Direct interaction of AGL24 and SOC1 integrates flowering signals in Arabidopsis

Chang Liu1,2, Hongyan Chen1,2, Hong Ling Er1, Hui Meng Soo3, Prakash P. Kumar1,2, Jin-Hua Han1, Yih Cherng Liou1 and Hao Yu1,2,*

During the transition from vegetative to reproductive growth, the shoot meristem of flowering plants acquires the inflorescence identity to generate flowers rather than vegetative tissues. An important regulator that promotes the inflorescence identity in Arabidopsis is AGAMOUS-LIKE 24 (AGL24), a MADS-box transcription factor. Using a functional estradiol-inducible system in combination with microarray analysis, we identified AGL24-induced genes, including SUPPRESSOR OF OVEREXPRESsion OF CO 1 (SOC1), a floral pathway integrator. Chromatin immunoprecipitation (ChIP) analysis of a functional AGL24-6HA-tagged line revealed in vivo binding of AGL24-6HA to the regulatory region of SOC1. Mutagenesis of the AGL24 binding site in the SOC1 promoter decreased ProsOC1:GUS expression and compromised SOC1 function in promoting flowering. Our results show that SOC1 is one of the direct targets of AGL24, and that SOC1 expression is upregulated by AGL24 at the shoot apex at the floral transitional stage. ChIP assay using a functional SOC1-9myc-tagged line and promoter mutagenesis analysis also revealed in vivo binding of SOC1-9myc to the regulatory regions of AGL24 and upregulation of AGL24 at the shoot apex by SOC1. Furthermore, we found that as in other flowering genetic pathways, the effect of gibberellins on flowering under short-day conditions was mediated by the interaction between AGL24 and SOC1. These observations suggest that during floral transition, a positive-feedback loop conferred by direct transcriptional regulation between AGL24 and SOC1 at the shoot apex integrates flowering signals.

KEY WORDS: Flowering time, MADS-box transcription factor, Transcriptional regulation, Chromatin immunoprecipitation, Gibberellin, Arabidopsis

INTRODUCTION

The transition from vegetative to reproductive development in Arabidopsis is mediated by multiple genetic pathways in response to developmental cues and environmental signals (Amasino, 2004; Balasubramanian et al., 2006; Blazquez et al., 2003; Cerdan and Chory, 2003; Halliday et al., 2003; Simpson and Dean, 2002). The photoperiod pathway perceives the light quantity and circadian clock, whereas the vernalization pathway responds to low temperatures. The autonomous pathway monitors endogenous cues from specific developmental states, which are independent of environmental signals. The gibberellin (GA) pathway particularly regulates flowering in non-inductive short-day conditions. In addition to these major genetic pathways, the pathways mediating the responses to various wavelengths of light and temperature alteration above a critical threshold have also been suggested to affect flowering. An intricate network of the above pathways promotes floral transition via transcriptional regulation of several floral pathway integrators including FLOWERING LOCUS T (FT), SUPPRESSOR OF OVEREXPRESSION OF CO 1 (SOC1; also known as AGL20 – TAIR) and LEAFY (LFY) (Boss et al., 2004; Mouradov et al., 2002; Parcy, 2005; Simpson and Dean, 2002).

MADS-box genes encode a large family of transcription factors in plants that share a highly conserved MADS-box domain, which recognizes the CC(A/T)6GG (CARg) box on target genes for binding (Riechmann et al., 1996; Shore and Sharrocks, 1995). In Arabidopsis, the MADS-box gene family is a major class of regulators mediating floral transition. AGAMOUS-LIKE 24 (AGL24) is one of the MADS-box genes found to promote flowering (Michaels et al., 2003; Yu et al., 2002). AGL24 expression is detectable in the vegetative shoot apex and is upregulated in the inflorescence apex during floral transition. Transgenic studies of 35S:AGL24 and AGL24 RNA interference lines have shown that the upregulated level of AGL24 expression corresponds to the degree of precocious flowering and that the reduction in AGL24 expression is related to the degree of late flowering, suggesting that AGL24 is a dosage-dependent promoter of flowering.

The expression of AGL24 is barely detectable in the center of emerging floral meristems and is present in floral reproductive organs at later stages (Yu et al., 2004). Overexpression of AGL24 promotes flowering and transforms floral meristems into inflorescence meristems, indicating that AGL24 specifically promotes inflorescence identity. Direct repression of AGL24 and two other flowering time genes, SOC1 and SHORT VEGETATIVE PHASE (SVP), by the floral meristem identity gene APETALA1 (AP1), prevents the continuation of the shoot developmental program, contributing to the specification of floral meristem identity (Liu et al., 2007; Yu et al., 2004). On the other hand, expression of AGL24 and SVP at an appropriate level in the floral meristem is also required for regulation of class B and C floral homeotic genes at a high temperature (Gregis et al., 2006). Therefore, AGL24 regulates both flowering time and flower development.

