

SEF, a New Protein Required for Flowering Repression in Arabidopsis, Interacts with PIE1 and ARP6^{1[W]}

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The SWR1/SRCAP complex is a chromatin-remodeling complex that has been shown to be involved in substitution of histone H2A by the histone variant H2A.Z in yeast (*Saccharomyces cerevisiae*) and animals. Here, we identify and characterize SERRATED LEAVES AND EARLY FLOWERING (SEF), an Arabidopsis (*Arabidopsis thaliana*) homolog of the yeast SWC6 protein, a conserved subunit of the SWR1/SRCAP complex. SEF loss-of-function mutants present a pleiotropic phenotype characterized by serrated leaves, frequent absence of inflorescence internodes, bushy aspect, and flowers with altered number and size of organs. *sef* plants flower earlier than wild-type plants both under inductive and noninductive photoperiods. This correlates with strong reduction of FLOWERING LOCUS C and MADS-AFFECTING FLOWERING4 transcript levels and up-regulation of FLOWERING LOCUS T and SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 gene expression. The *sef* phenotype is similar to that of the *photoperiod-independent early flowering1* (*pie1*) and the *actin-related protein 6* (*arp6*) mutants. PIE1 and ARP6 proteins are also homologs of SWR1/SRCAP complex subunits. Analysis of *sef pie1* double mutants demonstrates genetic interaction between these two genes. We also show physical interactions between SEF, ARP6, and PIE1 proteins. Taken together, our data indicate that SEF, ARP6, and PIE1 might form a molecular complex in Arabidopsis related to the SWR1/SRCAP complex identified in other eukaryotes.

Massive reprogramming of transcription associated to cell differentiation during development involves activation and silencing of hundreds of genes. This process requires extensive changes in chromatin structure as it has been evidenced by the identification of chromatin-remodeling factors whose mutation impairs normal development at many different levels (e.g. Muller and Leutz, 2001; Margueron et al., 2005; Reyes, 2006). Three main biochemical mechanisms have been reported to alter chromatin structure. The first involves the posttranslational covalent modification of the amino- and carboxy-terminal tails of histones. The pattern of chemical modifications of histones within a nucleosome (acetylation, methylation, phosphorylation, ubiquitination, and SUMOylation) seems to constitute a code that can be interpreted by other nuclear machineries (Jenuwein and Allis, 2001). The second consists in the ATP-dependent reorganization of interactions between DNA and histones, which provokes the destabilization of the nucleosome structure (Smith and Peterson, 2005). A third mechanism of

chromatin remodeling lies in the substitution of canonical histones of the octamer by histone variants, which confers new stability and interactions to the nucleosome (Kamakaka and Biggins, 2005). The recently identified homologous SWR1 and SRCAP complexes from yeast (*Saccharomyces cerevisiae*) and human, respectively, contain two of these basic activities (Krogan et al., 2003; Kobor et al., 2004; Mizuguchi et al., 2004; Cai et al., 2005; Ruhl et al., 2006). Thus, the SWR1 and SRCAP complexes seem to use the energy of ATP hydrolysis to destabilize protein-protein and protein-DNA interactions within the nucleosome, resulting in the substitution of a histone H2A-H2B dimer by a H2A.Z-H2B dimer. Histone H2A.Z is a universally conserved histone variant that is transcribed in a cell cycle-independent way and that is involved in transcription regulation and genome stability (for review, see Raisner and Madhani, 2006). Mutations in genes encoding components of the SWR1 complex result in identical phenotypes with null mutations in the *HTZ1* gene (structural gene for H2A.Z in yeast), indicating that the SWR1 complex is uniquely responsible for histone H2A.Z deposition in yeast (Kobor et al., 2004; Mizuguchi et al., 2004). The catalytic subunits of yeast and human complexes are the SNF2 family DNA-dependent ATPases Swr1 (Swi2/Snf2-related 1) and SRCAP (SNF2-related CBP activator protein), respectively. In addition to the ATPase, 10 to 13 subunits have been shown to co-purify with the SWR1 and SRCAP complexes, but their functions in remodeling activity remains unclear at present (Cai et al., 2005; Wu et al., 2005). SWR1 and SRCAP complexes share subunits with the yeast NuA4 and

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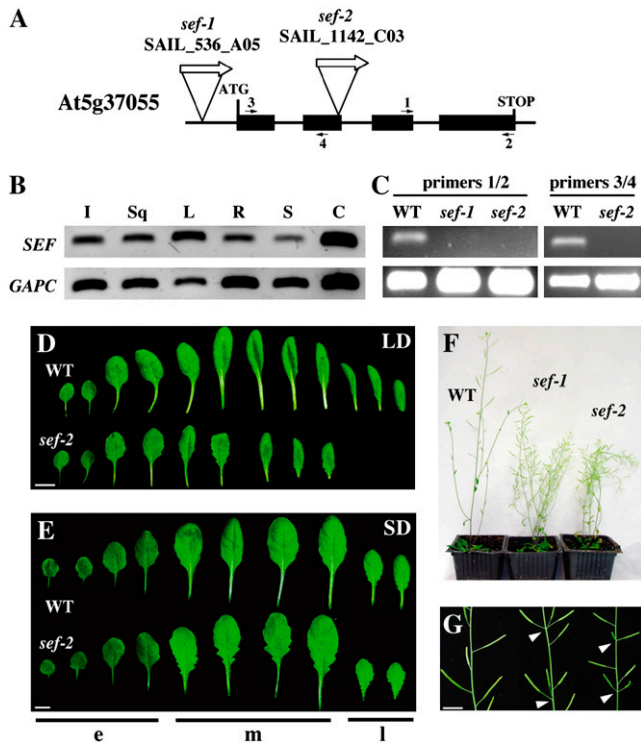


