Specification of Arabidopsis floral meristem identity by repression of flowering time genes

Chang Liu1,*, Jing Zhou1,*, Keren Bracha-Drori2, Shaul Yalovsky2, Toshiro Ito1 and Hao Yu1,†

Flowering plants produce floral meristems in response to intrinsic and extrinsic flowering inductive signals. In Arabidopsis, the floral meristem identity genes LEAFY (LFY) and APETALA1 (AP1) are activated to play a pivotal role in specifying floral meristems during floral transition. We show here that the emerging floral meristems require AP1 to partly specify their floral identities by directly repressing a group of flowering time genes, including SHORT VEGETATIVE PHASE (SVP), AGAMOUS-LIKE 24 (AGL24) and SUPPRESSOR OF OVEREXPRESSION OF CO1 (SOC1). In wild-type plants, these flowering time genes are normally downregulated in emerging floral meristems. By post-translational activation of an AP1-GR fusion protein and chromatin immunoprecipitation assays, we further demonstrate the repression of these flowering time genes by induced AP1 activity and in vivo AP1 binding to the cis-regulatory regions of these genes. These findings indicate that once AP1 is activated during the floral transition, it acts partly as a master repressor in floral meristems by directly suppressing the expression of flowering time genes, thus preventing the continuation of the shoot developmental program.

KEY WORDS: Floral meristem identity, APETALA1, Flowering time gene, Transcriptional regulation, Arabidopsis

INTRODUCTION

During floral transition in Arabidopsis, a complex regulatory network in response to endogenous and environmental signals mediates the activity of the floral meristem identity genes LEAFY (LFY) and APETALA1 (AP1) to specify floral meristems on the flanks of the shoot apical meristem (Bowman et al., 1993; Ferrandiz et al., 2000; Huala and Sussex, 1992; Irish and Sussex, 1990; Mandel and Yanofsky, 1995b; Parcy et al., 2002; Ratcliffe et al., 1999; Weigel et al., 1992). When the activity of either gene is lost or upregulated, meristems that would normally develop into flowers are partly converted into shoot-like structures or vice versa. This implies that both genes are crucial regulators mediating the specification of floral meristems. It has been suggested that LFY and AP1 antagonize the activity of the shoot identity gene TERMINAL FLOWER 1 (TFL1) to establish floral meristems, which might be affected indirectly by the TFL1 function in modulating the rate of shoot apical phase transitions in the Arabidopsis life cycle (Bradley et al., 1997; Liljegren et al., 1999; Parcy et al., 2002; Ratcliffe et al., 1998).

LFY plays dual roles in determining floral meristem identity and floral organ patterning via AP1 and other floral homeotic genes (Parcy et al., 1998; Wagner et al., 1999). AP1 is specifically expressed in young floral meristems, marking the start of flower development (Mandel et al., 1992). During floral transition, activation of AP1 by LFY and a complex of FLOWERING LOCUS T (FT), a flowering time integrator, and FD, a bZIP transcription factor, indicates an important regulatory function of AP1 in the specification of floral meristem identity (Abe et al., 2005; Huang et al., 2005; Wagner et al., 1999; Wigge et al., 2005).

It has been shown that AP1 is involved in the regulation of genes promoting either floral organ formation or inflorescence commitment in floral meristems (Hill et al., 1998; Ng and Yanofsky, 2001; Tilly et al., 1998; Yu et al., 2004). Several lines of evidence suggest that AP1 activates B class homeotic genes, especially APETALA3 (AP3), to determine the identity of petals and stamens. First, whereas AP3 expression is quite normal in ap1 mutants and reduced in lfy mutants, its expression is almost undetectable in lfy ap1 double mutants (Weigel and Meyerowitz, 1993), indicating that AP1 can function with LFY to regulate AP3 expression. Second, in vitro experiments have demonstrated the potential binding of AP1 protein to the AP3 cis-regulatory elements (Hill et al., 1998; Riechmann et al., 1996). Mutations in these elements abolish the AP3-specific expression (Tilly et al., 1998), suggesting that AP1 may directly regulate AP3 expression via these cis-acting regions. Lastly, expression of translational fusions of AP1 with the strong transcriptional activation domain of VP16 has revealed that AP1 can activate the expression of AP3 and another B class gene, PISTILLATA, in spatially specific domains through an F-box-containing protein, UNUSUAL FLORAL ORGANS, in early-arising flowers (Ng and Yanofsky, 2001). While these studies suggest that AP1 acts as transcriptional activator in floral meristem development, repression of AGAMOUS-LIKE 24 (AGL24), a promoter of inflorescence identity, by AP1 implies that AP1 could also be a repressor (Yu et al., 2004). Thus, AP1 may play dual roles in regulating the floral meristem development by activating or repressing different sets of genes that would determine the different fate of a floral meristem.

