Regulation of Floral Patterning by Flowering Time Genes

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DOI 10.1016/j.devcel.2009.03.011

SUMMARY

Floral patterning in Arabidopsis requires activation of floral homeotic genes by the floral meristem identity gene, LEAFY (LFY). Here we show that precise activation of expression of class B and C homeotic genes in floral meristems is regulated by three flowering time genes, SHORT VEGETATIVE PHASE (SVP), SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (SOC1), and AGAMOUS-LIKE 24 (AGL24), through direct control of a LFY coregulator, SEPELLATA3 (SEP3). Orchestrated repression of SEP3 by SVP, AGL24, and SOC1 is mediated by recruiting two interacting chromatin regulators, TERMINAL FLOWER 2/LIKE HETEROCHROMATIN PROTEIN 1 and SAP18, a member of SIN3 histone deacetylase complex. Our finding of coordinated regulation of SEP3 by flowering time genes reveals a hitherto unknown genetic pathway that prevents premature differentiation of floral meristems and determines the appropriate timing of floral organ patterning.

INTRODUCTION

Although flowers generated from different plant species are extensively diversified, the underlying genetic and molecular mechanisms that regulate flower development are highly conserved. In Arabidopsis, our understanding of the mechanisms controlling flower development is encapsulated in the “ABC” model, which describes how each whorl of floral organs is determined by a combinatorial action of the A, B, and C class floral homeotic genes (Bowman et al., 1991; Coen and Meyerowitz, 1991). Further discovery of the SEPELLATA (SEP) genes has led to a revised “ABCE” model, in which the E class SEP genes function redundantly with other homeotic genes in specifying floral organs (Ditta et al., 2004; Goto et al., 2001; Pelaz et al., 2000; Theissen and Saedler, 2001).

A key regulator of early floral patterning is the floral meristem identity gene, LEAFY (LFY), which is expressed throughout young floral meristems and activates various floral homeotic genes in combination with other regulators (Parcy et al., 1998; Weigel et al., 1992). LFY directly activates APETALA1 (AP1), which plays dual roles in specifying the floral meristem and acting as a class A gene to determine the identity of perianth organs (Man-del et al., 1992; Wagner et al., 1999). Activation of a class B gene, APETALA3 (AP3), which determines petals and stamens, requires the concerted action of LFY, AP1, and UNUSUAL FLORAL ORGANS, an F box gene (Ng and Yanofsky, 2001; Parcy et al., 1998). LFY also cooperates with a homeobox gene, WUSCHEL (WUS), to activate the class C gene, AGAMOUS (AG), which specifies the identity of stamens and carpels (Lenhard et al., 2001; Lohmann et al., 2001). These observations have demonstrated an indispensable role of LFY in mediating early floral patterning, which leads to the spatially restricted expression of floral homeotic genes described in the ABC model.

Although the expression of class B and C genes is reduced in lfy-6 null mutants, their expression is not abolished (Weigel and Meyerowitz, 1993), indicating that some other factors may also contribute to activation of floral homeotic genes. In this study, we report that a genetic pathway mediated by three flowering time genes, SHORT VEGETATIVE PHASE (SVP), SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (SOC1), and AGAMOUS-LIKE 24 (AGL24), is required for the regulation of early floral patterning in Arabidopsis. These three genes encode closely related MADS box transcription factors involved in the control of flowering time (Hartmann et al., 2000; Lee et al., 2000; Michaels et al., 2003; Yu et al., 2002). In the emerging floral meristems, the expression of these genes is normally downregulated by AP1 to prevent the reversion of floral meristems into various shoot structures (Liu et al., 2007; Yu et al., 2004). Their single and double mutants produce normal flowers under standard growth temperatures except for svp-41 agl24-2, in which several flowers at basal positions of the inflorescence show mild floral defects (Gregis et al., 2006). The defects are enhanced by growing at a higher temperature (e.g., 30°C) or in the background of ap1 mutants, indicating the involvement of AP1 and these flowering time genes in flower development.