Figure 1. *SEF* loss-of-function mutants display several developmental abnormalities. A, Molecular structure of the *SEF* locus and sites of T-DNA integration. Primers used for RT-PCR experiments are indicated as small arrows (1, 2, 3, and 4). B, *SEF* expression in different plant organs. RT-PCR analysis was performed with RNA isolated from adult plants grown in LD. *GAPC* transcript was determined as a loading control. I, Inflorescence; Sq, siliques; L, leaves; R, roots; S, seedlings; C, calli. C, RT-PCR analysis of the amount of *SEF* mRNA found in Columbia wild-type, *sef-1*, and *sef-2* plants. Sequence of primers 1, 2, 3, and 4 is provided in Supplemental Table S1. D, Rosette leaves of wild-type and *sef-2* plants grown under LD conditions 27 d after sowing. All rosette leaves are shown in order of production from the first true leaf at left. E, Rosette leaves of wild-type and *sef-2* plants grown under SD condition 52 d after sowing. Black bars indicate early (e), middle (m), and late (l) stage leaves. F, Forty-day-old wild-type, *sef-1*, and *sef-2* plants grown in soil under LD conditions. G, Inflorescences of wild type (left) and *sef-2* plants (middle and right). Arrowheads point to abnormal cluster of siliques on the *sef-2* mutant inflorescences. Scale bars: D, E, and G, 1 cm.

the human TRRAP/TIP60 acetyltransferase complexes, respectively (Kobor et al., 2004; Mizuguchi et al., 2004; Cai et al., 2005). Furthermore, a Tip60 complex has been also purified from *Drosophila* and human that seems to be the fusion of SRCAP and the TRRAP/TIP60 complexes (Cai et al., 2003; Doyon et al., 2004; Kusch et al., 2004), which links histone H2A.Z replacement and histone acetylation.

Two recently characterized *Arabidopsis* (*Arabidopsis thaliana*) genes encode proteins that show clear similarities with subunits of the SWR1 and SRCAP complexes. *PHOTOPERIOD-INDEPENDENT EARLY FLOWERING1* (*PIE1*) was originally identified as a suppressor of *FRIGIDA*-dependent late flowering. Thus, *PIE1* is an activator of the *FLOWERING LOCUS*

C (*FLC*) gene, a repressor of the transition from vegetative to reproductive phase. *PIE1* encodes a putative DNA-dependent ATPase of the SNF2 family (Noh and Amasino, 2003) closely related to yeast Swr1, human SRCAP, and *Drosophila* Domino proteins. Another protein of the SWR1 and SRCAP complexes is ACTIN-RELATED PROTEIN 6 (ARP6). Interestingly, mutations in the *Arabidopsis* *ARP6* ortholog gene (also called *SUF3* and *ESD1*) provoke developmental abnormalities that are similar to those displayed by *pie1* mutants, including early flowering and reduced levels of *FLC* expression (Choi et al., 2005; Deal et al., 2005; Martin-Trillo et al., 2006). Based on these phenotypic similarities, it has been suggested that *PIE1* and *ARP6* proteins may form part of a SWR1-like complex in plants, although physical or genetic interaction between these two factors has not been reported (Choi et al., 2005; Deal et al., 2005; Meagher et al., 2005; Martin-Trillo et al., 2006). Here, we identify *SERRATED LEAVES AND EARLY FLOWERING* (*SEF*) as a new positive regulator of *FLC*. Genetic and molecular approaches indicate that *SEF* forms a complex with *PIE1* and *ARP6*. Therefore, our data strongly support the existence in *Arabidopsis* of a SWR1/SRCAP-like complex similar to those identified in yeast and humans.

RESULTS

SEF Is Required for Normal Vegetative and Reproductive Development

A search for *Arabidopsis* protein homologs of yeast SWR1 and human SRCAP complex subunits led us to the identification of a protein related to the yeast Swc6 and to the human ZnF/HIT1 proteins (Mizuguchi et al., 2004; Cai et al., 2005; Supplemental Fig. S1), which we called *SEF*. This protein, encoded by the At5g37055 locus (Fig. 1A), was also closely related to *Nicotiana benthamiana* CIBP1, originally identified as a *Plum pox virus* cylindrical inclusion-interacting protein (accession no. AAW83129). This family of factors is characterized by the presence of a HIT-type zinc finger domain (pfam04438) in the carboxy terminus of the protein. The HIT-type zinc finger contains seven conserved Cys and one His that can potentially coordinate two zinc atoms (Supplemental Fig. S1). Semiquantitative reverse transcription (RT)-PCR experiments demonstrated that *SEF* transcript was present in all tested tissues (Fig. 1B). To investigate the role of *Arabidopsis* *SEF* in plant development, we decided to look for *sef* null mutants. A search for T-DNA insertion lines in different collections was carried out, and two different lines were originally identified from the SAIL collection (Fig. 1A) that we designed as *sef-1* and *sef-2*. Line SAIL_536_A05 (*sef-1*) contained a single T-DNA inserted in the putative promoter region, 30 bp upstream of the first nucleotide of the available largest cDNA and 83 bp upstream of the first translated nucleotide. RT-PCR analysis was unable to detect *SEF* mRNA in

homozygous *sef-1* plants (Fig. 1C). Line SAIL_1142_C03 (*sef-2*) had a single insertion in exon 2 of the At5g37055 locus, corresponding to nucleotide 200 of the coding region. RT-PCR analysis, using primers upstream and downstream of the T-DNA insertion point, demonstrated the absence of *SEF* mRNA in homozygous *sef-2* plants (Fig. 1C).

sef-1 and *sef-2* plants presented identical phenotypes and will be described simultaneously. Heterozygous plants displayed a wild-type phenotype indicating that *sef-1* and *-2* were recessive mutations. The progeny of self-fertilized heterozygous plants followed a normal Mendelian segregation. Homozygous plants exhibited different leaf development alterations depending on the photoperiod regime. Under long-day conditions (LD), *sef* leaves were smaller than those of the wild type and presented serrated margins. In contrast, under short days (SD) mutant leaf size was almost identical to that of the wild-type, but serrated phenotype was more severe (Fig. 1, D and E). Interestingly, a similar serrated phenotype has been described in *pie1* and *arp6* plants under SD conditions (Noh and Amasino, 2003; Choi et al., 2005; Deal et al., 2005; Martin-Trillo et al., 2006). The rate of leaf initiation was almost identical in *sef* mutants and wild type, both in LD and SD (data not shown).