Previous studies on the role of AGL24 in flowering time control have revealed that AGL24 and SOC1 affect expression of each other (Michaels et al., 2003; Yu et al., 2002), implying that these two MADS-box transcription factors might directly or indirectly interact to mediate flowering. However, AGL24 and SOC1 are differently regulated during floral transition in several aspects. First, although AGL24 expression is regulated by vernalization, it is independent of...
FLORENCING LOcus C (FLC), a potent repressor of flowering (Michaels et al., 2003). By contrast, FLC represses SOC1 expression in the meristem and also delays SOC1 expression by repressing FT, which encodes a protein acting as a long-distance floral signal moving from the leaf to the meristem (Corbesier et al., 2007; Hepworth et al., 2002; Searle et al., 2006). Second, in the photoperiod pathway, AGL24 is affected by CONSTANS (CO), but not by FT (Yu et al., 2002), whereas SOC1 is mainly regulated by FT and indirectly by CO via an unknown DNA-binding factor (Hepworth et al., 2002; Lee et al., 2000; Samach et al., 2000). Lastly, alteration of AGL24 activity determines flowering time partially independently of SOC1, and vice versa, indicating that they can promote flowering in independent pathways (Michaels et al., 2003; Yu et al., 2002). These observations suggest that AGL24 perceives flowering signals that are different from those integrated by SOC1. Therefore, what the exact relationship is between AGL24 and SOC1 and how they interact to affect flowering are essential questions for understanding the integration of flowering signals.

In this study we established and applied a functional estradiol-inducible AGL24 system in combination with microarray analysis to identify AGL24-induced genes including SOC1. We provide evidence that AGL24 and SOC1 directly regulate mutual transcription to integrate flowering signals from several genetic pathways, including the GA pathway. This direct interaction confers a positive-feedback regulation of the expression of AGL24 and SOC1 to a quantitative threshold required for the transition from vegetative to reproductive growth.

MATERIALS AND METHODS
Plant materials and growth conditions
Wild-type and transgenic Arabidopsis plants of the same Columbia ecotype were grown at 22°C under long-day (16 hours light/8 hours dark) or short-day (8 hours light/16 hours dark) conditions. GA treatment of plants was started with seedlings at 1 week after germination, and weekly application of 100 μM GA₃ was performed as published (Moon et al., 2003).

Plasmid construction and plant transformation
For the construction of pER22-AGL24, the AGL24 cDNA was amplified with primers (restriction sites underlined) AGL24-F1-Xhol (5’‐GGCTGGATCCAGCGGAGGAGTGTAGGGAATGTCGGG‐3’) and AGL24-R1-Apal (5’‐ATGGGCCCTTTCACAGGATGAAGAAC‐3’). The digested PCR products were cloned into the pER22 vector. The pER8 vector (Zuo et al., 2000) was cut with Apal and Spel, the cohesive ends filled in, and self-ligated to produce pER22. To construct 35S:AGL24-6HA, the AGL24 cDNA was amplified with primers AGL24-F1-Xhol and AGL24-R1-Apal. The digested PCR products were cloned into the pGreen-35S-HA vector to obtain an in-frame fusion of AGL24-6HA under the control of the 35S promoter. The pGreen-35S-6HA vector was generated by cloning six repetitive HA epitopes into the Spel site of pGreen-35S (Yu et al., 2004).

To construct 35S:SOc1-9myc, the SOc1 cDNA was amplified with primers SOc1-F1-Xhol (5’‐GGCTGGATCCAGCGGAGGAGTGTAGGGAATGTCGGG‐3’) and SOc1-R1-Xmal (5’‐GGCTGGATCCAGCGGAGGAGTGTAGGGAATGTCGGG‐3’). The digested PCR products were cloned into the pGreen-35S-9myc vector under the control of the 35S promoter. The pGreen-35S-9myc vector was generated by cloning nine repetitive myc epitopes into the Spel site of pGreen-35S.

To construct ProAGL24:GUS, the 4.7 kb AGL24 genomic sequence (Fig. 5F) was amplified with primers AGL24-P1-Xmal (5’‐AAGCTCTAGGCCTTTTAATGGGGATCTG‐3’) and AGL24-P4-Spel (5’‐GGACTAAGCTTTCCCAAGATGGAAAGATCCCC‐3’). The digested PCR products were cloned into pHY107. This construct was mutated to produce the mutated sites of M-2003 and M-2039 (Fig. 5F).

