**Running title:** SEF interacts with PIE1 and ARP6

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SEF, A NEW PROTEIN REQUIRED FOR FLOWERING REPRESSION IN ARABIDOPSIS, INTERACTS WITH PIE1 AND ARP6.

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ABSTRACT

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 and animals. Here we identify and characterize SERRATED LEAVES AND EARLY FLOWERING (SEF), an Arabidopsis 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 non-inductive photoperiods. This correlates with strong reduction of FLOWERING LOCUS C and MADS-AFFECTING FLOWERING4 transcript levels and upregulation of the FT and SOC1 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.
INTRODUCTION

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 (see for example (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 SUMOilation) 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 *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 the 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 (reviewed in (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 DNA-dependent ATPases of the SNF2 family 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 the 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 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 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 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 ARP6 (ACTIN RELATED PROTEIN 6). 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 SEF (SERRATED LEAVES AND EARLY FLOWERING) 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 supports 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 S. cerevisiae 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 data, Fig. S1), that we called SEF (SERRATED LEAVES AND EARLY FLOWERING)
FLOWERING). This protein, encoded by the At5g37055 locus (Fig. 1A), was also closely related to *Nicotiana benthamiana* CIBP1, originally identified as a plum pox virus CI interacting protein (accession number 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 7 conserved cysteines and one histidine that can potentially coordinate two zinc atoms (supplemental data, Fig. S1). Semiquantitative RT-PCR experiments demonstrated that *SEF* transcript was present in all tested tissues (Fig. 1B). In order 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 (WT) 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. 1D, 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 WT, both in LD and SD (data not shown).

Then we examined the development of inflorescence and flowers. Mutant plants produced two to three times more cpiolescence 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. 1F, G). As shown in Fig. 2 and Table I, sef flowers displayed several developmental abnormalities. They were smaller than WT flowers (Fig. 2A). Petals of mutant plants were about 25% smaller than WT 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 and Table I). Stamens were shorter than carpels (Fig. 2I) and most mutant flowers presented altered number of these organs (4 or 5), both under SD and LD. In contrast to the WT, all mutant stamens presented the same length. Mutant anthers were smaller than those of the WT 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 DAPI staining and microscopic observation (Fig. 2F-H and data not shown). sef gynoeciums were shorter and irregularly thickened in comparison to those of WT plants (Fig. 2D). Mutant siliques were also shorter and thicker (Fig. 2E) and contained unfertilized ovules or white aborted seeds (Fig. 2J, K), especially under SD conditions. In addition, distribution of seeds was more crowded in mutant siliques than in the WT siliques, which may explain the abnormal shape of many adult mutant seeds. The average number of seeds per mutant silique was 42.4 ± 4.4 in LD and 21.5 ± 9 in SD, while WT 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**

In order to explore the role of SEF in the transition from vegetative to reproductive development, we determined the flowering time of WT and mutant plants both under SD and LD conditions. Since pie1 mutants display similar morphological alterations than 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 Fig. 3A and 3B, sef mutants flowered with less leaves and earlier than the WT. Thus, sef plants flowered in LD with approximately 6 leaves while WT plants flowered with about 12 leaves. Under SD conditions sef plants flowered with around 21 leaves whereas the WT did it with 62 leaves approximately. pie1-5 plants flowered at a similar time and with a similar number of leaves than sef plants. Both sef and pie1-5 plants flowered earlier under LD than under SD conditions, indicating that they retained some sensitivity to photoperiod.

Then we 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-days or 12- and 15-days old seedlings, for LD or SD conditions, respectively. Transcript levels of the photoperiod pathway gene CONSTANS (CO) (Putterill et al., 1995), the floral repressor FLOWERING LOCUS C (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 Fig. 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 WT 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 both under SD and LD conditions. A similar although more severe deregulation of FLC, FT and SOC1 was also observed in pie1-5 plants (Fig. 3C and (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 downregulated in arp6 plants (Deal et al., 2005; Martin-Trillo et al., 2006). Fig. 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 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 Fig. 4A-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. 4E-F, Table II and 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 physically 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 analysed interaction with ARP6. To this end, we performed in vitro pull down experiments using GST-SEF and GST-ARP6 recombinant proteins and two in vitro translated PIE1 truncated polypeptides. As shown in Fig. 5A, both GST-SEF and GST-ARP6 proteins were able to interact with PIE522-1190 and PIE1813-1099 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 ARP6 and SEF proteins, respectively (Wu et al., 2005).