Then we examined the development of inflorescence and flowers. Mutant plants produced 2 to 3 times more coflorescence shoots than the wild type. This was accompanied by a shortening or absence of inflorescence internodes, resulting in a reduction of inflorescence length and thereby in a bushy appearance of mutant plants (Fig. 1, F and G). As shown in Figure 2 and Table I, *sef* flowers displayed several developmental abnormalities. They were smaller than wild-type flowers (Fig. 2A). Petals of mutant plants were about 25% smaller than wild-type petals and slightly wrinkled (Fig. 2C). In addition, mutant flowers often presented extra petals. This phenotype was more prominent in the first arising flowers under SD conditions, where extra sepals were also observed (Fig. 2B; Table I). Stamens were shorter than carpels (Fig. 2I), and most mutant flowers presented altered number of these organs (four or five), both under SD and LD. In contrast to the wild type, all mutant stamens presented the same length. Mutant anthers were smaller than those of the wild type and often presented a heart shape typical of immature anthers. The number of pollen grains was severely reduced, but its morphology and nuclei distribution was found normal after 4',6-diamino-phenylindole (DAPI) staining and microscopic observation (Fig. 2, F–H; data not shown). *sef* gynoeciums were shorter and irregularly thickened in comparison to those of wild-type plants (Fig. 2D). Mutant siliques were also shorter and thicker (Fig. 2E) and contained unfertilized ovules or white aborted seeds (Fig. 2, J and K), especially under SD conditions. In addition, distribution of seeds was more crowded in mutant siliques than in the wild-type siliques, which may explain the abnormal shape of many adult mu-

tant seeds. The average number of seeds per mutant silique was 42.4 ± 4.4 in LD and 21.5 ± 9 in SD, while wild-type siliques produced 62.2 ± 0.8 and 55.7 ± 2.2 in LD and SD, respectively (Table I). The partial reduction in fertility observed in *sef* plants may be a consequence of the small amount of pollen grains produced by stamens, especially under SD conditions. Otherwise, it could be due to reduction in stamen height.

Altogether, the phenotype of *sef* plants was strikingly similar to that of the *pie1* and *arp6* mutants (Noh and Amasino, 2003; Choi et al., 2005; Deal et al., 2005; Martin-Trillo et al., 2006; and see below).

SEF Represses Flowering by Positively Regulating *FLC* and *MAF4*

To explore the role of SEF in the transition from vegetative to reproductive development, we determined the flowering time of wild-type and mutant plants both under SD and LD conditions. Because *pie1* mutants display similar morphological alterations to *sef* mutants and *pie1* plants show an early flowering phenotype, we have also determined the flowering time of a *pie1-5* mutant for comparison. Flowering time was recorded as the number of rosette leaves at the time of bolting. As shown in Table II and Figure 3, A and B, *sef* mutants flowered with fewer leaves and earlier than the wild type. Thus, *sef* plants flowered in

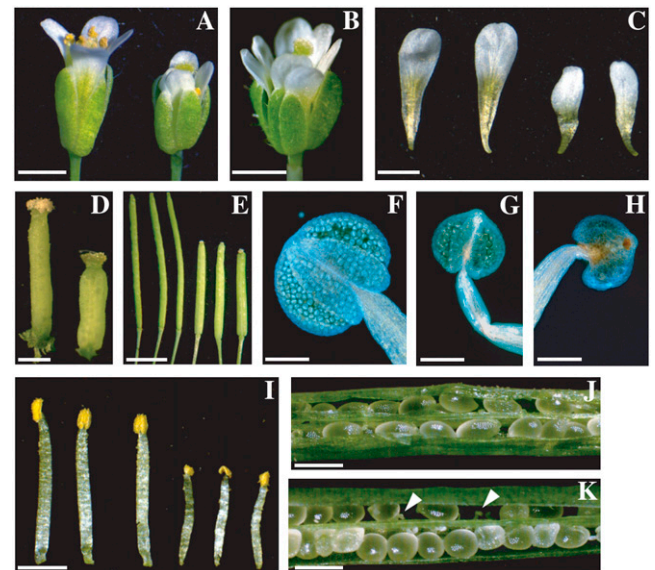


Figure 2. Flower morphology of *sef* mutant plants. Plants were grown under LD (A, C–K) or under SD (B) conditions. A, Side view of wild-type (left) and *sef-2* (right) flowers. B, Supernumerary petals and sepals in *sef-2* flowers. C, Wild-type petals (left) and short and wrinkled *sef-2* petals (right). D, Wild-type (left) and *sef-2* (right) flower gynoecium. E, Wild-type siliques (left) and *sef-2* siliques (right). F, DAPI staining of a wild-type anther. G and H, DAPI staining of *sef-2* anthers. I, Wild-type (left) and *sef-2* (right) stamens. J, Open siliques of self-fertilized wild-type plants. K, Open siliques of self-fertilized homozygous *sef-2* plants. Arrowheads indicate aborted ovules. Scale bars: A and C, 1 mm; E, 0.5 cm; D, I to K, 0.5 mm; F to H, 0.2 mm.