By investigating dramatic floral defects in the triple mutant soc1-2 agl24-1 svp-41, we have found that these three flowering time genes control floral patterning by directly preventing the ectopic expression of SEP3, a member of the class E genes, which acts with LFY to activate class B and C gene expression in stage 3 floral meristems. To maintain SEP3 chromatin in a silenced state, SVP interacts with TERMINAL FLOWER 2/LIKE HETEROCHROMATIN PROTEIN 1 (TFL2/LHP1) to modulate trimethylation of histone H3 lysine 27 (H3K27me3), while SOC1 and AGL24 interact with SAP18, a member of Sin3/histone deacetylase (HDAC) complex, to modulate histone H3 acetylation. Our results suggest that orchestrated repression of SEP3 by flowering time genes prevents premature differentiation of...
floral meristems and determines the timing of floral organ patterning.

RESULTS

SOC1, AGL24, and SVP Redundantly Regulate Flower Development

Our previous study on flowering time genes led to the generation of various combinations of mutants among soc1-2, agl24-1, and svp-41 (Li et al., 2008). Among all the single and double mutants generated, only flowers of agl24-1 svp-41 showed mild defects, including slightly reduced floral organs and occasional generation of deformed petals at the standard growth temperature (22°C) (Figures 1A–1D; see Table S1 available online), which was consistent with a previous observation (Gregis et al., 2006). However, the triple mutant soc1-2 agl24-1 svp-41 exhibited striking floral defects with loss of most floral organs and generation of various chimeric floral structures (Figures 1E and 1G–1M; Table S1). The severity of these phenotypes increased acropetally. In addition, each floral structure in soc1-2 agl24-1 svp-41 was subtended by a bract (Figure 1F). The floral phenotypes of soc1-2 agl24-1 svp-41 were rescued by the transgene containing SOC1, AGL24, or SVP (Figure S1), suggesting that these genes play redundant roles in regulating flower development.

Class B and C Genes Are Deregulated in soc1-2 agl24-1 svp-41

To examine whether the floral defects in soc1-2 agl24-1 svp-41 are due to deregulation of floral homeotic genes, we performed in situ hybridization to detect the expression of floral homeotic genes in inflorescence apices. For class A genes, APETALA2 (AP2) was expressed in a pattern similar to that in wild-type plants, while AP1 exhibited a similar expression pattern but with slightly reduced intensity (Figure S2), which is probably due to the repression by ectopic AG activity (see below) (Gustafson-Brown et al., 1994). However, two class B genes, AP3 and PISTILLATA (PI), and one class C gene, AG, were all ectopically expressed in floral anlagen in the inflorescence meristem and irregularly expressed in emerging floral meristems before stage 3 (Figures 1N–1P) (Smyth et al., 1990). This is in great contrast to their expression in wild-type plants, where they start to be expressed in stage 3 floral meristems, when they appear in stage 1 floral meristems immediately after floral patterning occurs. We further investigated whether class B and C genes are regulated by SOC1, SVP, and AGL24 in other developmental contexts. In soc1-2 agl24-1 svp-41, neither class B nor C genes were ectopically expressed during the vegetative phase, but they appeared in stage 1 floral meristems immediately after floral transition (Figure 2A). This indicates that deregulation of class B and C genes in soc1-2 agl24-1 svp-41 coincides with the reproductive growth. Interestingly, as the inflorescences of soc1-2 agl24-1 svp-41 bolted, ectopic AP3 expression remained mostly unchanged, whereas the domain and intensity of ectopic AG expression gradually increased in inflorescence apices (Figure S3). Such an expression profile was consistent with the observation that carpelloid structures increased acropetally in the inflorescences of the triple mutants (Table S1).

As carpelloid structures and homeotic transformation of sepals into petals were still observed in soc1-2 agl24-1 svp-41 ap3-3 and soc1-2 agl24-1 svp-41 ag-1, respectively (Figure S4), deregulation of class B and C genes in soc1-2 agl24-1 svp-41 was at least partially independent of each other. These results, together with the observation that concurrent activation of class B and C genes in soc1-2 agl24-1 svp-41 (Figure 2A; Figure S3), imply that a synchronized mechanism mediated by these flowering time...
genes could regulate both class B and C genes in floral meristems. Since chromatin immunoprecipitation (ChIP) assays did not reveal binding of SOC1, SVP, and AGL24 to the promoters of class B and C genes (Figure S5), their interaction could be mediated by other intermediate factor(s).