For the complementation test, the AGL24 genomic fragment was amplified with primers AGL24-P1-Xmal and AGL24-p-R-Xhol (5’‐CCCTCTAGATCATCTCTCCCAAGATGGAAAGCC‐3’), and the SOC1 genomic fragment was amplified with primers SOc1-P4-Xmal and SOc1-p-R-Xhol (5’‐CCCTCTAGATCATCTCTCCCAAGATGGAAAGCC‐3’). The digested PCR products were cloned into pHY105 (Liu et al., 2007). The constructs containing the mutated forms of the genomic AGL24 and SOC1 fragments were generated using the QuikChange II XL Site-Directed Mutagenesis Kit.

For the complementation test, the relevant constructs were introduced into agl24-1 or soc1-2, whereas other constructs were introduced into wild-type Columbia plants using the Agrobacterium-mediated floral dip method (Clough and Bent, 1998). Except for transgenic plants with the pER22-AGL24 construct that were selected on MS medium (Sigma) supplemented with hygromycin, transgenic plants with other constructs were selected by Basta.

β-estradiol induction of pER22-AGL24
To observe the phenotype of pER22-AGL24 plants upon β-estradiol induction, they were grown on solid MS medium supplemented with 1% sucrose at 22°C under long-day conditions before applying various treatments. Once we started the treatment, 10 μM β-estradiol was applied and replaced every 2 days. For examining the induction of AGL24 by estradiol, the seedlings at different developmental stages grown on solid MS medium were transferred into liquid MS medium supplemented with 10 μM β-estradiol. These seedlings were incubated in the liquid medium with gentle shaking for 1 to 24 hours. Mock treatment of transgenic plants was also performed for the above experiments in which the solvent dimethyl sulfoxide substituted for β-estradiol.

Microarray analysis
Isolation of total RNA, cDNA synthesis, cRNA labeling with the IVT Labeling Kit, and hybridization on the Arabidopsis ATH1 gene microarrays were performed following the manufacturer’s instructions (Affymetrix). Two biological replicates were tested for each treatment. The Affymetrix microarray suite software package (MAS 5.0) was used to scan and obtain signals. MAS-generated data files (.CEL files) were used as the input for preprocessing using the software package RMA to summarize probe sets and normalize signal intensities (Bolstad et al., 2003). Further analysis and filtering was performed using GeneSpring (Agilent). All samples were normalized per chip to the fiftieth percentile and per gene to median signals. For the Affymetrix flags, we filtered on ‘present’ value to appear in at least one sample. This reduced 22,746 total probe sets to 15,690 probe sets. The minimum expression value was set to 0.5 (log scale). Confidence in replicates was tested using standard deviation test with GeneSpring’s default cross-gene error model turned on. The filter for P-values was set to 0.01. One-color data with deviation from one as an error model gave an average base/proportional of 34.94. First, we compared the transcriptomes in pER22-AGL24 induced by estradiol relative to mock-treatment. Second, we compared the transcriptomes in estradiol-induced pER22-AGL24 relative to those in estradiol-induced wild-type seedlings. Only genes showing consistently altered expression (fold change ≥1.1) in these two comparisons were chosen as putative AGL24-regulated genes. The complete microarray data set is available as the accession number GSM69545 in the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo).

ChIP assay
About 300 mg of 9-day-old 35S::AGL24-6HA and 35S::SOC1-9myc seedlings were fixed at 4°C for 40 minutes in 1% formaldehyde under vacuum. Fixed tissues were homogenized, and chromatin was isolated and sonicated to produce DNA fragments shorter than 500 bp. The solubilized chromatin was incubated with anti-HA agarose beads (Sigma) for 90 minutes at 4°C or used as an input control. Beads were washed five times with IP buffer (50 mM HEPES, pH 7.5, 150 mM KCl, 5 mM MgCl₂, 10 μM...
ZnSO₄, 1% Triton X-100, 0.05% SDS), and then incubated with elution buffer (50 mM Tris, pH 8.0, 1% SDS, 10 mM EDTA) for 30 minutes at 65°C. The supernatant was collected and co-immunoprecipitated DNA was recovered according to a published protocol (Wang et al., 2002). An unrelated DNA sequence from the \textit{ACTIN2/7} (\textit{ACTIN}) gene that is constitutively expressed in \textit{Arabidopsis} was used as an internal control for normalization (Johnson et al., 2002). Primer sequences used for the ChIP enrichment test are listed in Table 1. All ChIP assays were repeated at least twice and representative data are presented. For identification of the precise binding sites of AGL24 and SOC1, DNA enrichment was evaluated by real-time quantitative PCR in triplicate. Relative enrichment of each fragment was calculated first by normalizing the amount of a target DNA fragment against the \textit{ACTIN} fragment, and then by normalizing the value for transgenic plants against the value for wild type as a negative control using the following equation: \(2^{(\text{Ct Transgenic Input} - \text{Ct Transgenic ChIP})/(\text{Ct WT Input} - \text{Ct WT ChIP})}\).