Table I. Phenotypic characteristics of *sef-2* mutant flowers

Variable	LD		SD		
	WT (n = 15) ^a	<i>sef-2</i> (n = 15)	WT (n = 15)	<i>sef-2</i> Early Flowers ^b (n = 10)	<i>sef-2</i> Late Flowers ^c (n = 10)
No. of sepals	4.0 ± 0.0 ^d	4 ± 0.0	4.0 ± 0.0	5.4 ± 0.5	4.4 ± 0.9
No. of petals	4.0 ± 0.0	4.3 ± 0.5	4.0 ± 0.0	5.9 ± 1.0	4.0 ± 0.9
No. of stamens	5.9 ± 0.3	4.4 ± 0.5	5.9 ± 0.1	5.5 ± 0.7	4.9 ± 0.3
No. of nondehiscent stamens per flower	0.0 ± 0.0	1.9 ± 0.2	0.0 ± 0.0	3.2 ± 2.3	0.6 ± 1.3
Petal length (mm)	2.9 ± 0.1	2.3 ± 0.2	3.0 ± 0.1	2.9 ± 0.1	3.0 ± 0.1
Seeds per silique	62.2 ± 0.8	42.4 ± 4.4	55.7 ± 2.2	n.d. ^e	21.5 ± 9.0
No. of aborted seeds per silique	1.2 ± 1.0	8.8 ± 4.0	2.1 ± 1.0	n.d.	21.2 ± 7.9

^an, Number of plants analyzed. ^bThe first five arising flowers were scored. ^cFlowers arising after the fifth flower were scored. ^dNumbers are means ± SD. ^en.d., Not determined.

LD with approximately six leaves, while wild-type plants flowered with about 12 leaves. Under SD conditions, *sef* plants flowered with around 21 leaves, whereas the wild type flowered with 62 leaves, approximately. *pie1-5* plants flowered at a similar time and with a similar number of leaves to *sef* plants. Both *sef* and *pie1-5* plants flowered earlier under LD than under SD conditions, indicating that they retained some sensitivity to photoperiod.

We then investigated changes in gene expression that might be responsible for the early flowering phenotype of the *sef* mutant. Transcript levels were determined by semiquantitative RT-PCR. Analyses were performed on 10- and 12-d-old or 12- and 15-d-old seedlings for LD or SD conditions, respectively. Transcript levels of the photoperiod pathway gene *CONSTANS* (*CO*; Putterill et al., 1995), the floral repressor *FLC* (Michaels and Amasino, 1999), and the flowering integrators *FLOWERING LOCUS T* (*FT*) and *SUPPRESSOR OF OVEREXPRESSION OF CO 1* (*SOC1*; Samach et al., 2000) were determined. As a control, we also analyzed transcript levels in *pie1-5* plants. As shown in Figure 3C, levels of the *CO* transcript were not significantly altered in the *sef-2* or *pie1-5* mutants. In contrast, *FLC* transcript levels in *sef-2* plants were reduced compared to wild-type plants. For instance, a 10- to 25-fold reduction was observed under SD conditions. It has been shown that the flowering positive

regulators *FT* and *SOC1* are negatively controlled by *FLC* (Samach et al., 2000; Michaels et al., 2005). As expected, reduced levels of the *FLC* transcript correlated with an increase in transcript levels of these two genes under both SD and LD conditions. A similar, although more severe, deregulation of *FLC*, *FT*, and *SOC1* was also observed in *pie1-5* plants (Fig. 3C; Noh and Amasino, 2003). Analogous deregulation of these three genes has also been shown in *arp6* mutant plants (Choi et al., 2005; Deal et al., 2005; Martin-Trillo et al., 2006). It has been reported that several *FLC* paralogs of the *MADS-AFFECTING FLOWERING* (*MAF*) gene family act as flowering repressors (Ratcliffe et al., 2003). Several members of this gene family are down-regulated in *arp6* plants (Deal et al., 2005; Martin-Trillo et al., 2006). Figure 3C shows that expression of the *MAF4* gene was also severely reduced in *sef* and *pie1-5* plants. *MAF1*, *MAF2*, and *MAF3* transcript levels were not altered in the mutant strains. Interestingly, *MAF5* mRNA levels were strongly reduced in *pie1-5* but not in *sef* plants (Fig. 3C), indicating that not all genes deregulated in the *pie1-5* mutant were also affected in the *sef* background.

SEF Genetically Interacts with PIE1

Our data indicate that the *sef* mutant displays a number of phenotypic characteristics similar to those

Table II. Bolting time of wild-type and *sef/pie1* mutant plants^a

Plant	LD		SD	
	Leaf No. ^b	Days ^c	Leaf No.	Days
Columbia (n = 16) ^d	12.4 ± 0.5	27.7 ± 0.5	62.0 ± 3.7	65.2 ± 2.4
<i>sef-1</i> (n = 16)	6.1 ± 0.5	21.4 ± 0.8	21.3 ± 1.8	48.4 ± 1.5
<i>sef-2</i> (n = 15)	5.7 ± 0.5	21.1 ± 0.9	21.4 ± 2.9	46.6 ± 2.6
<i>pie1-5</i> (n = 16)	5.4 ± 0.7	20.2 ± 0.9	21.0 ± 2.7	47.7 ± 1.2
<i>sef-2</i> +/- <i>pie1-5</i> +/- (n = 31)	8.0 ± 1.6	22.9 ± 1.3	47.9 ± 6.2	56.6 ± 2.7
<i>sef-2</i> -/- <i>pie1-5</i> -/- (n = 14)	5.2 ± 0.6	20.6 ± 0.9	20.6 ± 1.4	47.4 ± 2.4

^aData of the *sef* and *pie1* single and double mutants were significantly different from those of the wild type with a Student's *t* value of *P* < 0.0001. Data of the *sef-2*+/- *pie1-5*+/- plants were significantly different from those of the wild-type, single, and double homozygous mutants with a Student's *t* value of *P* < 0.0001. ^bNumber of rosette leaves at bolting ± SD. ^cNumber of days from planting to bolting ± SD. ^dn, Number of plants analyzed.