**SEP3 Is Repressed by SOC1, AGL24, and SVP**

To identify regulators that mediate the regulation of class B and C genes by SOC1, SVP, and AGL24, we examined the expression of those known regulators of B and C class genes, including LEUNIG (LUG) (Liu and Meyerowitz, 1995), SEUSS (SUE) (Franks et al., 2002), and SEP3 (Castillejo et al., 2005), and found that only SEP3 expression was ectopically expressed in both vegetative and inflorescence apices of the triple mutants (Figure S5). To further examine the expression of SEP3 in the triple mutants, we conducted ChIP assays to determine whether SOC1, SVP, and AGL24 directly control SEP3 expression. We scanned the SEP3 genomic sequence for the CC(A/T)6GG (CArG) motif, and found that only SEP3 was significantly upregulated in leaves and shoot apices of 9-day-old soc1-2 agl24-1 svp-41 seedlings at the floral transitional stage (Figure 2B). Further examination of 6-day-old seedlings revealed that SEP3 was upregulated in svp-41, and further strengthened in soc1-2 svp-41 (Figure 3A). As AGL24 was mainly expressed in the shoot apices of young seedlings (Liu et al., 2008), its loss-of-function effect on SEP3 was not observed in whole seedlings. On the contrary, overexpression of SOC1, SVP, or AGL24 all significantly suppressed SEP3 in both leaves and shoot apices (Figure 3B).

We further compared SEP3 expression in inflorescence apices of various mutants. In wild-type plants, SEP3 expression was first detected in the upper portion of late stage 2 floral meristems (Mandel and Yanofsky, 1998), which was comparable with its expression in soc1-2 svp-41 and soc1-2 agl24-1 (Figures 3C–3E and Figure S6). In agl24-1 svp-41, ectopic SEP3 expression was observed in stage 1 and 2 floral meristems of just bolting inflorescences, but not in the inflorescences 10 cm in height (Figures 3F and 3G and Figure S6). In soc1-2 agl24-1 svp-41, ectopic SEP3 expression was detectable in apical meristems and stage 1 floral meristems of just bolting inflorescences, and turned stronger in apical meristems of the inflorescences 10 cm in height, especially in floral anlagen (Figures 3H and 3I; Figure S6). The trend of changes in SEP3 expression patterns in bolting inflorescences was well correlated with the phenotype of agl24-1 svp-41 or soc1-2 agl24-1 svp-41, as floral defects were alleviated acropetally in the former, while aggravated in the latter (Table S1). These observations show that SEP3 is redundantly repressed by SOC1, SVP, or AGL24 and indicate that ectopic expression of SEP3 may contribute to the floral defects in soc1-2 agl24-1 svp-41.

**SOC1, AGL24, and SVP Repress SEP3 via Binding to a Common Promoter Region**

We performed further ChIP assays to examine whether SOC1, AGL24, and SVP directly control SEP3 expression. We scanned the SEP3 genomic sequence for the CG(A/T)6GG (CArG) motif,
a canonical binding site for MADS domain proteins, with a maximum of one nucleotide mismatch and designed primers near the identified motifs for measurement of DNA enrichment (Figure 3J). SVP-6HA was associated with the region near the SEP3-2 fragment (Figure 3K), while SOC1-myc and AGL24 were only associated with the same region in the absence of SVP (Figures 3L and 3M). This indicates that SVP is a primary suppressor of SEP3, while SOC1 and AGL24 function redundantly.

To test in vivo whether two CArG motifs near SEP3-2 serve as binding sites of SVP, SOC1, and AGL24 for repressing SEP3 expression (Figures 3A and 3B). Plant tissues in (B) were dissected as described in Figure 2B. The SEP3 expression level in wild-type is set as 1. Error bars indicate SD. (C–E) In situ localization of SEP3 expression in inflorescence apices of wild-type (G), soc1-2 svp-41 (D), and soc1-2 agl24-1 (E) plants. In these plants, SEP3 expression pattern remains consistent in inflorescences in different heights.

(F–I) In situ localization of SEP3 expression in inflorescence apices of agl24-1 svp-41 (H and I). (F and G) Apices of just bolting inflorescences. (G and I) Apices of the inflorescences 10 cm in height. The arrowhead in (I) indicates strong SEP3 expression in a floral anlagen. Asterisks in (C–I) indicate inflorescence meristems. Scale bars in (C–I), 100 μm.

(J) Schematic diagram of the SEP3 promoter. The bent arrow indicates a translational starting site. Exons and introns are shown by black and white boxes, respectively. The arrowheads indicate the sites containing either one mismatch or perfect match from the consensus binding sequence (CArG box) for MADS domain proteins. The hatched boxes represent the DNA fragments amplified in ChIP assays.

