Mutations in the *Arabidopsis SWC6* gene, encoding a component of the SWR1 chromatin remodelling complex, accelerate flowering time and alter leaf and flower development

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Abstract

Mutations affecting the *Arabidopsis SWC6* gene encoding a putative orthologue of a component of the SWR1 chromatin remodelling complex in plants have been characterized. *swc6* mutations cause early flowering, shortened inflorescence internodes, and altered leaf and flower development. These phenotypic defects resemble those of the photoperiod independent early flowering 1 (*pie1*) and early in short days 1 (*esd1*) mutants, also affected in homologues of the SWR1 complex subunits. SWC6 is a ubiquitously expressed nuclear HIT-Zn finger-containing protein, with the highest levels found in pollen. Double mutant analyses suggest that *swc6* abolishes the *FLC* mediated late-flowering phenotype of plants carrying active alleles of *FRI* and of mutants of the autonomous pathway. It was found that SWC6 is required for the expression of the *FLC* repressor to levels that inhibit flowering. However, the effect of *swc6* in an *flc* null background and the down-regulation of other *FLC*-like genes in *swc6* mutants suggest that flowering inhibition mediated by SWC6 occurs through both *FLC* and *FLC*-like gene-dependent pathways. Both genetic and physical interactions between SWC6 and ESD1 have been demonstrated, suggesting that both proteins act in the same complex. Using chromatin immunoprecipitation, it has been determined that SWC6, as previously shown for ESD1, is required for both histone H3 acetylation and H3K4 trimethylation of the *FLC* chromatin. Altogether, these results suggest that SWC6 and ESD1 are part of an *Arabidopsis* SWR1 chromatin remodelling complex involved in the regulation of diverse aspects of plant development, including floral repression through the activation of *FLC* and *FLC*-like genes.

Key words: *Arabidopsis*, chromatin remodelling, floral repression, HIT-Zn finger, phase transition, SWR1 complex.

Introduction

To ensure that flowering occurs in optimal conditions, plants integrate both environmental and endogenous signals before switching to reproductive development. To select the right season for flowering, plants rely fundamentally on environmental factors such as light and temperature that suffer predictable changes through the year. *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 (Baurle and Dean, 2006; Imaizumi and Kay, 2006; Schmitz and Amasino, 2007). The photoperiod pathway promotes flowering in response to LD through the activation of the floral integrators *FT* and *SUPPRESSOR OF OVEREXPRESSION OF CO 1* (*SOC1*). Indeed, FT protein has been recently proposed as an essential component of the systemic signal that mediates photoperiodic induction of flowering (Corbesier et al., 2007). The GA pathway is required for flowering in non-inductive photoperiods, and mutants with reduced...
GA levels are extremely delayed in flowering time under short days (SD) (Wilson et al., 1992). In addition, winter annuals require exposure to an extended period of cold (vernalization) to become flowering competent, thus preventing premature flowering in the autumn (Michaels and Amasino, 2000; Sung and Amasino, 2006). 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 suppresses the expression of FT and SOC1 genes, which function as integrators of flowering signals; therefore, FLC confers a flowering response to vernalization in Arabidopsis by repressing both the generation of flowering-inductive systemic signals and the meristem competence to respond to such signals (Searle et al., 2006). 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 them to flower (Michaels and Amasino, 1999; Sheldon et al., 1999).

Many summer annual accessions of Arabidopsis lack an active FRI allele (Johanson et al., 2000; Gazzani 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 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, 2001; Sheldon et al., 1999). Several genes have been classically ascribed to the autonomous pathway, including either factors involved in the binding and processing of mRNAs or proteins associated with chromatin remodelling processes (for reviews see Baurle and Dean, 2006; Schmitz and Amasino, 2007). FVE and FLOWERING LOCUS D (FLD) belong to the last group. The homologue of FVE in animals is found in nucleosome Remodelling Factor (NuRF) and histone deacetylase (HDAC) complexes and is likely to act as a histone chaperone (Auszin et al., 2004). FLD and related proteins, such as swp1, are highly homologous to human KIAA0601/lysine demethylase 1 (LSD1) (He et al., 2003; Krichevsky et al., 2007), also present in HDAC complexes (Lee et al., 2006). Consistent with the nature of these proteins, the increased expression of FLC in fve, fld, and swp1 mutants is correlated with hyperacetylation of histones H3 and H4 (He et al., 2003; Ausin et al., 2004; Krichevsky et al., 2007), a modification associated with transcriptionally active chromatin conformations.

During vernalization, histone modifications associated with active genes such as acetylation of histone H3 and H4 and methylation at H3K4 decrease in FLC chromatin but the level of repressive markers such as H3K9 and H3K27 trimethylation increase (Sung and Amasino, 2004; Sung et al., 2006). This vernalization-dependent repressed state of FLC is mitotically stable; upon passing to the next generation, FLC expression is reset to the active state, suggesting the involvement of a mechanism conferring cellular memory for remembering winter. VERNALIZATION INSENSITIVE 3 (VIN3) appears to be required for histone deacetylase in the FLC region following vernalization (Sung and Amasino, 2004) and none of the repressive markers associated with vernalization is present in vin3 mutants. A polycomb group (PcG) complex containing VERNALIZATION 2 (VRN2) and VIN3 may bring histone deacetylase and histone methyltransferase activities together at FLC chromatin, providing a coordinated mechanism for the epigenetic modifications associated with the vernalization-mediated repression of the FLC gene (Wood et al., 2006).