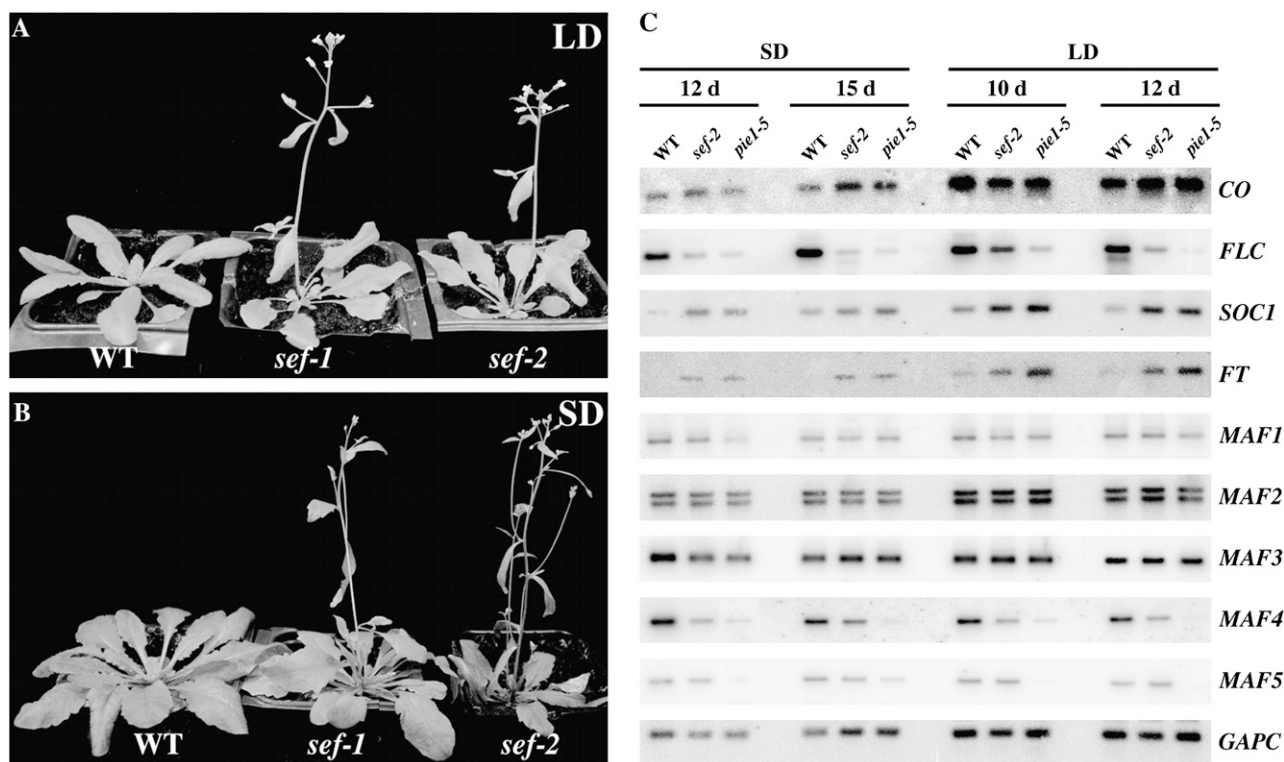


Figure 3. Flowering of *sef* mutants and expression of flowering time control genes in *sef* and *pie1* mutants. A, Twenty-five-day-old wild-type and *sef* plants grown under LD conditions. B, Sixty-two-day-old wild-type and *sef* plants grown under SD conditions. C, Semiquantitative RT-PCR analysis of the expression of flowering time control genes *CO*, *FLC*, *SOC1*, *FT*, *MAF1*, *MAF2*, *MAF3*, *MAF4*, and *MAF5*. Total RNA was isolated from seedlings collected 9 h after dawn, at 12 and 15 d of growth under SD conditions or at 10 and 12 d of growth under LD. GAPC transcript levels were also determined as a control for the amount of input cDNA.

of *pie1* plants (Noh and Amasino, 2003). One possibility is that SEF and PIE1 belong to different parallel pathways controlling similar processes; in contrast, SEF and PIE1 may act in the same pathway. To further investigate this aspect, we generated *sef-2 pie1-5* double mutants. As shown in Figure 4, A to D, *sef-2*^{-/-} *pie1-5*^{-/-} plants were indistinguishable from *pie1-5*^{-/-} plants, suggesting that SEF and PIE1 act in the same pathway. Time of flowering of *sef-2*^{-/-} *pie1-5*^{-/-} double mutants was also identical to that of *pie1-5*^{-/-} plants (Table II). Interestingly, double heterozygous *sef-2*^{+/-} *pie1-5*^{+/-} plants presented similar alterations to those observed in the *sef-2*^{-/-} mutants and never observed in the single heterozygous plants, such as shortened inflorescence internodes, flowers with five stamens, and a reduction in flowering time (Fig. 4, E–F; Table II; data not shown). These results suggest that the haploinsufficiency of *PIE1* behaves as an enhancer mutation of the *sef-2*^{+/-} phenotype.