(K) ChIP analysis of SVP binding to the SEP3 promoter. Inflorescence apices of svp-41 SVP:SVP-6HA (Li et al., 2008) were harvested for the ChIP assay.

(L) ChIP analysis of SOC1 binding. Inflorescence apices of soc1-2 SOC1:SOC1-myc, which exhibited phenotypes like wild-type plants, were harvested for the ChIP assay. To test whether SVP affects SOC1 binding, a ChIP assay of soc1-2 svp-41 SOC1:SOC1-myc was also performed.

(M) ChIP analysis of AGL24 binding. Inflorescence apices of wild-type plants were harvested for the ChIP assay. To test whether SVP affects AGL24 binding, a ChIP assay of svp-41 was also performed.

(N) Schematic diagram of the SEP3:GUS construct where a 4.7 kb SEP3 genomic fragment including its coding region was fused with the GUS gene. Two native CarG boxes near SEP3-2 were mutated as indicated.

(O) GUS staining of inflorescence apices of the transformants containing SEP3:GUS (top panel) and its mutated construct (bottom panel). Arrowheads in the last picture indicate bracts subtending floral meristems in soc1-2 agl24-1 svp-41. Asterisks indicate inflorescence meristems.
(Figure 4A), we created SEP3:GUS and its derived mutant constructs, in which two CArG motifs near SEP3-2 were mutagenized (Figure 3N). Transgenic plants bearing SEP3:GUS exhibited a staining pattern similar to that of endogenous SEP3 expression (Figures 3O and 3P). In most of transgenic lines generated, mutagenesis of single CArG motif (m-502 or m-287) did not alter the GUS staining pattern, while mutagenesis of two CArG motifs (m-502/-287) caused dramatic ectopic GUS staining in whole plants including inflorescence apices (Figures 3O and 3P). Furthermore, introducing the m-502/-287 reporter line into soc1-2 agl24-1 svp-41 did not enhance GUS staining (Figure 3P). These observations suggest that SVP, SOC1, and AGL24 specifically bind to both CArG motifs near SEP3-2 to repress SEP3 expression.

**Ectopic SEP3 Activity Results in Ectopic Expression of Class B and C Genes**

To test whether ectopic SEP3 expression is relevant to ectopic expression of class B and C genes in soc1-2 agl24-1 svp-41, we created soc1-2 agl24-1 svp-41 sep3-2. This mutant exhibited significantly alleviated floral phenotypes (Figure 4A), indicating that SEP3 contributes to the floral defects in soc1-2 agl24-1 svp-41.

As members of class E homeotic regulators, including SEP3, form protein complexes with other floral homeotic proteins to specify the floral organ identity (Honma and Goto, 2001), suppression of the floral defects in soc1-2 agl24-1 svp-41 by sep3-2 could be due to the removal of SEP3 from homotic protein complexes rather than altered expression of class B and C genes. We thus compared the gene expression in soc1-2 agl24-1 svp-41 sep3-2 and soc1-2 agl24-1 svp-41, and found that class B and C genes...
were significantly downregulated in terms of scope and intensity in the quadruple mutants (Figure 4B). These results suggest that ectopic expression of class B and C genes induced by ectopic SEP3 expression is responsible for the phenotypes in soc1-2 agl24-1 svp-41.

**SEP Genes Activate the Expression of Class B and C Genes**

The results from soc1-2 agl24-1 svp-41 reminded us of a previous study showing ectopic activation of class B and C genes by over-expressing SEP3 (Castillejo et al., 2005). Furthermore, initial SEP3 expression in the apical region of late stage 2 floral meristems (Mandel and Yanofsky, 1998) covers the region where class B and C genes are activated (Goto and Meyerowitz, 1994; Jack et al., 1992; Yanofsky et al., 1990). These prompted us to hypothesize that SEP3 together with other SEP genes may play a role in activating class B and C genes in wild-type plants.