Then, we analysed the SEF-ARP6 interaction. Fig. 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 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 Fig. 5C, yeast cells co-expressing the GAD-ARP6 and GDB-SEF fusion proteins were able to grow in selective medium without histidine, 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 result 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 FRI-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, other 12 subunits copurify 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 which 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 downregulation 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 (see Fig. 5 and (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) and our observations). pie1 plants present a stronger downregulation of FLC and MAF4 transcript 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 than 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 lost of other two 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; Ausin et al., 2004; Bastow et al., 2004; He et al., 2004; Sung and Amasino, 2004; Zhao et al., 2005). Martin-Trillo et al. have recently reported that arp6 mutants present low levels of histone H3 acetylation and H3K4 methylation in the FLC locus (Martin-Trillo et al., 2006), 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 a recent 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 S. cerevisiae, 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, that 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 thaliana* wild type and mutants (Columbia ecotype) were grown in long-day (16 h light/8 h dark) or short-day (10 h light/14 h dark) conditions (130 µE m-2 s-1) at 23ºC (day)/20ºC (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 Singenta 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 LBA primer were used for amplification of wild type or T-DNA insertion alleles, respectively (LBA SALK 5’-TGGTTCACGTAGTGGGCCATCG-3’; LBA SAIL 5’-TTCATAACCAATCTCGATACAC-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 µg 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. 15 to 25 cycles were typically used and products were detected by Southern blot. Number of cycles were set up for each experiment to keep amplification within the quantitative range. For quantification of radioactive areas, an InstantImager Electronic Autoradiography apparatus (Packard Instrument Company) was used. Specific primers for *SEF, CO, FT, SOC1, FLC, GAPC* and *MAF1-MAF5* are described in supplemental data, Table S1.

Yeast two-hybrid
Yeast two-hybrid interaction analyses were conducted in the MaV203 strain with the PROQUEST Two-Hybrid System (Invitrogen). pDBLeu or pPC86 vectors were used for Gal4 activation domain or Gal4 DNA binding domain fusion constructs, respectively. cDNAs for SEF, ARP6 and PIE1 were obtained by standard PCR techniques and cloned into the SalI-NotI sites of the above vectors. Selection was performed on SC minimal medium without His, Leu and Trp (Bio101 Systems), supplemented with 10 to 50 mM 3-amino-1,2,4-triazole (3-AT).

**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 *E. coli* DH5α. Proteins were purified on Gluthatione 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 µl of buffer1 (20 mM Tris-HCl pH 7.0, 100 mM NaCl, 1 mM EDTA, 10% glycerol, 0.01% Nonidet P-40) with 1 µg of purified protein or 5 µl of the TNT reaction and rinsed with buffer1 and buffer1 supplemented with 500 mM NaCl. Samples were boiled in the presence of Laemmli buffer and analysed 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 over night at 4°C in coloration buffer. Coloration buffer contained equal volumes of extraction buffer (0.1% Nonidet P40, 10% DMSO, 5 mM ethylene glycol bis(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) (pH 7.5), 50 mM piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES) pH 6.9) and DAPI solution (1 mg DAPI / ml DMSO).

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LITERATURE CITED


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remodelling complexes that positively regulates FLC accumulation in Arabidopsis. Development 133: 1241-1252


FIGURE LEGENDS

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 long days. GAPC (glyceraldehyde-3-phosphate dehydrogenase) 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 (WT), sef-1 and sef-2 plants. Sequence of primers 1, 2, 3 and 4 is provided in supplemental data (Table S1). D, Rosette leaves of WT and sef-2 plants grown under LD conditions 27 days after sowing. All rosette leaves are shown in order of production from the first true leave at left. E, Rosette leaves of WT and sef-2 plants grown under SD condition 52 days after sowing. Black bars indicate early (e), middle (m) and late (l) stage leaves. F, Forty-day-old WT, sef-1, and sef-2 plants grown 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, G, 1 cm.

**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 WT (left) and sef-2 (right) flowers. B, Supernumerary petals and sepals in sef-2 flowers. C, WT petals (left) and short and wrinkled sef-2 petals (right). D, WT (left) and sef-2 (right) flower gynoecium. E, WT siliques (left) and sef-2 siliques (right). F, DAPI staining of a WT anther. G,H, DAPI staining of sef-2 anthers. I, WT (left) and sef-2 (right) stamens. J, Open siliques of self-fertilized WT plants. K, Open siliques of self-fertilized homozygous sef-2 plants. Arrowheads indicate aborted ovules. Scale bars: A, C, 1 mm; E, 0.5 cm; D, I-K, 0.5 mm; F-H, 0.2 mm.

**Figure 3.** Flowering of sef mutants and expression of flowering time control genes in sef and pie1 mutants. A, Twenty-five-day-old WT and sef plants grown under LD conditions. B, Sixty-two-day-old WT 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 hours after dawn, at 12 and 15 days (d) of growth under SD conditions or at 10 and 12 days of growth under LD. GAPC transcript levels were also determined as a control for the amount of input cDNA.
Figure 4. Genetic interaction of SEF and PIE1. A, Phenotype of 28-day-old WT 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-D, Petals (B), siliques (C) or carpel (D) of WT (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 WT (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, F, 1 cm.

Figure 5. SEF, ARP6 and PIE1 proteins interact in vitro. A, Schematic representation of truncated PIE1 proteins (upper panel) used in pull down assays (lower panel). GST, GST-SEF and GST-ARP6 fusion proteins bound to Gluthatione Sepharose 4B beads were incubated separately or combined as indicated with 35S-Met labelled, 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. 20% of the input 35S-Met labelled 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 non-selective (SC-L-T) or selective media with 25 mM 3AT (SC-L-T-H+3AT).
Table I. Phenotypic characteristics of *sef-2* mutant flowers.