SEF Physically Interacts with ARP6 and PIE1

Taken together, the above results suggest that SEF and PIE1 might act in the same genetic pathway or form part of the same molecular complex. Therefore, we decided to investigate whether these proteins phys-

ically interacted. Given that mutations in *SEF* provoke similar phenotypes to those in *PIE1* or *ARP6*, and *ARP6* has been proposed to associate with *PIE1* in a SWC/SRCAP-like complex in Arabidopsis (Choi et al., 2005; Deal et al., 2005; Martin-Trillo et al., 2006), we also analyzed interaction with *ARP6*. To this end, we performed in vitro pull-down experiments using glutathione *S*-transferase (GST)-SEF and GST-*ARP6* recombinant proteins and two in vitro-translated *PIE1* truncated polypeptides. As shown in Figure 5A, both GST-SEF and GST-*ARP6* proteins were able to interact with *PIE1*_{522–1190} and *PIE1*_{813–1099} proteins, suggesting that SEF and *ARP6* proteins interact with the region comprised between the SNF2_N (pfam00176) and the HELICc (pfam00271) domains of *PIE1*. This is consistent with previous results from yeast in which deletion of a region between the SNF2_N and the HELICc domains of Swr1 resulted in loss of several subunits from the complex, including Arp6 and Swc6, the yeast homologs of the *ARP6* and *SEF* proteins, respectively (Wu et al., 2005).

Then, we analyzed the SEF-*ARP6* interaction. Figure 5B shows that a GST-SEF recombinant protein interacted with a HA-tagged *ARP6* protein. To further confirm this interaction, we turned to the two-hybrid analysis. Full-length SEF protein was expressed as a

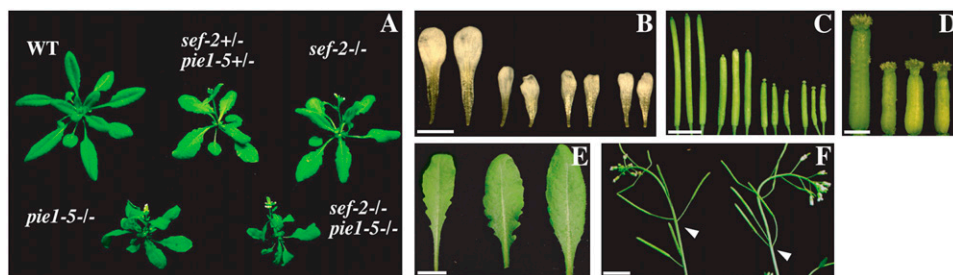


Figure 4. Genetic interaction of *SEF* and *PIE1*. A, Phenotype of 28-d-old wild-type Columbia, double heterozygous *sef-2+/- pie1-5+/-*, single *sef-2*, and *pie1-5* mutants and double *sef-2 pie1-5* mutant plants grown under LD conditions. B to D, Petals (B), siliques (C), or carpel (D) of wild-type (left), single *sef-2* (middle left), single *pie1-5* (middle right), and double *sef-2 pie1-5* (right) mutants. E, Phenotype of middle stage rosette leaves of *sef-2* (left), *sef-2+/- pie1-5+/-* (middle), and wild-type (right) plants grown in LD. F, Inflorescences of double heterozygous *sef-2+/- pie1-5+/-* plants. Arrowheads point to abnormal clusters of siliques. Scale bars: B, 1 mm; C, 0.5 cm; D, 0.5 mm; E and F, 1 cm.

bait in a fusion with the GAL4 DNA binding domain (GBD) and full-length ARP6 protein as the prey, fused to the GAL4 activation domain (GAD). As shown in Figure 5C, yeast cells co-expressing the GAD-ARP6 and GBD-SEF fusion proteins were able to grow in selective medium without His, due to the activation of the *GAL1::HIS3* reporter gene.

Therefore, these results indicate that SEF interacts with ARP6 and that both proteins are also able to interact with PIE1.

DISCUSSION

In this study, we have identified a new factor, SEF, which represses flowering at least in part by positively regulating the expression of the *FLC* and *MAF4* genes. The phenotypic analysis of *sef* mutants indicates that this protein also controls other developmental processes such as leaf and flower morphology, indicating that SEF regulates other genes involved in plant development. One of the most prominent abnormalities of *sef* plants, especially under SD conditions, is the presence of leaves with serrated margins. Serrated leaves are a symptom of defects in cell proliferation along the margins of leaf primordia. For instance, overexpression of cyclin-dependent kinase inhibitors results in serrated leaves (De Veylder et al., 2001). This suggests that SEF might play a role in the control of cell cycle progression or cell proliferation. Mutations in the *SERRATE* (*SE*) gene also provoke serrated leaf morphology. Interestingly, *se* mutants show other phenotypic similarities with *sef* mutants, such as irregular length or absence of internodes between adjacent flowers, flowers with extra sepals and petals, and fewer stamens (Prigge and Wagner, 2001). Furthermore, *SE* is a positive regulator of *FLC*, and *se* mutations are suppressor of FRIGIDA-mediated late flowering (Bezerra et al., 2004). Unlike *sef*, *se* mutants display alterations in the rate of leaf production and the number of juvenile leaves. RT-PCR assays showed that expression of *SE* was not altered in *sef* mutants (data not shown). *SE* encodes a C2H2-type zinc-finger

protein that has been suggested to regulate gene expression by modification of chromatin structure (Prigge and Wagner, 2001). Therefore, one possibility is that SEF cooperates with SE to control gene expression at the chromatin level. However, further investigation is required to verify this hypothesis.