In this study, we show that the emerging floral meristems require AP1 to directly repress a group of flowering time genes to partly specify their floral identities. Without AP1 activity, the ectopic expression of these genes transforms floral meristems into various shoot structures. Therefore, AP1 partly acts as a repressor in the floral meristem to suppress the genes required for the control of flowering time. As AP1 expression in floral stage 1 indicates the outcome of the integration of flowering inductive signals (Mandel
et al., 1992). *API* stands out to be a key coordinator providing feedback regulation of flowering time genes in the switch from vegetative to reproductive growth in *Arabidopsis*.

**MATERIALS AND METHODS**

**Plant materials**

All transgenic plants or mutants of the same Columbia background were used for calculating flowering time, while those in the Landsberg *erecta* (Ler) background were used for the examination of floral phenotypes. The Ler near-isogenic *svp-41* line was obtained by three backcrosses of the *ŕvp-41* Columbia line (Hartmann et al., 2000) into *Ler* line was obtained by three backcrosses of the *Ler* background were used for the examination of floral phenotypes. The Ler near-isogenic *svp-41* line was obtained by three backcrosses of the *spar-41* Columbia line (Hartmann et al., 2000) into *Ler* background were used for the examination of floral phenotypes.

**Primer pairs used for ChIP assays**

<table>
<thead>
<tr>
<th>Primer pairs used for ChIP assays</th>
<th>5'-AACTGGTTTTGTTATGTTGTTTACAT-3'</th>
</tr>
</thead>
</table>

**Table 1. Primers used in this study**

<table>
<thead>
<tr>
<th>Primers used for genotyping, cloning and expression analyses</th>
<th>5'-GGATGCAACCTCCTTTCATGAG-3'</th>
</tr>
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</table>

**Plasmid construct**

To produce 35S: *SVP* construct, the coding region of *SVP* was amplified using primers P1 and P2 (Table 1). The restriction enzyme *NheI* cleaved only the mutant DNA, generating two fragments, of 105 and 374 bp.

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the target gene and the Ct of TUB2 (ΔCt=ΔCt_target gene–ΔCt_TUB2) was used to obtain the normalized expression of target genes, which corresponds to 2^−ΔΔCt. The expression of AGL24, SVP, SOC1 and TUB2 was examined by the primer pairs P15 and P16, P5 and P6, P7 and P8, and P13 and P14, respectively (Table 1).

**In situ hybridization and GUS activity analysis**

Non-radioactive in situ hybridization was carried out according to a published protocol (Long and Barton, 1998). For synthesis of SOC1 and SVP RNA probes, the 3′ end gene-specific regions were amplified by P9 and P10 primers and P11 and P12 primers (Table 1), respectively, and cloned into pGEM-T Easy vector (Promega) to produce plasmids pHY303 (SOC1) and pHY305 (SVP). They were used as templates for in vitro transcription by the DIG RNA Labeling Kit (Roche Molecular Biochemicals). In situ localization of GUS activity was performed as previously described (Yu et al., 2002).

**ChIP assays**

A peptide EQWDQQNQGHNMPPPLPPQQ corresponding to amino acid residues 184–203 of AP1 was synthesized in the Peptide Synthesis Facility of the Weizmann Institute of Science, Rehovot, Israel. Synthetic peptide was conjugated to keyhole limpet hemocyanin (KLH) with the linker maleimide (Pierce) and used as an antigen for generating the anti-AP1 serum in rabbits, conjugated to keyhole limpet hemocyanin (KLH) with the linker maleimide (Pierce) and used as an antigen for generating the anti-AP1 serum in rabbits, and for affinity purification of the antibody.

Chromatin immunoprecipitation (ChIP) assays were carried out as described previously (Ito et al., 1997; Wang et al., 2002) with minor modifications. Inflorescence tissues were collected and fixed with 1% formaldehyde for 40 minutes under vacuum. Chromatin was isolated and sonicated to produce DNA fragments under 500 bp. The solubilized chromatin was pre-cleared by incubating with normal rabbit IgG (sc-2027, Santa Cruz Biotecntology) and Protein G-Plus agarose beads (sc-2002, Santa Cruz Biotecntology) for 1 hour at 4°C. After centrifugation, the supernatant was divided equally into two parts. One part was incubated with anti-AP1 serum for 4 hours, while the other part was mixed with normal rabbit IgG as a negative control. Protein G-Plus agarose beads were then added for incubation for another hour under the same conditions. The beads were washed five times and incubated with the elution buffer supplemented with 1 μg RNase A (1 mg/ml) for 30 minutes at 37°C. Precipitated DNAs were subsequently recovered and used for enrichment test by real-time PCR assays. For ChIP assay of mock- or DEX treated samples, inflorescences of ap1-1 35S:AP1-GR containing floral buds of stages 1-10 were collected 4 hours after a single treatment.