The establishment of the winter-annual habit of Arabidopsis requires that FLC is expressed at high levels in the first growing season to block flowering before winter. High levels of acetylation of histone H3 and H4 and H3K4 methylation contribute to an active chromatin conformation at the FLC locus during initial stages of development (He et al., 2004; Sung and Amasino, 2004; Kim et al., 2005; Martin-Trillo et al., 2006; Sung et al., 2006). The isolation of mutants capable of flowering early in winter-annual backgrounds has led to the identification of genes required to activate FLC at the beginning of the life cycle and that encode components of putative chromatin remodelling complexes. Most of these mutants can be classified into two different groups, affecting putative orthologues of either the SWR1 or the PAF1 complexes. Mutations in genes encoding proteins related to components of the yeast transcriptional-activating PAF1 complex [early flowering 7 (elf7), elf8, and vernalization independent 4 (vip4)] (He et al., 2004; Oh et al., 2004) cause an acceleration of flowering. In yeasts, this complex interacts with SET1 and SET2 histone methyltransferases involved in methylation of H3K4 and H3K36, respectively (Krogan et al., 2003). Mutants in the Arabidopsis histone methyltransferase EARLY FLOWERING IN SHORT DAYS (EFS/SDG8) also flower early and display reduced levels of FLC expression, like PAF1 complex mutants (Kim et al., 2005; Zhao et al., 2005), suggesting that the PAF1 complex and EFS may act directly on FLC to maintain high levels of expression. Consistent with this, two different studies have provided evidence that this protein is required for high levels of either H3K4me3 or H3K36me2 in the region of FLC (Kim et al., 2005; Zhao et al., 2005).

In the same way, mutations in putative orthologues of the yeast SWR1 complex, including EARLY IN SHORT
DAYS 1/SUPPRESSOR OF FRIGIDA 3/ACTIN RELATED PROTEIN 6 (ESD1/SUF3/ARP6) (Choi et al., 2005; Deal et al., 2005; Martin-Trillo et al., 2006), the SWI/SNF ATPase PHOTOPERIOD INDEPENDENCE1 (PIE1) (Noh et al., 2003), and SEF (SERRATED AND EARLY FLOWERING)/AtSWC6 (Choi et al., 2007; March-Diaz et al., 2007) have been described recently. The SWR1 complex in yeast catalyses the replacement of nucleosomal H2A with the H2A.Z variant, ensuring full activation of underlying genes. Recently H2A.Z was identified within FLC and FLC-like chromatin (Deal et al., 2007). Loss of H2A.Z from FLC chromatin in esd1/suf3/arp6 and pie1 mutants results in reduced FLC expression and premature flowering, indicating that this histone variant is required for a high level of expression of FLC (Deal et al., 2007). In addition, H2A.Z interacts with both PIE1 and AtSWC2, and knockdown of the H2A.Z genes by RNA interference or artificial microRNA caused a phenotype similar to that of esd1/suf3/arp6 (Choi et al., 2007). These observations support the existence of a SWR1-like complex in plants that is targeted to different loci including FLC, and show that H2A.Z can enhance transcriptional activation in plants. The fact that H2A.Z remains associated with chromatin throughout mitosis suggests that it may serve as an epigenetic memory function by marking active genes and poising silenced genes for reactivation (Deal et al., 2007).

This work reports the characterization of SWC6, a putative component of the SWR1 complex of Arabidopsis, required for the maintenance of FLC expression. swc6, like mutations in other putative components of this complex, causes early flowering mainly through the reduction of FLC expression, although it also appears to affect flowering through other FLC-like repressors. It is demonstrated here that SWC6 interacts both genetically and physically with another crucial subunit of the complex such as ESD1/SUF3/ARP6 and that both proteins are needed to achieve the levels of both H3 acetylation and H3K4me3 required for high FLC expression. Taken together, the data indicate that SWC6 and ESD1/SUF3/ARP6 might form a molecular complex in Arabidopsis related to the SWR1/SRCAP complex identified in other eukaryotes, which regulates diverse aspects of plant development, including floral repression.

Materials and methods

Genetic stocks and growth conditions

Mutant seed stocks used were in the Columbia (Col) genetic background, and were obtained from the Arabidopsis Biological Resource Center (ABRC) of Ohio State University (Columbus, USA) and personal donations. The fve-3 mutant was described by Ausin et al. (2004); fce-3 was described by Michaels and Amasino (2001); esd1-10 was described by Martin-Trillo et al. (2006); the Col FRI-Sf2 lines were described by Lee and Amasino (1995). The origin of the swc6-1 and swc6-2 alleles is described in the text. The same alleles were identified previously and denoted as sef-2 and sef-1, respectively (March-Diaz et al., 2007). swc6-1 and swc6-2 mutations were confirmed to be 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 (~120 μE m⁻² s⁻¹). LD conditions consisted of 16 h of light followed by 8 h of darkness; SD conditions consisted of 8 h of light followed by 16 h 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; for each experiment the average flowering time of at least 20 plants ± SE is error is given (Martin-Trillo et al., 2006). cauline, adult, and juvenile leaves were scored independently. Rosette leaves lacking abaxial trichomes were considered as juvenile leaves (Telfer et al., 1997).

Genetic analysis

Double mutants were generated by crossing the monogenic swc6-1 mutant with lines carrying the fve-3 (Ausin et al., 2004), fce-3 (Michaels and Amasino, 2001), and esd1-10 (Martin-Trillo et al., 2006) mutations and with Col FRI Sf-2 (Lee and Amasino, 1995). Double mutants were isolated from selfed F2 progeny using molecular markers associated with each mutation.

Molecular characterization of the swc6 alleles

The T-DNA insertion swc6-1 (SAIL_1142_C03) and swc6-2 (SAIL_536_A05) mutant lines were obtained from NASC. Two specific primers or one specific primer and a T-DNA left border (LBA SAIL) primer were used for amplification of wild-type or T-DNA insertion alleles, respectively (LBA SAIL, 5'-TTCATAAC-CAATCTCGATACAC-3'). T-DNA borders were determined by sequencing PCR products obtained with T-DNA border primers and gene-specific primers.