<table>
<thead>
<tr>
<th>Variable</th>
<th>LD</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT (n = 15) b</td>
<td><em>sef-2</em> (n = 15)</td>
</tr>
<tr>
<td>Number of sepals</td>
<td>4.0±0.0</td>
<td>4±0.0</td>
</tr>
<tr>
<td></td>
<td>4.0±0.0</td>
<td>5.4 ±0.5</td>
</tr>
<tr>
<td></td>
<td>4.4±0.9</td>
<td>4.4±0.9</td>
</tr>
<tr>
<td>Number of petals</td>
<td>4.0±0.0</td>
<td>4.3±0.5</td>
</tr>
<tr>
<td></td>
<td>4.0±0.0</td>
<td>5.9±1.0</td>
</tr>
<tr>
<td></td>
<td>4.0±0.9</td>
<td>4.0±0.9</td>
</tr>
<tr>
<td>Number of stamens</td>
<td>5.9±0.3</td>
<td>4.4±0.5</td>
</tr>
<tr>
<td></td>
<td>5.9±0.1</td>
<td>5.5±0.7</td>
</tr>
<tr>
<td></td>
<td>4.9±0.3</td>
<td>4.9±0.3</td>
</tr>
<tr>
<td>Number of non-dehiscent stamens per flower</td>
<td>0.0±0.0</td>
<td>1.9±0.2</td>
</tr>
<tr>
<td></td>
<td>0.0±0.0</td>
<td>3.2±2.3</td>
</tr>
<tr>
<td></td>
<td>0.0±0.0</td>
<td>0.6±1.3</td>
</tr>
<tr>
<td>Petal length (mm)</td>
<td>2.9±0.1</td>
<td>2.3±0.2</td>
</tr>
<tr>
<td></td>
<td>3.0±0.1</td>
<td>2.9±0.1</td>
</tr>
<tr>
<td></td>
<td>3.0±0.1</td>
<td>3.0±0.1</td>
</tr>
<tr>
<td>Seeds per silique</td>
<td>62.2±0.8</td>
<td>42.4±4.4</td>
</tr>
<tr>
<td></td>
<td>55.7±2.2</td>
<td>n.d. e</td>
</tr>
<tr>
<td></td>
<td>21.5±9.0</td>
<td>21.2±7.9</td>
</tr>
<tr>
<td>Number of aborted seeds per silique</td>
<td>1.2±1.0</td>
<td>8.8±4.0</td>
</tr>
<tr>
<td></td>
<td>2.1±1.0</td>
<td>n.d. e</td>
</tr>
<tr>
<td></td>
<td>21.2±7.9</td>
<td>21.2±7.9</td>
</tr>
</tbody>
</table>

- **a** n, number of plants analyzed.
- **b** numbers are means ± standard deviation.
- **c** The first five arising flowers were scored.
- **d** Flowers arising after the fifth flower were scored.
- **e** n.d., not determined.
Table II. Bolting time of wild-type and sef/pie1 mutant plants a.

<table>
<thead>
<tr>
<th></th>
<th>LD</th>
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<th>SD</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>Leaf number</td>
<td>Days</td>
<td>Leaf number</td>
<td>Days</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Columbia (n = 16)b</td>
<td>12.4 ± 0.5</td>
<td>27.7 ± 0.5</td>
<td>62.0 ± 3.7</td>
<td>65.2 ± 2.4</td>
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<tr>
<td>sef-1  (n = 16)</td>
<td>6.1 ± 0.5</td>
<td>21.4 ± 0.8</td>
<td>21.3 ± 1.8</td>
<td>48.4 ± 1.5</td>
</tr>
<tr>
<td>sef-2  (n = 15)</td>
<td>5.7 ± 0.5</td>
<td>21.1 ± 0.9</td>
<td>21.4 ± 2.9</td>
<td>46.6 ± 2.6</td>
</tr>
<tr>
<td>pie1-5 (n = 16)</td>
<td>5.4 ± 0.7</td>
<td>20.2 ± 0.9</td>
<td>21.0 ± 2.7</td>
<td>47.7 ± 1.2</td>
</tr>
<tr>
<td>sef-2+/- pie1-5+/-  (n = 31)</td>
<td>8.0 ± 1.6</td>
<td>22.9 ± 1.3</td>
<td>47.9 ± 6.2</td>
<td>56.6 ± 2.7</td>
</tr>
<tr>
<td>sef-2-/- pie1-5-/-  (n = 14)</td>
<td>5.2 ± 0.6</td>
<td>20.6 ± 0.9</td>
<td>20.6 ± 1.4</td>
<td>47.4 ± 2.4</td>
</tr>
</tbody>
</table>

aData of the sef and pie1 single and double mutants were significantly different from those of the WT 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 WT, single and double homozygous mutants with a Student’s t value of P < 0.0001.

bn, number of plants analyzed.
ncNumber of rosette leaves at bolting ± standard deviation.
ddNumber of days from planting to bolting ± standard deviation.