We performed two fully independent ChIP experiments using samples collected separately. For each ChIP experiment, real-time PCR assay of immunoprecipitated DNAs with selected primer pairs (Table 1) was conducted in triplicate. To calculate ChIP fold enrichment, the relative amount of a target DNA fragment was first normalized against a TUB2 genomic fragment to get the difference between the cycle threshold (ΔCt=ΔCt_target gene–ΔCt_TUB2). The fold enrichment was then obtained by comparing the values between DNAs immunoprecipitated by anti-AP1 serum and IgG (ΔΔCt=ΔCt_Anti-AP1–ΔCt_IgG). The enrichment of another unrelated DNA sequence from the ACTIN 2/7 (ACT7 – TAIR) gene (Johnson et al., 2002) that is constitutively expressed in Arabidopsis was also used as a negative control. The results of the first set of ChIP experiments are shown in Fig. 6, which were also replicated in the second set of ChIP experiments (data not shown).

**RESULTS**

**Ectopic expression of flowering time genes mimics ap1-1 phenotypes**

To elucidate the regulatory networks during floral transition, we performed a series of genetic crossings among a group of known flowering time genes. Three members of the MADS-box family of DNA-binding transcription factors, SHORT VEGETATIVE PHASE (SVP) (Hartmann et al., 2000), AGL24 (Michaels et al., 2003; Yu et al., 2002) and SUPPRESSOR OF OVEREXPRESSSION OF CO1 (SOC1; also known as AGL20 – TAIR) (Borner et al., 2000; Lee et al., 2000; Samach et al., 2000), exhibit close relationships in terms of flowering time control. While AGL24 and SOC1 regulate each other (Michaels et al., 2003; Yu et al., 2002), almost complete suppression of agl24-1 and 35S:AGL24 by svp-41 and 35S:SVP (Gregis et al., 2006) (data not shown), respectively, suggests that SVP is an important repressor located downstream of AGL24 in the regulatory network mediating floral inductive signals from multiple promotion pathways.

In addition to their effects on flowering time, SVP, AGL24 and SOC1 were distinguished from most other flowering time genes because transgenic plants overexpressing these three genes singly or in combination showed significant defects in floral meristem development. 35S:AGL24 plants often generated a central primary flower with extra secondary flowers in the axes of leaf-like sepals (Fig. 1A). The base of the ovaries of 35S:AGL24 flowers elongated...
like an inflorescence stem, and ectopic inflorescences eventually formed in the swollen ovaries (Yu et al., 2004). These phenotypes indicate the partial conversion of the floral meristem into the inflorescence meristem. 35S::SOC1 flowers were relatively normal with light green sepaloid petals (Fig. 1B). 35S::SVP flowers usually initiated shoot-like structures with chimeric characteristics of a vegetative shoot and a flower (Fig. 1C,D). Continuous growth of 35S::SVP flowers demonstrated that leaves emerged continuously on the substantially elongated internodes in either a whorled or a spiral mode (Fig. 1D), and stamens occasionally arose in the axes of leaves (Fig. 1C). The elongated 35S::SVP flowers eventually terminated with a mixture of leaves, carpeloid leaves and stamens without clear carpel structures (Fig. 1E). These phenotypes demonstrated that when ectopically expressed in flowers, SVP promoted the shoot identity. A comparison of the average number of flowers produced in each pedicel or peduncle of 35S::AGL24, 35S::SVP and 35S::SOC1 revealed that overexpression AGL24 produced more ectopic floral structures in floral meristems than SVP and SOC1, suggesting that AGL24 plays a main role in promoting the inflorescence characteristics (Table 2).

Further combinations of the above single transgenic plants showed that the interactions among AGL24, SVP and SOC1 transformed floral meristems into various shoot-like structures that were partially similar to the inflorescence-like floral structures in loss of function of AP1 (Bowman et al., 1993). Flowers of double transgenic plants for 35S::AGL24 35S::SVP developed like inflorescence shoots with the continuous production of leaves and the corresponding secondary flowers in their axes on the elongated internodes (Fig. 1F), which partially mimicked the severe defects observed in floral structures arising at basal positions of the inflorescence of ap1-1 (Fig. 1G). Although 35S::SOC1 produced nearly normal flowers, it enhanced the production of secondary flowers in floral meristems at basal positions of the inflorescence of 35S::SOC1 35S::AGL24 (Fig. 1H), which mimicked the intermediate defects observed in floral structures arising at median positions of the inflorescence of ap1-1 (Fig. 1I). Flowers of 35S::SOC1 35S::SVP developed like the initial floral structures of 35S::SVP (Fig. 1C). The internode in 35S::SOC1 35S::SVP was not elongated and generation of secondary floral structures were only sometimes observed (data not shown). The phenotypes described above indicate that AP1 may be a potential upstream regulator of these genes, and that misexpression of AGL24, SVP and SOC1 in floral meristems affects normal flower development.