Generation of transgenic plants

Transgenic plants expressing SWC6 and ESD1/SUF3/ARP6 full-length cDNAs under the control of the 35S cauliflower mosaic virus promoter or expressing a promoter fragment of the SWC6 gene fused to the GUS gene (353 bp upstream of the ATG) were generated following Agrobacterium tumefaciens-mediated transformation using the floral-dip method (Clough and Bent, 1998). The Agrobacterium strain used was C58C1. Transformant plants were selected on GM medium containing appropriate antibiotics. Levels of overexpressed genes were tested by northern blots using SWC6- and ESD1/SUF3/ARP6-specific probes. As loading controls, a 305 bp EcoRI fragment of the cauliflower 18S rDNA gene was used.

Histochemical β-glucuronidase assays

GUS activity in pSWC6:GUS plants was revealed by incubation in 100 mM NaPO₄ (pH 7.2), 2.5 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide, 0.5 mM K₃Fe(CN)₆, 0.5 mM K₄Fe(CN)₆ and 0.25% Triton X-100. Plant tissue was incubated at 37 °C overnight. After staining, chlorophyll was cleared from the samples by degradation through ethanol.
Whole-mount anther preparation for microscopy

Anthers were collected and incubated overnight at 4 °C in coloration buffer, containing equal volumes of extraction buffer (0.1% Nonidet P40, 10% dimethyl sulphoxide, 5 mM EGTA, pH 7.5, 50 mM PIPES, pH 6.9) and DAPI solution (1 mg DAPI ml−1 dimethyl sulphoxide).

Yeast two-hybrid analysis

Yeast two-hybrid interaction analyses were conducted in the Y190 strain with the MatchMaker two-hybrid system (Clontech). pGBT-8 or pGAD plasmids were used for GBD or GAD fusion constructs, respectively. cDNAs for SWC6 and ESD1/SUF3/ARP6 were obtained by standard PCR techniques and cloned into the above-mentioned vectors using Gateway recombinant technologies (Clontech). Selection was performed on synthetic complete (SC) minimal medium without His, Leu, and Trp, supplemented with 5−25 mM 3-amino-1,2,4-triazole (3-AT).

Protein expression, purification, and pull-down assays

The SWC6 expression construct was expressed in the pGEX-6P-3 vector (Amersham Biosciences) and expressed in Escherichia coli BL21 Rosetta. Standard PCR techniques were used for GST tagging of SWC6. Proteins were purified on glutathione 4B Sepharose beads (GE Amersham) and kept on beads as GST–SWC6 or GST alone. In vitro transcription/translation ESD1/SUF3/ARP6 reactions were performed with the TNT Quick Coupled Transcription/Translation system (Promega) in the presence of [35S]methionine (Amersham Biosciences). For pull-down assays, 500 ng of GST or GST–SWC6 bound to beads were incubated in 200 μl of binding buffer (20 mM TRIS-HCl, pH 7.0, 100 mM NaCl, 1 mM EDTA, 10% glycerol, 0.01% Nonidet P-40) with 15 μl of the TNT reaction and rinsed with binding buffer supplemented with 500 mM NaCl. Samples were boiled with Laemmli buffer and analysed by SDS–PAGE.

Expression analysis

Total RNA was isolated using TRIzol (Invitrogen-Gibco). cDNA was prepared by reverse transcription of total RNA from Arabidopsis roots, stems, rosette and cauline leaves, floral buds, and flowers, according to described procedures (Martin-Trillo et al., 2006). SWC6 transcript levels were assayed by reverse transcription (RT)-PCR, with specific primers, 5′-ATGGAGGAAGAGATGC-3′ and 5′-CGAGATCATCATCTCATCAAGAG-3′, designed to amplify the N-terminal end of the coding region. For the rest of genes analysed, such as FLC, the MAF family, FT RT–PCR and SOCI genes, was performed, according to described procedures (Scortecci et al., 2001; Pinedo et al., 2003; Ratcliffe et al., 2003; Martin-Trillo et al., 2006). UBQUITIN 10 (UBQ10) was used as the control in these experiments.

Chromatin immunoprecipitation (ChiP) assays and PCR

ChiP assays were carried out as described (Ausz et al., 2004). Chromatin proteins and DNA were cross-linked in 10-d-old Col, esd1-10, and swc6-1 seedlings by formaldehyde fixation. After chromatin isolation, the H3 acetylated and methylated fractions were immunoprecipitated using specific antibodies; one of them recognizes both acetylated K9 and K14 residues, and the second one recognizes K4me3 residues (06-599 and 07-473 from Upstate Biotechnology, respectively). Cross-links were reversed by incubations at 65 °C for 2 h, and DNA was purified with QIAquick spin columns (Qiagen) and eluted in 40 μl of TE (pH 8.0). Semi-quantitative PCR was used to amplify two different fragments of the FLC gene as described previously (Martin-Trillo et al., 2006; details and primer sequences are available on request). All PCR and quantification of the amplified DNA were done as described previously (Martin-Trillo et al., 2006). Three independent experiments were carried out. UBQ10 served as an internal control for the ChiP analysis. To calculate the fold decrease 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.

Gene sequences described in this article can be found in GenBank under accession numbers NM_123064 (SWC6) and NM_114070 (ESD1/SUF3/ARP6).