The SEP family consists of four homologs in Arabidopsis. While their single mutants only exhibit subtle phenotypes, simultaneous loss of their function transforms all floral organs into leaf-like tissues (Ditta et al., 2004; Pelaz et al., 2000), demonstrating a crucial and redundant role of SEP genes in flower development. In situ hybridization revealed significantly reduced expression of class B and C genes at early stage 3 floral meristems of sep1 sep2 sep3 sep4 (Figure 4C; Figure S7B). In stage 5 floral meristems of sep1 sep2 sep3 sep4, the expression domain of AP3 or AG was restricted to a smaller region, while LFY was completely misexpressed in the center of the meristems (Figures 4C; Figure S7B). Notably, the expression of the well-known activator of floral homeotic genes, LFY, was not altered in sep1 sep2 sep3 sep4 (Figure S7A). These results indicate that SEP genes are required for activating the expression of class B and C genes at early stages even in the presence of LFY.

**SEP3 and LFY Act in Concert to Activate the Expression of Class B and C Genes**

We noticed that in soc1-2 agl24-1 svp-41, although SEP3 was ectopically expressed in whole seedlings, class B and C genes were not expressed only until the emergence of floral primordia (Figure 2). This result implies that SEP3 requires certain floral-specific cofactor(s) in activating class B and C genes. LFY is the most possible coregulator, because of its known function in activating class B and C genes and its expression throughout young floral meristems (Parcy et al., 1998; Weigel et al., 1992). In soc1-2 agl24-1 svp-41, LFY was highly expressed in emerging floral meristems (Figure S8), where SEP3 was also ectopically expressed (Figures 2A, 3H, and 3I). To investigate whether transcriptional activation of class B and C genes by SEP3 is dependent on LFY, we crossed soc1-2 agl24-1 svp-41 with ify-2, in which LFY function was partially lost (Schultz and Haughn, 1993). In soc1-2 agl24-1 svp-41 ify-2, ectopic expression of class B and C genes was greatly reduced (Figure 4B). Accordingly, soc1-2 agl24-1 svp-41 ify-2 showed significantly rescued floral phenotypes as compared with soc1-2 agl24-1 svp-41 (Figure 4A). These results suggest that SEP3 and LFY function in concert in activating the expression of class B and C genes in soc1-2 agl24-1 svp-41.

To further test the concerted effect of SEP3 and LFY on flower development, we created ify-2 sep3-2. Flower development was almost normal in sep3-2, while ify-2 showed mild defects with a slightly reduced number of petals and stamens (Figure 4D). On the contrary, ify-2 sep3-2 showed dramatic floral defects, such as loss of most floral organs and homeotic transformation of stamens and petals into leaf-like structures (Figure 4D), confirming that LFY and SEP3 synergistically act to regulate class B and C genes. This raises the possibility of direct interaction between LFY and SEP3, which was supported by a GST pull-down assay showing their physical interaction in vitro (Figure 4E).

**SOC1 and AGL24 Interact with SAP18**

We further sought to elucidate how SVP, SOC1, and AGL24 repress SEP3 expression. Protein sequence alignment revealed a conserved C-terminal motif in SOC1, SVP, AGL24, and another MADS box protein, AGL15 (Figure 5A). This conserved C-terminal motif, together with the K domain, of AGL15 was found to mediate the interaction between AGL15 and SAP18, a member of Sin3/HDAC complex (Hill et al., 2008; Silverstein and Ekwall, 2005). Thus, we tested whether SVP, SOC1, and AGL24 could also interact with SAP18. A GST pull-down assay revealed that SAP18 interacted with both SOC1 and AGL24, but not SVP (Figure 5B). Coimmunoprecipitation analyses further showed the in vivo interaction of SOC1 and AGL24 with SAP18 (Figures 5C and 5D). Moreover, bimolecular fluorescence complementation (BiFC) analysis, which detects protein-protein interactions through monitoring the fluorescence emitted by reconstitution of an enhanced yellow fluorescent protein from two fragments fused to two interacting proteins, revealed the direct interaction of SAP18-SOC1 (Figure 5E) and SAP18-AGL24 (Figure 5F) in the nuclei of living plant cells. These results strongly suggest that AGL24 and SOC1 interact with SAP18 in the nuclei. As mutating the conserved C-terminal motif only abolished the protein interaction between SAP18 and SOC1, but not AGL24 (Figures 5G and 5H), AGL24 interaction with SAP18 might rely on other domain(s) rather than the C-terminal motif.

