLC* paralogs *MAF1*, *MAF4* and *MAF5* are targets of ARP6-containing chromatin-remodelling complexes, and that some components of the autonomous pathway might affect the activity of such complexes. Moreover, the pleiotropic phenotype observed for *esd1* mutants suggests a crucial role for the *Arabidopsis* ARP6 protein in the regulation of several leaf and flower development stages, probably through chromatin-mediated regulation of gene expression. Further functional studies, such as the identification of the proteins within ARP6-containing complexes, as well as the identification of additional genes regulated by these complexes, will help us to understand the crucial role of ARP6 in *Arabidopsis* development.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/133/7/1241/DC1

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