Results

swc6 mutants are early flowering and display pleiotropic defects in both vegetative and reproductive development

Previously, esd1, an Arabidopsis early flowering mutant affected in an orthologue of ACTIN-RELATED PROTEIN 6 (ARP6), had been identified (Martin-Trillo et al., 2006). The yeast ARP6 protein is a component of the SWR1 complex, which consists of 13 subunits including the ATPase component SWR1 and SWC6/VSP71 (Kobor et al., 2004; Mizuguchi et al., 2004). A physical interaction between ARP6 and SWC6 has been proposed in yeast (Wu et al., 2005). A search for an Arabidopsis protein homologue of the yeast SWC6/VSP71 subunit led to the identification of a related protein, encoded by the At5g37055 gene. To investigate the role of Arabidopsis SWC6 in plant development, T-DNA insertion lines were searched in different collections, and two different lines were identified (Fig. 1A) and designated as swc6-1 and swc6-2. Line SAIL_1142_C03 (swc6-1) bore an insertion in exon 2 of the At5g37055 locus, in a position corresponding to nucleotide 146 of the coding region. RT-PCR analysis, using primers forward (F) and reverse (R), upstream of the T-DNA insertion, demonstrated no expression of SWC6 mRNA in homozygous swc6-1 plants (Fig. 1B). In the same way, line SAIL_536_A05 (swc6-2) contained a T-DNA inserted in the promoter region, upstream of the 5′ UTR of SWC6 mRNA. RT-PCR analysis was unable to detect SWC6 mRNA in swc6-2 plants (data not shown). Because both alleles produced a similar array of phenotypes, swc6-1 was chosen to carry out all the genetic and phenotypic analyses. Heterozygous plants displayed a wild-type phenotype, indicating that both swc6-1 and -2 were recessive. Plants homozygous for swc6-1 mutations were early flowering mainly under non-inductive SD photoperiods (Fig. 2A, B, Table 1). The fact that swc6-1 mutants flower earlier under inductive photoperiods indicates that this mutation does not abolish the flowering photoperiodic response. Earliness was associated with a reduction in the length of all developmental phases of the plant (Fig. 2C), based on leaf shape and leaf trichome distribution (Telfer et al., 1997).
This reduction was more dramatic for adult rosette leaves, which were highly reduced in swc6 mutant plants under both LD and SD (Fig. 2C). This behaviour is similar to that exhibited by other early flowering mutants such as esd1, esd4, and ebs, which also show a major reduction in the adult vegetative phase (Gomez-Mena et al., 2001; Reeves et al., 2002; Martin-Trillo et al., 2006).

Apart from their flowering-time phenotype, swc6 mutants also displayed complex pleiotropic alterations of both vegetative and reproductive development. Mutant plants produced more coflorescence shoots than Col. This was accompanied by a shortening of inflorescence internodes, resulting in a reduction in inflorescence length and apical dominance (Fig. 2D). Furthermore, swc6 leaves are smaller and more curled than wild-type leaves, and frequently have serrated margins (Fig. 2E, F). As shown in Fig. 2G–J, swc6 flowers displayed several developmental abnormalities, including a reduction in size as compared with wild-type flowers. Petals of mutant plants were smaller than wild-type petals and slightly wrinkled (Fig. 2H); mutant anthers were also smaller than those of the wild type and often presented a heart shape characteristic of immature anthers (Fig. 2I); indeed the swc6-1 mutant showed a reduced fertility associated with a reduction in the amount of pollen. In the same way, mutant carpels (Fig. 2J) and siliques (Fig. 2K) were approximately half the length in swc6 mutants of those in wild-type plants. In addition, swc6 flowers frequently bear extra perianth organs. This phenotype was more extreme under SD, where swc6 flowers contained 5.8±0.8 sepals and 5.7±0.9 petals per flower (Fig. 2G). Similar phenotypes have been described in pie1 and esd1/suf3/arp6 mutant plants, suggesting that these proteins may form part of a complex that regulates multiple aspects of Arabidopsis development.

To complement the mutant swc6 phenotype, an At5g37055 cDNA driven by the 35S promoter was introduced into swc6-1 mutants. Several transgenic lines of 35S:SWC6 were generated (Fig. 3A). Northern blot analysis showed high accumulation of SWC6 mRNA in the transgenic lines that complemented all the developmental defects observed in the mutant plants (Fig. 3A, Table 1). It is significant that overexpression of SWC6 in swc6 or overexpression of ARP6 in esd1 (Fig. 3B) did not cause an additional delay in flowering time. Consistently,
the SWC6 or the ARP6 overexpression lines in the Col genetic background did not show any additional flowering phenotype.

**SWC6 encodes a HIT-type zinc-finger protein**

To confirm the genomic structure of SWC6, a cDNA of 516 bp was identified and sequenced. SWC6 is a single gene in Arabidopsis; it possesses four exons and encodes a nuclear HIT-type zinc-finger protein of 171 amino acids (Choi et al., 2007), whose homologues, SWC6 and ZNHIT1, are subunits of the yeast SWR1 and mammalian SRCAP (SWI2/SNF2-related CBP activator protein) complexes, respectively (Mizuguchi et al., 2004; Cai et al., 2005). AtSWC6 is also closely related to Nicotiana benthamiana CIBP1, identified as a Plum pox virus cylindrical inclusion-interacting protein, and to a SWC6 rice protein (OsSWC6). All these proteins have seven cysteines and one histidine highly conserved in a C-terminal region, which are part of a HIT-type zinc finger domain (Fig. 4A).

Semi-quantitative RT-PCR experiments demonstrated that SWC6 transcript was present at variable levels in all the tissues tested (Fig. 4B). AtSWC6 expression was more strongly detected in roots, flowers, and flower buds (Fig. 4D). Similar expression profiles of SWC6 are obtained from Genevestigator (http://www.genevestigator.ethz.ch; Zimmermann et al., 2004). In the transgenic plants expressing a 353 bp transcriptional fusion of the AtSWC6 promoter region with GUS (AtSWC6p:GUS), GUS expression was detected in actively dividing cells such as root and shoot apices, lateral root primordia, trichomes, inflorescences, flowers, etc. (Fig. 4D). Interestingly, GUS expression was particularly high in anthers (Fig. 4D). By contrast, GUS expression was rarely detected in stems, leaves, seeds, and siliques (data not shown). Analysis of the SWC6 promoter region fused to GUS shows the presence of various cis-acting elements, including sequences known to confer anther/pollen-specific gene expression (Fig. 4C).