### Inflorescence character of ap1-1 is reduced by agl24, svp and soc1

We then tested if the floral phenotypes of loss of function of AP1 were partially caused by the activity of SVP, AGL24 and SOC1. The ap1-1 strong mutants exhibited at least two types of defects in floral meristem specification and perianth organ specification. The disturbed specification of floral meristems in ap1-1 was manifested by the phenotypes showing that flowers arising at basal positions of the ap1-1 inflorescence generated secondary flowers or inflorescences in the axes of the leaf-like first whorl organs on the elongated internodes, whereas flowers arising at median or apical positions generated fewer or no secondary flowers in the axes of first whorl organs without internode elongation (Bowman et al., 1993). The generation of secondary flowers or inflorescences in floral structures arising from individual floral meristems at basal positions of the main inflorescence was significantly reduced in the double mutants of ap1-1 agl24-1, ap1-1 svp-41 and ap1-1 soc1-2 compared with that in ap1-1 single mutants (Table 2 and Fig. 2B,D,F,H), and the phenotype of supernumerary inflorescence of ap1-1 was suppressed accordingly (Fig. 2A,C,E,G). In ap1-1 agl24-1 and ap1-1 svp-41, most of the flower meristems developed as single flowers occasionally with secondary flowers, but without internode elongation (Fig. 2D,F). In the flowers of ap1-1 soc1-2, the number of secondary floral structures was reduced compared with that in ap1-1, but these floral structures usually developed like inflorescences with internode elongation (Table 2 and Fig. 2H). A close examination of the mean number of floral structures produced in each pedicel or peduncle of ap1-1 agl24-1, ap1-1 svp-41, and ap1-1 soc1-2 showed that loss of these genes function caused almost similar effect on reducing the ectopic floral structures in ap1-1 (Table 2). Triple mutants created by genetic crossing of the above double mutants further decreased the mean number of floral structures in each pedicel or peduncle (Table 2). These observations indicate that all these three genes partly contribute to the shoot characteristics in ap1-1 floral meristems, and that AP1 activity may be required for the regulation of expression of these genes.

Another striking phenotype in ap1-1 was the disruption of perianth floral organ development (Bowman et al., 1993). The first whorl sepals of ap1-1 flowers developed into bract-like structures, whereas the second whorl petals were usually absent, especially in the floral structures derived from basal positions of the inflorescence. The sepal defect of ap1-1 was not obviously rescued by agl24-1, svp-41 and soc1-2, because bract-like structures are still present in the double mutants. However, agl24-1, rather than svp-41 and soc1-2, alleviated the second whorl defect of ap1-1 flowers as one or two petals were often observed in the flowers at basal positions of the ap1-1 agl24-1 inflorescence (Fig. 2D,F,H).

#### Ectopic expression of SVP and SOC1 in ap1-1 floral meristems

In wild-type plants, AP1 was strongly expressed in emerging floral meristems and perianth organ primordia of floral meristems after stage 3 (Fig. 3A). To examine if AP1 acts as a repressor of flowering time genes in floral meristems, we compared the expression patterns

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**Table 2. Number of flowers per pedicel/peduncle* in mutants and transgenic plants**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Basal position of flowers after cauline leaf production</th>
<th>No. of plants scored</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1-5</td>
<td>6-10</td>
</tr>
<tr>
<td>Wild type (Ler)</td>
<td>1.0±0.2</td>
<td>1.0±0.2</td>
</tr>
<tr>
<td>ap1-1</td>
<td>7.8±1.5</td>
<td>3.7±1.2</td>
</tr>
<tr>
<td>soc1-2</td>
<td>1.0±0.2</td>
<td>1.0±0.2</td>
</tr>
<tr>
<td>svp-41</td>
<td>1.0±0.2</td>
<td>1.0±0.2</td>
</tr>
<tr>
<td>agl24-1</td>
<td>1.0±0.2</td>
<td>1.0±0.2</td>
</tr>
<tr>
<td>ap1-1 agl24-1</td>
<td>2.8±0.6</td>
<td>1.7±0.5</td>
</tr>
<tr>
<td>ap1-1 soc1-2</td>
<td>3.2±0.4</td>
<td>1.6±0.3</td>
</tr>
<tr>
<td>ap1-1 svp-41</td>
<td>3.6±0.8</td>
<td>1.8±0.4</td>
</tr>
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<td>ap1-1 soc1-2 svp-41</td>
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<td>1.2±0.3</td>
</tr>
<tr>
<td>ap1-1 soc1-2 agl24-1</td>
<td>2.5±0.4</td>
<td>1.4±0.4</td>
</tr>
<tr>
<td>ap1-1 svp-41 agl24-1</td>
<td>2.3±0.2</td>
<td>1.4±0.1</td>
</tr>
<tr>
<td>35S::SOC1</td>
<td>1.0±0.2</td>
<td>1.0±0.2</td>
</tr>
<tr>
<td>35S::AGL24</td>
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<td>1.9±0.6</td>
</tr>
<tr>
<td>35S::SVP</td>
<td>1.7±0.7</td>
<td>1.3±0.4</td>
</tr>
</tbody>
</table>