Interaction of AGL24 and SOC1 with SAP18 raises the possibility that both SOC1 and AGL24 may repress SEP3 transcription by recruiting an HDAC complex. We therefore analyzed histone acetylation status at the SEP3 locus in various mutants. In general, hyperacetylation of histone H3 and H4 is associated with promoter regions of actively transcribed genes (Li et al., 2007). For SEP3 chromatin, histone H3, but not H4, was hyperacetylated in soc1-2 svp-41 and soc1-2 agl24-1 svp-41 seedlings (Figure S9), in which SEP3 was highly expressed (Figure 3A). This observation, together with the ChIP results (Figures 3L and 3M), supports the role of SOC1 and AGL24 in preventing H3 acetylation of SEP3 in the absence of SVP.

As SAP18 did not interact with SVP, these two proteins may involve different mechanisms to repress SEP3 transcription. To test this, we created SAP18 knockdown lines by artificial microRNA interference (Schwab et al., 2006), and crossed a representative AmiR-sap18 line with svp-41. As expected, svp-41 AmiR-sap18 had higher SEP3 expression than svp-41 and AmiR-sap18 (Figure 5I). Consequently, svp-41 amir-sap18 exhibited significant floral defects (Figure 5J), which partially mimicked those of soc1-2 agl24-1 svp-41. We further found that H3 acetylation of SEP3 in AmiR-sap18 increased in the svp-41 background (Figure S9). These results suggest that...
SAP18 recruited by AGL24 and SOC1 contributes to H3 deacetylation of SEP3 in the absence of SVP.

**SVP Interacts with TFL2**

To understand how SEP3 is repressed by SVP, we performed yeast two-hybrid screening to identify its protein partners. By using the SVP sequence as a bait, we found the sequences encoding TFL2/LHP1, the only Arabidopsis homolog of HP1 of metazoans and S. pombe (Gaudin et al., 2001; Kotake et al., 2003). Previous studies have suggested that TFL2 suppresses genes involved in various developmental processes by recognizing H3K27me3 (Larsson et al., 1998; Turck et al., 2007; Zhang et al., 2007). In yeast TFL2 interacted with SVP but not its closest homolog AGL24 (Figure 6A). The interaction between SVP and TFL2 was confirmed by GST pull-down assays (Figures 6B and 6C). Their interaction required the chromoshadow domain of TFL2 and the conserved C-terminal motif of SVP (Figures 6A and 6D and Figure S10). BIFC analysis further revealed in vivo interaction of these two proteins in the nuclei (Figure 6E). These results suggest that SVP interacts with TFL2 in the nuclei.

To investigate the role of SVP in guiding TFL2 to the SEP3 promoter, we performed ChIP analysis using 35S:TFL2-3HA transgenic lines, which fully rescued tfl2-1 loss-of-function mutants (data not shown). In agreement with previous data of genome-wide analysis of TFL2 binding (Zhang et al., 2007), we found that TFL2-3HA was associated with the SEP3 locus (Figure 6F). Importantly, TFL2-3HA and SVP-6HA bound to the same genomic region (SEP3-2) with the highest enrichment fold (Figures 3K and 6F). In svp-41, the enrichment of TFL2-3HA binding to SEP3-2 was significantly decreased (Figure 6F). These results demonstrate that SVP plays an important role in guiding TFL2 to the SEP3-2 region.

We further found that H3K27me3 at the SEP3 locus was almost completely lost in tfl2-1 (Figure S11). This may partly explain the significantly increased SEP3 expression in tfl2-1 loss-of-function mutants (data not shown). In agreement with previous data of genome-wide analysis of TFL2 binding (Zhang et al., 2007), we found that TFL2-3HA was associated with the SEP3 locus (Figure 6F). Importantly, TFL2-3HA and SVP-6HA bound to the same genomic region (SEP3-2) with the highest enrichment fold (Figures 3K and 6F). In svp-41, the enrichment of TFL2-3HA binding to SEP3-2 was significantly decreased (Figure 6F). These results demonstrate that SVP plays an important role in guiding TFL2 to the SEP3-2 region.

We further found that H3K27me3 at the SEP3 locus was almost completely lost in tfl2-1 (Figure S11). This may partly explain the significantly increased SEP3 expression in tfl2-1 (Kotake et al., 2003), indicating that TFL2 represses SEP3 by modulating H3K27me3. In svp-41, where localization of TFL2 to the SEP3 locus was partially compromised, H3K27me3 at the SEP3 locus was also reduced (Figure S11). Thus, SVP at least guides TFL2 to the SEP3 locus, repressing SEP3 by influencing H3K27me3.
As SVP function is associated with TFL2, we reasoned that lack of TFL2 in sot1-2 agl24-1 might produce certain floral phenotypes like those in sot1-2 agl24-1 svp-41. sot1-2 agl24-1 tfl2-1 showed an enhanced determinate inflorescence with only two or three terminal flowers. These flowers developed sepaloid stamens and stamenooid petals in outer two whorls (Figure 6G), indicating the ectopic activity of class B and C genes. This result further supports that TFL2, which interacts with SVP, acts with SOC1 and AGL24 to regulate class B and C genes.