**Table 1. Flowering time of swc6, double mutants with swc6, and 35S::SWC6 transgenic plants**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Long days</th>
<th>Short days</th>
</tr>
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<tbody>
<tr>
<td>Col</td>
<td>13.3 ± 1.5</td>
<td>69.5 ± 4.5</td>
</tr>
<tr>
<td>swc6-1</td>
<td>9.5 ± 0.6</td>
<td>38.5 ± 4.6</td>
</tr>
<tr>
<td>esd1-10</td>
<td>8.9 ± 0.7</td>
<td>31.8 ± 8.3</td>
</tr>
<tr>
<td>swc6-1 esd1-10</td>
<td>7.9 ± 0.3</td>
<td>31.3 ± 6.3</td>
</tr>
<tr>
<td>fve-3</td>
<td>34.3 ± 3.9</td>
<td>&gt;110</td>
</tr>
<tr>
<td>swc6-1 fve-3</td>
<td>13.7 ± 1.6</td>
<td>62.9 ± 5.3</td>
</tr>
<tr>
<td>FRI</td>
<td>34.7 ± 7.1</td>
<td>&gt;110</td>
</tr>
<tr>
<td>swc6-1 FRI</td>
<td>21.2 ± 3.7</td>
<td>79.3 ± 9.1</td>
</tr>
<tr>
<td>flc-3</td>
<td>12.1 ± 1.4</td>
<td>62.7 ± 4.1</td>
</tr>
<tr>
<td>swc6-1 flc-3</td>
<td>8.7 ± 0.7</td>
<td>40.5 ± 3.3</td>
</tr>
<tr>
<td>35S::SWC6 swc6-1(3-1-6)</td>
<td>14.6 ± 1.2</td>
<td>ND</td>
</tr>
<tr>
<td>35S::SWC6 swc6-1(4-1-6)</td>
<td>8.9 ± 0.5</td>
<td>ND</td>
</tr>
<tr>
<td>35S::ESD1 esd1-10(3-2-4)</td>
<td>12.7 ± 0.8</td>
<td>ND</td>
</tr>
<tr>
<td>35S::ESD1 esd1-10(6-1-2)</td>
<td>8.7 ± 0.9</td>
<td>ND</td>
</tr>
</tbody>
</table>

**The swc6-1 mutation suppresses the late flowering of FRI and autonomous pathway mutants**

The early-flowering phenotype of swc6 mutants suggested that SWC6 could negatively interact with a flowering-promoting pathway or alternatively interact positively with a flowering-repressor pathway in Arabidopsis.

**Fig. 3. Phenotype of SWC6 and ESD1/SUF3/ARP6 overexpression lines.** (A) Col, swc6-1, and 35S::SWC6 swc6-1 transgenic Arabidopsis (line 3-1-6) plants grown for 2 weeks under LD conditions. In the left panel a northern blot hybridization shows the level of expression of SWC6 in these plants and in the 35S::SWC6 swc6-1 4-1-6 line, a representative that did not show complementation of the swc6 mutant phenotype. (B) Col, esd1-10, and 35S::ESD1 esd1-10 transgenic Arabidopsis (line 3-2-4) plants grown for 2 weeks under LD conditions. In the left panel a northern blot hybridization shows the level of expression of ESD1 in these plants together with the 35S::ESD1 esd1-10 6-1-2 line, which did not show complementation of the esd1 mutant phenotype. 18S ribosomal probe was used as a control of loading and integrity of RNAs in the northern blots.
Mutations affecting ESD1, another member of the SWR1 complex, suppress the late flowering of FRI and autonomous pathway mutants (Martin-Trillo et al., 2006) and, for that reason, the genetic analysis was focused initially in combinations between swc6 and these genotypes. To test the possible interaction between SWC6 and autonomous pathway genes, the flowering phenotype of the swc6 fve-3 double mutant was analysed (Fig. 5A). Under LD, some of the swc6 fve-3 double mutants were indistinguishable from swc6, although, on average, swc6 fve-3 produced a few more leaves than swc6 (Table 1); this result indicates that the late-flowering phenotype of fve mutations requires SWC6.

The quasi-epistatic interaction of swc6 with fve mutations suggests that swc6 might cause early flowering, either by increasing the activity of the autonomous pathway downstream of FVE or by bypassing the requirement for the autonomous pathway causing a reduction of FLC expression. When the swc6 mutation was introduced into FRI-containing Col (Col:FRI SF2, referred to as FRI below) (Michaels and Amasino, 1999), which displays a very late-flowering phenotype, swc6 partially suppressed FRI-mediated late flowering (Fig. 5B, Table 1). The plants harbouring the swc6 FRI combination showed much earlier flowering than FRI (Table 1).