*The term pedicel/peduncle is used to define the floral stem for either true flowers or the flowers bearing ectopic flowers or inflorescences. A floral-like structure terminated by a gynoecium is considered a flower (Bowman et al., 1993).

†Each value represents the mean±d.d.

‡Ectopic floral-like structures generated from the floral meristems at basal positions of the main inflorescences usually developed like secondary inflorescences.

§In our culture conditions, 35S::SOC1 inflorescences terminated after producing less than ten flowers.

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of SVP and SOC1 in floral meristems arising at basal positions of the inflorescences of wild-type and ap1-1 plants by in situ hybridization. In wild-type plants during floral transition, SVP was expressed in the shoot apex and the corresponding cauline leaf (Hartmann et al., 2000). SVP expression was barely detectable in the inflorescence meristem, but strongly localized in the stage 1 floral meristem (Fig. 3B,C). Its expression was mainly confined to a lower part of the stage 2 floral meristem (Fig. 3B,C) and not detectable in the floral meristems after stage 3 (Fig. 3D,E). In ap1-1 mutants, by comparing in situ hybridization results with SVP sense (Fig. 3F) and antisense (Fig. 3G-I) probes, we detected strong SVP expression in both stage 1 and stage 2 floral meristems. In ap1-1 floral meristems at stages 3 and 4, ectopic SVP expression was detectable in the adaxial surface of the first whorl organs (Fig. 3J-L). At later stages of floral development, SVP was ectopically expressed in certain regions that might potentially emerge as new shoot meristems or floral meristems in ap1-1 basal flowers (Fig. 3J).

The ectopic expression of SOC1 was also observed in ap1-1 floral meristems. In wild-type plants, SOC1 expression was strong in the inflorescence meristem, but almost absent in the floral meristems before stage 3 (Fig. 4A-D). Its expression was again detectable in the centre of the floral meristem after stage 3 (Fig. 4E) and in the stamen and carpel primordia of later-stage floral meristems (Fig. 4F). These expression patterns are comparable to previously published results showing the subtle change of SOC1 expression in early-stage floral meristems (Borner et al., 2000; Lee et al., 2000; Samach et al., 2000). In ap1-1, in situ hybridization with SOC1 sense (Fig. 4G) and antisense (Fig. 4I-N) probes revealed that SOC1 was ectopically expressed throughout young floral meristems from stages 1 to early stage 3 compared with its expression in wild-type floral meristems (Fig. 4A-D). SOC1 expression was also detectable with the antisense probe in the whole zone of the floral meristems at stage 3 (Fig. 4O) or later stages (Fig. 4P) compared with background staining with the sense probe (Fig. 4G,H). These results, together
with the previous report showing the ectopic expression of *AGL24* in the floral meristems of *ap1-1* (Yu et al., 2004), suggest that AP1 may be required for the repression of a group of flowering time genes in floral meristems, at least including *AGL24*, *SVP* and *SOC1*.

**Induced AP1 activity represses the expression of SVP and SOC1**

In a previous study, we have established a transgenic line of *ap1-1 35S:AP1-GR*, where the biologically functional *AP1-GR* fusion could rescue *ap1-1* phenotypes in a steroid-dependent manner (Yu et al., 2004). By using this line, we found that *AGL24* expression in inflorescence apices was repressed by induced AP1 activity even in the presence of cycloheximide, an efficient inhibitor of protein synthesis, suggesting that *AGL24* is an immediate target of transcriptional repression by AP1.

To test whether AP1 activity is also able to repress the expression of *SVP* and *SOC1*, we used the same transgenic line of *ap1-1 35S:AP1-GR* to quantify the expression levels of both genes upon the induction of AP1 activity. Quantitative real-time RT-PCR analyses showed that dexamethasone treatment of inflorescence apices of *ap1-1 35S:AP1-GR* for 2 hours or longer resulted in continuous reduction of *SVP* and *SOC1* RNA levels relative to mock-treated controls (Fig. 5A,B). In the experiment with combined treatment of dexamethasone and cycloheximide at the 4 hour time point, the repression of *SVP* and *SOC1* by induced AP1 activity was not blocked by cycloheximide (Fig. 5C). These results suggest that both *SVP* and *SOC1*, like *AGL24*, are immediate targets of transcriptional repression by AP1.