SEF is the only Arabidopsis protein homolog of Swc6, a component of the yeast SWR1 complex (Krogan et al., 2003; Kobor et al., 2004; Wu et al., 2005). The catalytic core of the yeast complex is Swr1, an ATPase of the SNF2 family. In addition to Swr1 and Swc6, 12 other subunits co-purify with the SWR1 complex. One of these proteins is Arp6, an actin-related protein conserved from yeast to humans. The closest Arabidopsis homologs of Swr1 and Arp6 are the *FLC* gene activators PIE1 and ARP6, respectively (Noh and Amasino, 2003; Choi et al., 2005; Deal et al., 2005; Martin-Trillo et al., 2006). Here, we present results that strongly support that PIE1, ARP6, and SEF act in the same pathway, probably forming a molecular complex similar to the SWR1 complex. This conclusion is supported by the following data. First, *sef*, *arp6*, and *pie1* mutants present obvious phenotypic similarities, such as leaf and flower morphology, bushy aspect, and down-regulation of *FLC* and *MAF4* genes. Second, mutations in *pie1* are epistatic to mutations in *sef*, indicating that both genes act in the same genetic pathway. Third, combined haploinsufficiency of *sef* and *pie1* provokes a phenotype similar to that of *sef* homozygous plants. Fourth, PIE1 physically interacts with SEF and ARP6, and SEF also interacts with ARP6.

The absence of SEF, PIE1, or ARP6 does not result in identical abnormalities. Thus, some phenotypes are more dramatic in *pie1* plants than in *sef* and *arp6* plants. For instance, while the three single mutants present almost identical flower characteristics (Fig. 5; Noh and Amasino, 2003; Choi et al., 2005; Deal et al., 2005; Martin-Trillo et al., 2006), the *pie1* mutants in Columbia background display a stronger reduction of fertility, a very remarkable reduction of primary inflorescence elongation, and smaller and deformed leaves (Noh and Amasino, 2003; this article). *pie1* plants present a stronger down-regulation of *FLC* and *MAF4* transcript

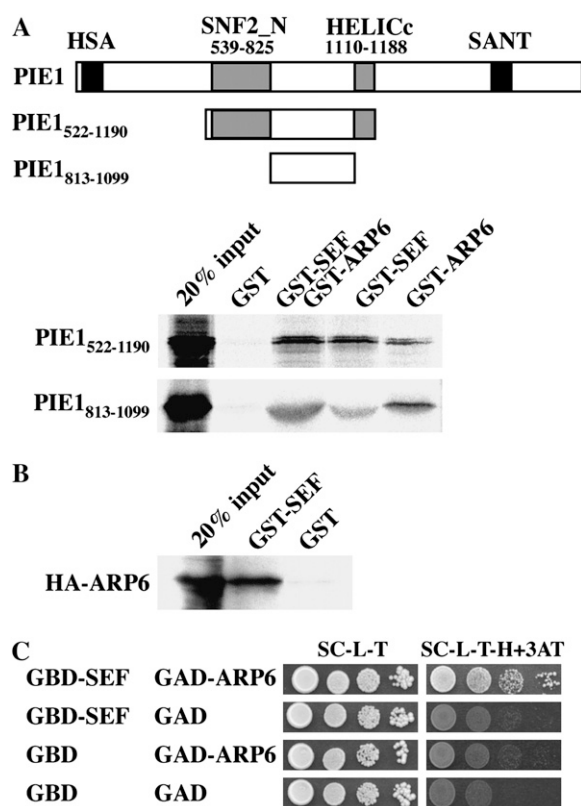


Figure 5. SEF, ARP6, and PIE1 proteins interact in vitro. **A**, Schematic representation of truncated PIE1 proteins (top) used in pull-down assays (bottom). GST, GST-SEF, and GST-ARP6 fusion proteins bound to glutathione Sepharose 4B beads were incubated separately or combined as indicated with ³⁵S-Met-labeled, in vitro-translated, PIE1 truncated proteins. **B**, Beads-bound GST or GST-SEF fusion proteins were incubated with HA-tagged ARP6. Retained protein was revealed with an anti-HA antibody. Twenty percent of the input ³⁵S-Met-labeled protein (**A**) and the HA-tagged ARP6 (**B**) are also shown. **C**, Full-length SEF and ARP6 proteins were fused to GAL4 DNA binding and activation domains, respectively (GBD-SEF, GAD-ARP6). Yeast transformed with these constructs or empty vectors (GBD, GAD) as indicated, were grown in nonselective (SC-L-T) or selective media with 25 mM 3AT (SC-L-T-H + 3AT).

levels than the *sef* plants (Fig. 3C). Furthermore, we found that the *MAF5* gene was deregulated in the *pie1-5* mutant but not in the *sef-2* mutant. This is consistent with the fact that *PIE1* encodes a DNA-dependent ATPase that may constitute the enzymatic core of the putative PIE1 complex. We rationalize that the absence of the enzymatic core of the complex should provoke a stronger phenotype than the absence of accessory subunits. The finding that *sef pie1* double mutants show identical phenotype to single *pie1* mutants suggests that the putative complex is inactive in the absence of the ATPase, and, therefore, inactivation of additional subunits does not have further consequences. Biochemical characterization of the yeast SWR1 complex indicates that removal of either *arp6* or *swc6* in single-mutant strains 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 to Swr1 (Wu et al., 2005). Similarly, Arabidopsis ARP6 and SEF, together with other not-yet identified factors, may form a subcomplex that associates to PIE1. Again, this is consistent with a very similar phenotype of the *sef* and *arp6* mutants but a slightly different phenotype of the *pie1* mutant.