In order to test if swc6 suppresses the effect of the autonomous pathway mutations and FRI by reducing FLC mRNA levels, the abundance of the FLC mRNA in wild-type and swc6 seedlings was compared. FLC transcript levels were reduced by the swc6 lesion (Fig. 5D), suggesting that SWC6 is required to maintain high FLC expression levels, either as promoted by FRI or by mutations that impair the autonomous pathway. In agreement with this scenario, the expression of the floral integrator genes FT and SOC1, normally repressed by FLC (Moon et al., 2003), was up-regulated in the swc6 mutants under SD conditions where the early-flowering phenotype of the mutant is more conspicuous (Fig. 5D).
Although the effects of swc6 mutations on flowering time are more readily observed in the late-flowering FRI and fve mutant backgrounds, as discussed above, the fact that swc6 mutants also flower earlier than the rapid-flowering wild-type strain Col (Fig. 2A, Table 1) suggests that, in addition to regulating FLC expression, SWC6 plays other roles in the control of flowering time. To determine the fraction of the swc6 early-flowering phenotype that is independent of FLC, the phenotypic effect of the swc6-1 mutation in an flc null (flc-3) background (Michaels and Amasino, 1999) was examined. When combined with flc-3, the swc6 mutation reduces the number of leaves produced by flc-3 (Fig. 5C, Table 1). In addition, as for ESD1, loss of function of SWC6 also resulted in down-regulation of some other members of the FLC/MAF gene family, particularly MAF4 and MAF5 (Fig. 5D), suggesting that these MAF genes represent additional regulatory targets of SWC6 and confirming that swc6 mutations have an FLC-independent effect on flowering time.

Genetic and physical interaction between SWC6 and ESD1/SUF3/ARP6

Previous observations indicate that the swc6 mutant displays a number of phenotypic characteristics similar to those of esd1 plants (Fig. 1) (Martin-Trillo et al., 2006). One possibility is that SWC6 and ESD1/SUF3/ARP6 may act in the same pathway or alternatively participate in different parallel pathways controlling similar processes. To investigate this aspect further, swc6 esd1 double mutants were generated. As shown in Fig. 6A, swc6 esd1 plants were indistinguishable from esd1 plants. The flowering time of swc6 esd1 double mutants was identical to that of esd1 plants (Table 1), and the expression of floral integrator genes in swc6 esd1 plants was in general similar to that observed for each parental mutant (Fig. 5D). In addition, vegetative and reproductive phenotypes of the swc6 esd1 double mutant were quite similar to those observed in esd1 mutants (Fig. 6B). Taken together, the above results are consistent with SWC6 and ESD1 acting in the same genetic pathway.

Since yeast homologues of these proteins are part of the SWR1 complex and several lines of evidence have suggested the existence of this complex in plants, a possible physical interaction between these Arabidopsis proteins was analysed by yeast two-hybrid assays. To do this, full-length SWC6 protein was expressed as bait, fused to the GAL4 DNA binding domain (GBD), and full-length ESD1/SUF3/ARP6 protein as the prey, fused to the GAL4 activation domain (GAD). As shown in Fig. 6C, yeast co-expressing the GAD–ESD1 and GBD–SWC6 fusion proteins were able to grow in selective medium without His plus 3-AT, due to the activation of the GAL1::HIS3 reporter gene. To confirm this interaction further, in vitro pull-down experiments using glutathione
S-transferase (GST)–SWC6 and in vitro–translated ESD1 protein were performed. As shown in Fig. 6D, GST–SWC6 was able to interact with ESD1, but not GST alone. Together, the results show interaction among SWC6 and ESD1, providing further evidence for the existence of a SWR1 complex in Arabidopsis.

**SWC6 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 (Schmitz and Amasino, 2007). Recent work has demonstrated the role of histone modification in the regulation of FLC expression through FRI, the autonomous, the vernalization, and the PAF1 pathways (He et al., 2003, 2004; Ausin et al., 2004; Bastow et al., 2004; Sung and Amasino, 2004; He and Amasino, 2005; 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, 2004; Ausin et al., 2004; Bastow et al., 2004; Sung and Amasino, 2004) and transcriptional regulation (Gendall et al., 2001; Sheldon et al., 2002). Because swc6 suppresses the late-flowering phenotype of...
five autonomous pathway mutants and FVE represses FLC transcription through a histone deacetylation mechanism, it was speculated that SWC6 could be required for the acetylation of histones necessary to activate FLC expression. In fact, another putative component of the SWR1 complex, ESD1/SUF3/ARP6, has been previously reported to be required for setting this epigenetic marker in FLC chromatin (Martin-Trillo et al., 2006).

To determine whether SWC6 promotes histone acetylation of the FLC chromatin, ChIP assays were performed (Fig. 7). Chromatin of Col, esd1-10, and swc6-1 plants was immunoprecipitated by using antibodies against acetylated H3, and PCR was used to amplify two DNA fragments spanning regions of the promoter and the first intron of FLC, respectively, from the precipitated chromatin (Fig. 7A). These probes were among those that consistently showed the biggest differences in previous experiments involving the swc6 mutant (Martin-Trillo et al., 2006). For the probes assayed, FLC-amplified sequences were consistently more abundant in DNA from precipitated chromatin of Col than from chromatin of the swc6 and esd1 mutant plants (Fig. 7B), indicating that both SWC6 and ESD1/SUF3/ARP6 affect the levels of H3 acetylation of FLC. Therefore, both proteins are required to activate FLC expression through a mechanism involving the histone acetylation of FLC chromatin.

This assay was extended to explore further if SWC6 also has an effect on histone methylation at the FLC locus, as does ESD1. It has been shown recently that H3K4 hypertrimethylation is associated with actively transcribed FLC chromatin (He et al., 2004), and we wondered whether SWC6 was required for the setting of this epigenetic marker on FLC chromatin. Compared with wild-type plants, the trimethylated H3K4 levels in the FLC probes assayed were lower in swc6 and esd1 mutant plants than in Col (Fig. 7C), indicating that SWC6 is also required for the hypertrimethylation of H3K4 in FLC chromatin. Although in swc6 background a decrease in both H3 acetylation and H3K4 trimethylation was consistently observed with the probes assayed, this effect was always less pronounced than that observed in esd1 mutants, suggesting a stronger involvement of ESD1 in these modifications as compared with SWC6.