We further compared the expression of *AGL24*, *SVP* and *SOC1* in wild-type and *ap1-1* inflorescence apices containing floral buds of stage 1-10 and found that the expression of these three genes was much elevated in *ap1-1* (Fig. 5D). This substantiates that AP1 may transcriptionally repress these three genes in young floral meristems.

**AGL24, SVP and SOC1 are direct targets of AP1**

To determine whether AP1 is a direct repressor of these flowering time genes, we performed ChIP assays to detect the in vivo binding of AP1 protein to the regulatory regions of *AGL24*, *SVP* and *SOC1*. We have found that a *P_{AGL24}:GUS* reporter gene containing 4.7 kb of sequence upstream of the *AGL24* stop codon and a *P_{SVP}:GUS* reporter gene containing 5.1 kb of sequence upstream of the *SVP* stop codon could recapitulate the endogenous *AGL24* and *SVP* mRNA expression patterns (C.L., D. Li and H.Y., unpublished). Also, it has been reported that a *SOC1* genomic DNA fragment including 1.4 kb of sequence upstream of the transcriptional start site was able to complement the *soc1* mutation (Samach et al., 2000). Thus, we scanned the sequences encompassing the above regulatory regions of *AGL24*, *SVP* and *SOC1* for the CC(A/T)6GG (CArG) motif (Riechmann and Meyerowitz, 1997), a canonical binding site for MADS-domain proteins, with a maximum of one nucleotide mismatch (Fig. 6A). Gene-specific primers flanking the regions near the identified CArG motifs (Table 1) were designed for quantification of the enrichment of the DNA sequences associated with the AP1 or AP1-GR fusion proteins, both of which were specifically precipitated by the anti-AP1 serum in ChIP assays (Fig. 6B-D). Real-time PCR assay of ChIP-enriched DNAs revealed that the fragments of *AGL24-4*, *SVP-4*, *SOC1-1* and *SOC1-2* were more significantly enriched than other fragments by the specific anti-AP1 serum over IgG in wild-type inflorescences, while all the tested DNA fragments of *AGL24*, *SVP* and *SOC1* as well as a control genomic fragment of *ACTIN2/7* gene were not enriched in *ap1-1* (Fig. 6E-G).
mock-treated inflorescences of *ap1-1 35S:AP1-GR*, none of the fragments tested was enriched by the specific anti-AP1 serum over IgG, whereas in dexamethasone-treated samples, four fragments (AGL24-4, SVP-4, SOC1-1 and SOC1-2) demonstrated again highest enrichment (Fig. 6E-G). These results show that both the endogenous AP1 protein and the biologically functional AP1-GR protein interact directly with the regulatory sequences of *AGL24*, *SVP* and *SOC1* genes.

To evaluate whether the CArG motif near the identified AP1-binding site is responsible for the regulation of *AGL24* expression in floral meristems, we made a translational fusion between an *AGL24* genomic fragment containing 4.7 kb of sequence upstream of the stop codon and the GUS reporter gene (Fig. 7A). This construct provided a similar pattern of GUS staining to endogenous *AGL24* expression (Fig. 7B) (Michaels et al., 2003; Yu et al., 2004), which is strong in the inflorescence shoot apical meristem but decreased in young floral meristems. Based on this construct, we created another reporter gene cassette where the CArG motif near the identified AP1-binding site was mutated (Fig. 7A). The transformants bearing this construct exhibited ectopic GUS staining in young floral meristems (Fig. 7C). These results confirm that AP1 directly binds to the tested site to repress *AGL24* expression in young floral meristems.

**DISCUSSION**

Here we have shown that ectopic expression of several flowering time genes partially mimics the phenotypes of *ap1-1* floral structures (Fig. 1). Overexpression of *AGL24* transformed the floral meristem into the inflorescence meristem that had the potential to generate new floral meristems, while overexpression of *SOC1* enhanced the production of secondary flowers in flower meristems of 35S:*AGL24*. Overexpression of *SVP* produced chimeric floral structures bearing the typical features of vegetative shoots, such as the continuous generation of leaves instead of floral organs. These phenotypes suggest that *SVP* functions in the maintenance of vegetative shoot identity, while *AGL24*, enhanced by *SOC1*, mainly promotes inflorescence identity. This conclusion is consistent with the respective function of *AGL24* and *SOC1* as flowering activators and of *SVP* as a flowering repressor (Bornert et al., 2000; Hartmann et al., 2000; Lee et al., 2000; Michaels et al., 2003; Samach et al., 2000; Yu et al., 2002).