We show that SEF is required to obtain the maximum expression level of the *FLC* gene. Similar results have been shown for ARP6 and PIE1 proteins (Noh and Amasino, 2003; Choi et al., 2005; Deal et al., 2005; Martin-Trillo et al., 2006). 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 *arp6* mutants present low levels of histone H3 acetylation and H3K4 methylation in the *FLC* locus; however, whether ARP6 is directly involved in setting these epigenetic marks or whether these alterations are secondary consequences is unclear. Therefore, how the putative PIE1 complex may control gene expression of the *FLC* gene remains unknown at present. The yeast SWR1 complex catalyzes the replacement of histone H2A for the histone variant H2A.Z. Histone H2A.Z has been associated with gene activation and limiting of telomeric silencing in yeast, and heterochromatin formation and chromosome stability in metazoans (for review, see 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. A recent phylogenetic analysis of the histone H2A gene family in Arabidopsis suggests the existence of at least three genes, HTA8, HTA9, and HTA11, which cluster together with H2A.Z variants from other organisms (Yi et al., 2006). Therefore, one obvious possibility is that SEF, ARP6, and PIE1 are required to catalyze the exchange of the canonical histone H2A for some of these H2A.Z variants in the *FLC* promoter and regulatory regions, which may facilitate the recruiting of other chromatin factors or the general transcriptional machinery.

MATERIALS AND METHODS

Plants and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) wild type and mutants (Columbia ecotype) were grown in LD (16 h light/8 h dark) or SD (10 h light/14 h dark) conditions (130 $\mu\text{E m}^{-2} \text{s}^{-1}$) at 23°C (day)/20°C (night) and 70% relative humidity. The T-DNA insertion mutant SALK_096434 was obtained from the SALK collection (<http://signal.salk.edu/>; Alonso et al., 2003). It consisted of a T-DNA insertion at exon 9 of the *PIE1* gene. RT-PCR analysis confirmed the lack of wild-type *pie1* mRNA in the homozygous line. As other *pie1* mutant

alleles have already been described (Noh and Amasino, 2003), we named this new allele *pie1-5*. The T-DNA insertion mutants *sef-1* (SAIL_536_A05) and *sef-2* (SAIL_1142_C03) were obtained from the Syngenta Arabidopsis Insertion Library. Lines were genotyped by PCR. DNA for PCR was extracted from leaves as previously described (Murray and Thompson, 1980). Two specific primers or one specific primer and a T-DNA left border A (LBA) primer were used for amplification of wild-type or T-DNA insertion alleles, respectively (LBA SALK, 5'-TGGTTCACGTAGTGGGCCATCG-3'; LBA SAIL, 5'-TTC-ATAACCAATCTCGATACAC-3'). T-DNA borders were determined by sequencing PCR products obtained with T-DNA border primers and gene-specific primers. Mutant lines were backcrossed twice to wild-type Columbia before analysis of phenotypes. The double mutant *sef-2 pie1-5* was generated by pollinating *sef-2* flowers with pollen from *pie1-5* plants.

Gene Expression Analysis

RNA was isolated using the RNeasy Plant Mini kit (Qiagen). cDNA was synthesized from 5 μ g of total RNA with the SuperScript First-Strand Synthesis system for RT-PCR (Invitrogen). One-tenth of the reaction was used for PCR amplification with specific primers spanning an intron to control for DNA contamination. Fifteen to 25 cycles were typically used, and products were detected by Southern blot. Number of cycles was set up for each experiment to keep amplification within the quantitative range. For quantification of radioactive areas, an InstantImager Electronic Autoradiography apparatus (Packard Instrument) was used. Specific primers for *SEF*, *CO*, *FT*, *SOC1*, *FLC*, *GAPC* (glyceraldehyde-3-phosphate dehydrogenase), and *MAF1-MAF5* are described in Supplemental Table S1.

Yeast Two-Hybrid

Yeast (*Saccharomyces cerevisiae*) two-hybrid interaction analyses were conducted in the MaV203 strain with the PROQUEST two-hybrid system (Invitrogen). pDBLeu or pPC86 vectors were used for GBD or GAD fusion constructs, respectively. cDNAs for *SEF*, *ARP6*, and *PIE1* were obtained by standard PCR techniques and cloned into the *Sall*-*NotI* sites of the above vectors. Selection was performed on synthetic complete (SC) minimal medium without His, Leu, and Trp (Bio101 Systems), supplemented with 10 to 50 mM 3-amino-1,2,4-triazole.

Protein Expression, Purification, and Pull-Down Assays

All expression constructs were prepared in the pGEX-6P-3 vector (Amersham Biosciences). Standard PCR techniques were used for HA tagging of ARP6. Constructs were expressed in *Escherichia coli* DH5 α . Proteins were purified on glutathione 4B Sepharose beads (Amersham Biosciences) and kept on beads as GST fusions or excised from GST by using PreScission Protease (Amersham Biosciences). In vitro transcription/translation 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-fusion proteins bound to beads were incubated in 200 μ L of buffer 1 (20 mM Tris-HCl, pH 7.0, 100 mM NaCl, 1 mM EDTA, 10% glycerol, 0.01% Nonidet P-40) with 1 μ g of purified protein or 5 μ L of the TNT reaction and rinsed with buffer 1 and buffer 1 supplemented with 500 mM NaCl. Samples were boiled in the presence of Laemmli buffer and analyzed by SDS-PAGE. Rat anti-HA antibodies (Roche) were used for detection of HA-ARP6.

Whole-Mount Anthers Preparation for Microscopy

Anthers were teased apart and incubated overnight at 4°C in coloration buffer. Coloration buffer contained equal volumes of extraction buffer (0.1% Nonidet P40, 10% dimethyl sulfoxide, 5 mM EGTA, pH 7.5, 50 mM PIPES, pH 6.9, and DAPI solution (1 mg DAPI/mL dimethyl sulfoxide).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AY279398 (*PIE1*), NM_123064 (*SEF*), and NM_114070 (*ARP6*).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Alignment of SEF-related proteins.

Supplemental Table S1. Primers used in gene expression analysis.

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