DISCUSSION

Control of Floral Patterning by Flowering Time Genes

Regulation of floral homeotic genes that specify floral organ identity is a key event for proper patterning of floral organs. Our findings have revealed a hitherto unknown genetic pathway that determines the timely expression of class B and C homeotic genes in floral meristems (Figure 7). The central regulators of this pathway are three MADS box transcription factors, SVP, SOC1, and AGL24, which were identified early as flowering time genes. These genes are redundantly required to prevent precocious expression of class B and C genes in emerging floral meristems through repression of SEP3. In floral meristems before late stage 2, class B and C genes are not expressed because SEP3 is repressed by SVP, SOC1, and AGL24. As floral meristems proceed to late stage 2, direct repression of SVP, SOC1, and AGL24 by the floral meristem identity gene AP1 (Liu et al., 2007; Yu et al., 2004) gradually derepresses SEP3. This, in the apical region of early stage 3 floral meristems, SEP3 and LFY function together to activate the expression of class B and C genes.

As SEP3 is ectopically expressed in whole seedlings of sot1-2 agl24-1 svp-41, suppression of SEP3 by SVP, SOC1, and AGL24 is likely a constitutive event. This suppression in emerging floral meristems is vital for flower development, as it secures a normal expansion of floral anlagen into large floral meristems that contain sufficient cells for proper patterning of whorled organs by floral homeotic genes. Complete removal of this suppression in sot1-2 agl24-1 svp-41 activates class B and C genes early in floral anlage, which in turn causes premature differentiation of floral meristems, thus producing a limited number of chimeric floral structures. In wild-type plants, AP1 plays a progressive role in overcoming this suppression by repressing SVP, SOC1, and AGL24 within a short, but crucial time window in young floral meristems before stage 3 (Liu et al., 2007; Yu et al., 2004). This leads to the timely derepression of SEP3, which in turn acts with LFY to activate class B and C genes in stage 3 floral meristems. Thus, consistent with previous studies showing concerted effects of LFY and AP1 on regulating class B and C genes (Weigel et al., 1992; Weigel and Meyerowitz, 1993), our results propose a genetic pathway in which AP1 contributes to floral
Regulation of SEP3 Expression by SOC1, AGL24, and SVP through Recruiting of Different Chromatin Factors

To unravel the underlying mechanisms by which SEP3 is repressed by SOC1, SVP, and AGL24, we have demonstrated a typical scenario in which different chromatin factors relevant to various histone modifications are guided by three transcription factors to a specific locus. To maintain SEP3 chromatin in a silenced state, SVP recruits TFL2 to modulate H3K27me3, while SOC1 and AGL24 interact with SAP18 to modulate histone acetylation in the absence of SVP (Figure 7).

Previous studies on TFL2 have suggested that it specifically associates with genome regions marked with H3K27me3 and is involved in maintaining gene repression (Turck et al., 2007; Zhang et al., 2007). SEP3 has been identified as one of the potential targets of TFL2 through microarray and genome-wide ChIP analyses (Kotake et al., 2003; Zhang et al., 2007). Our results reveal the specific transcription factor, SVP, that plays a role in guiding the general chromatin factor TFL2 to the SEP3 locus, thus repressing SEP3 at least by affecting H3K27me3. Although comparison of H3K27me3 distribution in Arabidopsis Chromosome 4 in tfl2 and wild-type plants has suggested that TFL2 may not be involved in the deposition of H3K27me3 (Turck et al., 2007), the mammalian homolog of TFL2, HP1, functions to not only decipher histone code, but it also encodes it (Kourmouli et al., 2005). H3K27me3 at the SEP3 locus is almost completely lost in tfl2-1, and also reduced in agl24-1 and soc1-2 where TFL2 binding to the SEP3 locus is compromised (Figure S1), demonstrating a close link between TFL2 and the level of H3K27me3.