AP1 activity is required for repressing the ectopic expression of *AGL24*, *SVP* and *SOC1* at different floral developmental stages. Both *AGL24* (Yu et al., 2004) and *SOC1* (Fig. 4I-N) are ectopically expressed throughout the emerging *ap1-1* floral meristems, which coincides with the loss of AP1 activity in the same regions (Mandel et al., 1992), indicating that AP1 represses *AGL24* and *SOC1* early in emerging floral meristems. However, *SVP* expression was regulated in a different pattern. In wild-type plants, *SVP* expression is still detectable in stage 1 and 2 floral meristems despite the presence of AP1 activity (Fig. 3B,C). In *ap1-1*, *SVP* expression slightly increases in the stage-2 floral meristem and is only detectable in the adaxial surface of the first whorl floral organs or the regions that could potentially emerge as new meristems in floral meristems after stage 2 (Fig. 3J-L). Thus, AP1 specifically represses *SVP* mainly in floral meristems after stage 2, which may be temporally and spatially mediated by other AP1 co-factors. In support of the suggestion that AP1 is necessary for repression of these flowering time genes, loss of function of *AGL24*, *SVP* and *SOC1* or their combinations reduces the inflorescence character of *ap1-1* flowers at various degrees (Table 2, Fig. 2).
By post-translational activation of AP1-GR, we further demonstrated the repression of SVP and SOC1 by induced AP1 activity (Fig. 5A,B). Moreover, downregulation of SVP and SOC1 by dexamethasone treatment of AP1-GR inflorescence apices was not affected by cycloheximide, indicating that repression of both genes by AP1 is independent of protein synthesis (Fig. 5C). These results, together with the previous finding (Yu et al., 2004), suggest that AGL24, SVP and SOC1 are all early targets of transcriptional repression by AP1. ChIP assays using specific anti-AP1 antibodies further revealed in vivo AP1 binding to the cis-regulatory regions of these genes (Fig. 6), thus suggesting that AP1 acts as a direct regulator repressing a group of flowering time genes, including AGL24, SVP, and SOC1 in the floral meristem. A further experiment by promoter mutagenesis substantiates the idea that AP1 represses AGL24 expression in young floral meristems by directly binding to its genomic region (Fig. 7). In our previous study (Yu et al., 2004), AGL24 was suggested as an early target of transcriptional repression by AP1. However, this study could not establish whether or not AP1 is a real transcriptional repressor, because the effect of AP1 on AGL24 could be mediated by other molecules such as miRNAs, which could not be revealed by applying the translation inhibitor cycloheximide in our AP1-GR inducible system. The results shown here demonstrate that AP1 at least functions as a transcriptional repressor in wild-type floral meristems and directly represses three flowering time genes to prevent the reversion of floral meristems into shoot meristems.

Although AP1 and LFY function together as major floral meristem identity genes, LFY and AP1 may specify floral meristem identity by distinct mechanisms. In a previous study, we have suggested that LFY could repress indirectly AGL24 expression in young floral meristems by directly binding to its genomic region (Fig. 7). In our previous study (Yu et al., 2004), AGL24 was suggested as an early target of transcriptional repression by AP1. However, this study could not establish whether or not AP1 is a real transcriptional repressor, because the effect of AP1 on AGL24 could be mediated by other molecules such as miRNAs, which could not be revealed by applying the translation inhibitor cycloheximide in our AP1-GR inducible system. The results shown here demonstrate that AP1 at least functions as a transcriptional repressor in wild-type floral meristems and directly represses three flowering time genes to prevent the reversion of floral meristems into shoot meristems.
Specification of floral meristem

DEVELOPMENT

may provide an essential channel to coordinate these two events

(Fig. 6E was mutated. (B, C) GUS staining in inflorescence apices of the transformants containing ProAGL24:GUS (B) and its derived construct with the mutated CArG box (C). At least 12 independent lines for each construct were analyzed and representative images are shown. Scale bars: 100 µm for B, C.