Discussion

In Arabidopsis, flowering time is regulated by a complex genetic network where the floral repressor FLC has a pivotal role integrating the autonomous and vernalization pathways and down-regulating the expression of FT and SOC1 floral integrators (Searle et al., 2006). The expression level of these integrators is mainly responsible for the correct flowering time (Baurle and Dean, 2006). Transcriptional regulation of FLC is a central checkpoint in both winter and summer annual accessions of Arabidopsis. Recently, the regulation of FLC through chromatin modifications has been intensively demonstrated (reviewed by Reyes, 2006; Sung and Amasino, 2006).

In this work, Arabidopsis swc6 mutants that are affected in a putative orthologue of the SWR1 chromatin remodelling complex have been characterized. SWC6 is the only Arabidopsis gene homologue of yeast SWC6/VPS73 (Krogan et al., 2003; Kobor et al., 2004; Wu et al., 2005). Recently, the function of the ATP-dependent chromatin remodelling complex SWR1 has been intensively studied in yeast (Wu et al., 2005). The subunits
of SWR1 and of the mammalian homologue SRCAP complexes have been biochemically identified and analysed (Kobor et al., 2004; Mizuguchi et al., 2004; Cai et al., 2005; Wu et al., 2005), and the evidence for the presence of a homologous complex in plants has been provided (Choi et al., 2007; Deal et al., 2007). In addition, how SWR1 homologues affect development in higher eukaryotes remains largely unknown. Homologues of most SWR1 components are present in Arabidopsis, and thus the function of this complex is being genetically dissected.

Phenotypical analyses of swc6 mutants revealed a complex array of pleiotropic defects affecting vegetative and reproductive development, including a reduction in flowering time and phase length (Fig. 2). swc6 causes early flowering mainly through the reduction in FLC expression (Fig. 5), suggesting a role for the SWR1 complex in the regulation of flowering time. Genetic analyses have revealed that the early flowering phenotype of swc6 mutants is almost completely epistatic over the flowering time delay caused by fve mutation in the autonomous pathway, and that swc6 partially suppresses the late-flowering phenotype conferred by active FRI alleles (Fig. 5). These genetic interactions correlate at the molecular level with a decrease in the steady-state levels of FLC mRNA in swc6 mutant alleles (Fig. 5D). Together, these results indicate that SWC6 is required for the proper expression of FLC. The phenotypic analysis of swc6 mutants indicates that this protein also controls other developmental processes such as leaf and flower morphology (Fig. 2), suggesting that SWC6 regulates other genes involved in plant development. For instance, swc6 plants show smaller leaves with serrated margins, which might reflect defects in cell proliferation along the margins of leaf primordia. In addition, swc6 mutant flowers display extra floral perianth organs, suggesting a role for the SWR1 complex in the control of floral development.

Mutations in different Arabidopsis homologues of SWR1 components such as PIE1, ESD1/SUF3/ARP6, and SWC6/SEF cause similar developmental defects (Noh and Amasino, 2003; Choi et al., 2005, 2007; Martin-Trillo et al., 2006; March-Diaz et al., 2007; this study), and these genes display similar expression patterns, although SWC6 is more highly expressed in anthers (Fig. 4D). This may be explained by the presence of various cis-acting elements (AGAAA and GTGA) in the SWC6 promoter region, known to confer anther/pollen-specific gene expression (Gupta et al., 2007), and is consistent with a role for SWC6 in anther and/or pollen development (March-Diaz et al., 2007; this study) (Fig. 4C). All three mutants cause suppression of late flowering in autonomous pathway mutants as well as in FRI-containing lines and other developmental defects including leaf serration, weak apical dominance (bushy aspect), flowers with extra petals, and short siliques. They also show earlier flowering than an flc-null mutant, suggesting that flowering inhibition mediated by these proteins occurs through both FLC- and FLC-like gene-dependent pathways (Noh and Amasino, 2003; Choi et al., 2005, 2007; Martin-Trillo et al., 2006). However, some phenotypes are more dramatic in piel plants than in swc6/sef and esd1/suf3/arp6 plants (Noh and Amasino, 2003). piel displays a stronger reduction in fertility, a very notable reduction in primary inflorescence elongation, and smaller and deformed leaves (Noh and Amasino, 2003; March-Diaz et al., 2007), phenotypes that were not obvious in esd1/suf3/arp6 or in swc6/sef (Noh and Amasino, 2003; Choi et al., 2005, 2007; Deal et al., 2005; Martin-Trillo et al., 2006; March-Diaz et al., 2007). Moreover, piel plants show a stronger down-regulation of FLC and MAF4 transcript levels than the esd1 and swc6 plants (Deal et al., 2007; March-Diaz et al., 2007). Furthermore, the MAF3 gene was deregulated in the piel, esd1/suf3/arp6, and swc6/sef mutant (Fig. 5D; Martin-Trillo et al., 2006; March-Diaz et al., 2007). Altogether, these observations suggest that PIE1 might fulfill functions that are at least partially independent from those of SWC6 and ESD1; a tempting possibility is that PIE1 may participate in other chromatin remodelling complexes besides SWR1.