SAP18 is so far not well characterized, but is generally considered to be a structural protein that stabilizes the Sin3/HDAC complex and its interacting nonconstitutive components (Silverstein and Ekwall, 2005). SAP18 has been shown to interact with HDA19, an Arabidopsis histone deacetylase, and link the HDAC complex to transcriptional repressors that bind to specific chromatin regions (Hill et al., 2008; Song and Galbraith, 2006). In this study, we found that in the absence of SVP, SOC1 and AGL24 modulate H3 acetylation at the SEP3 locus, suggesting that SOC1 and AGL24 repress SEP3 by recruiting the HDAC complex.

Coordinated repression of SEP3 by SOC1, SVP, and AGL24 through recruiting different chromatin factors demonstrates the flexibility of chromatin regulation during plant development. As SEP3 is ultimately relevant to reproductive growth, it should be continuously repressed only until the conditions for flower development are appropriate. Although the expression trend of SVP is opposite to that of SOC1 and AGL24 during floral transition (Hartmann et al., 2000; Lee et al., 2000; Michaels et al., 2003; Yu et al., 2002), their capacity in recruiting different chromatin factors enables them to continuously create a nonpermissive chromatin environment for SEP3 expression. This developmental plasticity allows plants to progress normally to the reproductive stage even if some of the redundant regulators are lost.

EXPERIMENTAL PROCEDURES

Plant Materials and Growth Conditions

All Arabidopsis plants were grown at 22°C under long days (16 hr light/8 hr dark). The mutants soc1-2, svp-41, agl24-1, ify-2, sep3-2, and tfl2-1 are in...
The coding regions of SVP, AGL24, and TFL2 were amplified and cloned into pGBK7T and pGADT7 (Clontech), respectively. Subsequent yeast two-hybrid assays were carried out using the Yeastmaker Yeast Transformation System 2 according to the manufacturer’s instructions (Clontech). For library screening, BD-SVP was used as bait to screen an inflorescence cDNA library (CD4-30 from ABRC). Yeast transformants were selected on the SD medium lacking histidine, tryptophan, and leucine (SD–His−/−Trp−/−/Leu) and supplemented with 0.2 mg/ml X-a-gal. The prey plasmids were recovered with the E.Z.N.A. Yeast Plasmid Kit (Omega Bio-Tek). For directly testing protein interactions, yeast AH109 cells were cotransformed with specific bait and prey constructs, and plated onto the selective SD medium (SD–Trp−/−Leu or SD–His−/−Trp−/−Leu).

In Vitro Pull-Down Assay

The cDNAs encoding LFY, LAP18, and TFL2 were cloned into pGEX-4T-1 vector (Pharmacia). These expression vectors were transformed into E. coli Rosetta (DE3) (Novagen), and protein expression was induced by IPTG. The soluble GST fusion proteins were extracted and immobilized onto glutathione sepharose sepharose (Amersham Biosciences), and subsequently used for GST pull-down assays. HA-tagged SEP3, SOC1, SVP, and AGL24 proteins and their relevant mutant forms were synthesized as previously described (Li et al., 2008). These epitope-tagged proteins were incubated with the immobilized GST and GST fusion proteins. Proteins retained on the beads were resolved by SDS-PAGE and detected with anti-HA or anti-myc antibody (Santa Cruz Biotechnology).

Coimmunoprecipitation Experiments

Plant materials were harvested and nuclear proteins were extracted according to the ChIP protocol, but without tissue fixation. SOC1-myc or AGL24 protein was immunoprecipitated by anti-myc agarose conjugate (Sigma) or anti-AGL24 antibody bound to Protein G PLUS agarose (Santa Cruz biotechnology), respectively. Proteins bound by the beads were resolved by SDS-PAGE and detected with anti-FLAG antibody (Sigma).

BIFC Analysis

The cDNAs of SOC1, AGL24, SAP18, SVP, and TFL2 were cloned into serial pSAT1 vectors. The resulting cassettes including fusion proteins and constitutive promoters were cloned into pGreen binary vector HY105 and transformed into Agrobacterium. For BIFC experiments, 3-week-old tobacco (Nicotiana benthamiana) leaves were coinfected with Agrobacterium as previously described (Sparkes et al., 2006).

SUPPLEMENTAL DATA

The Supplemental Data include twelve figures and two tables and can be found with this article online at http://www.cell.com/regulation/.