Fig. 7. Mutagenesis of AP1-binding site causes ectopic expression of AGL24 in young floral meristems. (A) Schematic of the ProAGL24::GUS construct where the 4.7 kb Arabidopsis AGL24 genomic sequence was translationally fused with the GUS gene. The native CArG box near the AGL24-4 fragment identified in Fig. 6E was mutated. (B, C) GUS staining in inflorescence apices of the transformants containing ProAGL24::GUS (B) and its derived construct with the mutated CArG box (C). At least 12 independent lines for each construct were analyzed and representative images are shown. Scale bars: 100 µm for B, C.

transformants containing ProAGL24:GUS

suggesting that different threshold levels of AP1 are required for repression of different target genes. As LFY directly upregulates AP1 (Wagner et al., 1999), LFY may partly specify floral meristem identity via mediating the expression levels of AP1. It has recently been shown that AP1 is activated by a flowering complex of FT and FD that is independent of LFY activity (Abe et al., 2005; Huang et al., 2005; Wigge et al., 2005). Activation of AP1 for direct repression of flowering time genes in the floral meristem could be a key regulatory pathway that is parallel with activation of LFY for promoting floral organ identity genes (Pарть et al., 1998; Weigel et al., 1992). Direct regulation of AP1 by LFY may provide an essential channel to coordinate these two events during the specification of the floral meristem identity.

CAULIFLOWER (CAL) and FRUITFULL (FUL; also known as AGL8 – TAIR) are other two regulators involved in floral meristem formation, as ap1 ful mutants show complete transformation of floral meristems into inflorescence meristems and ap1 cal ful mutants show even stronger phenotypes with more vegetative traits in the transformed meristems (Bowman et al., 1993; Ferrandiz et al., 2000; Mandel and Yanofsky, 1995a). It is possible that the flowering time genes in this study are controlled redundantly by AP1 and CAL, because AP1 and CAL have overlapping expression patterns and act redundantly to specify floral meristems (Bowman et al., 1993; Kemph et al., 1995). On the contrary, FUL may not be directly involved in the regulation of flowering time genes, as it is not expressed in floral meristems at early stages (Mandel and Yanofsky, 1995a).

Interestingly, AP1 has shown dual functions as either an activator or a repressor in the floral meristem. Previous studies have revealed that AP1 acts as a transcriptional activator mediating the specification of petals by regulating B class homeotic genes (Hill et al., 1998; Ng and Yanofsky, 2001), and the current study has uncovered a new facet of AP1 as an important transcriptional repressor in preventing the reversion of floral meristems into shoot meristems. The fascinating variety of activities ascribed to AP1 implies that it may be a part of different protein complexes or subject to various post-translational modifications that lead to different developmental regulations. One example is that AP1 protein could be farnesylated both in vitro and in vivo and that the non-prenylated form of AP1 could generate novel phenotypes when ectopically expressed in Arabidopsis (Yalovsky et al., 2000), implying that protein farnesylation plays a role in modulating AP1 function.

It is noteworthy that yeast two-hybrid assays have revealed broad protein interactions between three flowering time regulators examined in this study (SOC1, AGL24 and SVP) and some floral organ identity genes (de Folter et al., 2005). In particular, the protein interaction of AP1 and AGL24 or AP1 and SVP may mediate flower development at early stages (de Folter et al., 2005; Gregis et al., 2006; Pelaz et al., 2001). When the double mutantssvp agl24 were grown at 30°C, their flowers exhibited homeotic transformation in all four whorls of floral organs due to ectopic expression of function B and C homeotic genes. The similar floral defects were also observed under normal growth conditions (22°C) in ap1svp agl24 (Gregis et al., 2006). These phenotypes were similar to those observed in the single or double mutants of leunig (lug) and seuss (sen) (Franks et al., 2002; Liu and Meyerowitz, 1995). In vitro assays further revealed that the MADS-box dimers AP1-AGL24 and AP1-SVP weakly interacted with the LUG-SEU co-repressor in yeast, indicating that AP1, together with AGL24 and SVP, is involved in the recruitment of LUG-SEU repressor complex for the regulation of flower development (Gregis et al., 2006). Transcriptional regulation of flowering time genes by AP1 mediates the specification of floral meristems, and possibly affects the components involved in the protein interactions required for further floral organ development. An intriguing aspect is to investigate whether recruitment of different components into an AP1 protein complex would cause distinct setting of transcriptional activities of AP1.

The repressive function of AP1 seems crucial for determining the identities of perianth floral organs, because ectopic expression of several flowering time genes in the absence of AP1 is sufficient to transform perianth organs into new flowers or inflorescences with or without internode elongation. This significantly affects the structure of floral perianth organs. The orthologs of Arabidopsis AP1, termed euAP1 gene clade, are only present in the core eudicots that comprise the majority of extant angiosperm species (Litt and Irish, 2003). The fixed floral perianth structures in these plants are in contrast to the plastic ones in non-eudicot and non-core eudicot species. It will be interesting to examine if the orthologs of the flowering time genes revealed in this study are normally expressed in the flowers of non-eudicot and non-core eudicot species that lack euAP1 genes. This will be important for addressing the puzzle of whether repression of flowering time genes by AP1 orthologs contributes to the variation of floral perianth structures in flowering plants.

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