SWC6 and ARP6 yeast homologues are tightly associated in SWR1C, being necessary for the interaction with the SWC2 subunit and for nucleosome binding (Wu et al., 2005). According to this, Arabidopsis SWC6 and ESD1/SUF3/ARP6 have a similar developmental function. The phenotypes of swc6 and esd1 mutants are quite comparable, and the esd1 swc6 double mutant has the same phenotype and causes similar alterations in gene expression as any single mutant (Figs 5D, 6B), indicating that both genes act in the same genetic pathway. Together with the absence of any obvious developmental phenotype in plants overexpressing either ESD1 or SWC6, the results described in this study strongly suggest that ESD1 and SWC6 act together as a protein complex. The protein interaction analyses confirmed that both proteins can physically interact (Fig. 6C, D). Biochemical characterization of the yeast SWR1 complex indicates that removal of either arp6 or swc6 results in the reciprocal loss of the other subunit from the complex and also in the loss of two other proteins, Swc2 and Swc3, suggesting that Arp6, Swc6, Swc2, and Swc3 form a subcomplex associated with Swr1 (Wu et al., 2005). Similarly, Arabidopsis ARP6 and SWC6, together with SWC2 and other unidentified factors, may form a subcomplex that associates with PIE1 (Choi et al., 2007; March-Diaz et al., 2007). Again, this is consistent with a very similar phenotype of the swc6 and esd1/suf3/arp6 mutants but a slightly different phenotype of the piel mutant. The SWR1 complex in yeast catalyses the replacement of nucleosomal H2A with the H2A.Z variant, ensuring full activation of underlying genes (Guillemette et al.,
In promoter DNA (reviewed in Raisner and Madhani, 2006). Recent studies have shown that two H2A.Z nucleosomes flank a nucleosome-free region containing the transcription initiation site in promoters of both active and inactive genes in yeast and that H2A.Z-bearing nucleosomes facilitate transcription activation through their susceptibility to loss, thereby helping to expose promoter DNA (reviewed in Raisner and Madhani, 2006). In Arabidopsis, the histone variant H2A.Z has been identified within FLC, MAF4, and MAF5 chromatin, occupying regions near both the transcription start and termination sites on the three genes examined (Deal et al., 2007). In addition, H2A.Z interacts with both PIE1 and AtSWC2, and knockdown of H2A.Z caused a phenotype similar to that of pie1, esd1/suf3/arp6, and swc6 (Choi et al., 2007). Loss of H2A.Z from FLC chromatin in esd1/suf3/arp6 and pie1 mutants results in reduced FLC expression and premature flowering, indicating that this histone variant is required for a high level of expression of FLC (Deal et al., 2007). These observations support the existence of an SWR1-like complex in plants that is targeted to different loci including FLC, and show that H2A.Z can poise transcriptional activation in plants. Interestingly, the spatial distribution and the overall levels of H2A.Z on FLC was the same in samples that had a 10-fold higher level of FLC expression (Deal et al., 2007), suggesting that H2A.Z by itself does not activate FLC gene expression and that the replacement of nucleosomal H2A with H2A.Z may form a variant nucleosome with unique tails that might bind specific regulatory proteins to help promote FLC gene activation.

High levels of FLC expression are correlated with H3 and H4 hyperacetylation and trimethylation of H3K4 and H3K36 at the FLC locus (He et al., 2003, 2004; Ausin et al., 2004; Bastow et al., 2004; Sung and Amasino, 2004; Zhao et al., 2005). Martin-Trillo et al. (2006) have recently reported that esd1/suf3/arp6 mutants present low levels of histone H3 acetylation and H3K4 methylation in the FLC locus; this work demonstrates a comparable behaviour in the swc6 mutant, although the effect was consistently more conspicuous for esd1 (Fig. 7). Whether ARP6 and SWC6, and, consequently, the Swr1 complex are directly involved in setting these epigenetic markers or whether these alterations are secondary consequences is still unclear. Moreover, it remains to be determined whether the effect of esd1/suf3/arp6 and swc6 on the expression of other MAF genes takes place through similar mechanisms.

A human H2A.Z sequence variant to the yeast Swr1 complex has histone acetyltransferase activity (Owen-Hughes and Bruno, 2004), and the Swr1 complex shares several subunits with the NuA4 histone acetyltransferase. Furthermore, mutants of these two complexes share several phenotypes, suggesting that they may work together, which might help to understand the role of ESD1/SUFI3/ARP6 and SWC6 in histone acetylation. In the same way, the fact that components of the Swr1 complex were found to interact genetically with the PAF1 complex in yeast might explain the role of ESD1 and SWC6 in the trimethylation of H3K4 in FLC chromatin (Mueller and Jaehning, 2002; Squazzo et al., 2002; Krogan et al., 2003, 2004). Like the yeast PAF1 complex, the PAF1-like complex in Arabidopsis may also recruit an H3K4 methyl transferase to FLC to regulate its expression (Kim et al., 2005). Indeed, mutations in Arabidopsis homologues of the components of the PAF1 complex cause a decrease in the trimethylation of H3K4 in FLC chromatin, and provoke early flowering and small leaves, similar to the esd1 and swc6 mutations (He et al., 2004), raising the possibility that all of these genes are in the same pathway and regulate similar targets.

We propose that the H2A.Z variant may serve to poise the FLC gene, and maybe other related genes, in a state competent for activation by other factors, rather than activating transcription directly (Deal et al., 2007). This may reflect the ability of H2A.Z to facilitate nucleosome remodelling (Santisteban et al., 2000) and/or to recruit the transcription machinery (Adam et al., 2001) or other chromatin remodelling complexes to allow high-level transcription under certain conditions. Thus, in the absence of H2A.Z in swc6, esd1/suf3/arp6, and pie1 mutants, FLC levels remain low even in the presence of strong activators such as FRI (Noh and Amasino, 2003; Choi et al., 2005; Deal et al., 2005, 2007; Martin-Trillo et al., 2006; this study), resulting in early flowering.

Biochemical characterization of the SWR1C homologue and functional studies using transcriptomic analyses and ChIP-to-chip hybridization will help to identify additional genes regulated by this complex and to understand the crucial role of the SWR1 complex plant homologue in chromatin remodelling processes related to leaf and flower development and to the control of flowering time.

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