### RESULTS

#### Generation of an estradiol-inducible AGL24 system

To identify target genes that are regulated by AGL24 during floral transition, we generated a functional pER22-AGL24 transgenic line in which overexpression of AGL24 is controlled by an estradiol-induced XVE system (Zuo et al., 2000). To test the dose response of the XVE inducible system, we examined the time-course of AGL24 expression in seedlings from a selected transgenic pER22-AGL24 line at different developmental stages (3, 6, 9, 12 and 15 days after germination).

#### In situ hybridization and GUS expression analysis

Non-radioactive in situ hybridization and GUS staining were carried out as previously described (Jefferson et al., 1987; Liu et al., 2007).

### Table 1. Primer pairs used for ChIP assays

<table>
<thead>
<tr>
<th>Primer pair</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>For ChIP assays of protein binding on the SOC1 sequence</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>GGATGCAACCTTCTTCTCATGAG, ATATGGGTTTGGTTTCATTTGG</td>
</tr>
<tr>
<td>2</td>
<td>AAAAACCTAAACCGAGGAGAGGAGGATCTTCCTCC, TTACCAAGGGGAGGATATG</td>
</tr>
<tr>
<td>3</td>
<td>GCAAAAAGAATCTTCTTCTCAGAG, AGGAGAGAGGAGGATATG</td>
</tr>
<tr>
<td>4</td>
<td>TGGACGCTTGAAACACTCATCCT, GGAGGGGAGGAGGATATG</td>
</tr>
<tr>
<td>5</td>
<td>AGTTGAGGAGAATTGCTTGA, TTACCAAGGGGAGGATATG</td>
</tr>
<tr>
<td>6</td>
<td>TCTCTGCTCATATGACCACCATT, TTATCTGCTTTGGGATGAG</td>
</tr>
<tr>
<td>7</td>
<td>GAGGCTGAGAGAGGAGGAGGAGGAGGATCTTCCTCC, TTACCAAGGGGAGGATATG</td>
</tr>
<tr>
<td>8</td>
<td>TATATGGGAGGAGGAGGAGGAGGATCTTCCTCC, TTACCAAGGGGAGGATATG</td>
</tr>
<tr>
<td>9</td>
<td>ATCAACATTCCTCTGAGGGATCTTCCTCC, TTACCAAGGGGAGGATATG</td>
</tr>
<tr>
<td>10</td>
<td>TGTTTCAGACATTTGCTTCAGCC, AGGTTCGTCTATCTCCCTCCTT</td>
</tr>
</tbody>
</table>

### Table 2. Primer pairs used for gene expression analysis

<table>
<thead>
<tr>
<th>Gene amplified</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>For quantitative real-time PCR</td>
<td></td>
</tr>
<tr>
<td>AGL24</td>
<td>GAGGCTTGGGAGGACGAGGATGTCGTGA, AGATGGGAAAGCCAAGCTCAGGGGA</td>
</tr>
<tr>
<td>SOC1</td>
<td>AGCTGCGAGAAACCAAAAGGAAGGAGGATCTTCCTCC, TTACCAAGGGGAGGATATG</td>
</tr>
<tr>
<td>AP1</td>
<td>CAGGCTGAGAGAGGAGGAGGAGGAGGATCTTCCTCC, TTACCAAGGGGAGGATATG</td>
</tr>
<tr>
<td>TUB2</td>
<td>AAGGACCTACTTCTGTTGATGAG, GCCTCCACCAATGTTAGATG</td>
</tr>
<tr>
<td>For semi-quantitative PCR</td>
<td></td>
</tr>
<tr>
<td>AGL24</td>
<td>AAGGAAAGGAGGACGAGGAGGAGGAGGATCTTCCTCC, TTACCAAGGGGAGGATATG</td>
</tr>
<tr>
<td>TUB2</td>
<td>ATCCGCTGAAAGAGTACCAGATC, TCACCTCTTCTCACCAGAG</td>
</tr>
</tbody>
</table>
Development 135 (8) 1484

RESEARCH ARTICLE

Fig. 1. Generation of a functional estradiol-inducible AGL24 expression system. (A) Induction of AGL24 expression in 9-day-old pER22-AGL24 Arabidopsis seedlings mock-treated (M) or treated with 10 μM β-estradiol (E) for 0, 1, 2, 4, 8, 12 or 24 hours. TUB2 expression was used as a control. (B) The estradiol-inducible AGL24 system is biologically functional. The pER22-AGL24 plants (right) initially treated with β-estradiol at 9 days after germination show earlier flowering than mock-treated plants (left). (C) Upregulation of AGL24 during floral transition is sufficient to promote flowering. β-estradiol treatment did not affect the flowering of wild-type plants, whereas initial treatment of pER22-AGL24 with β-estradiol before or at the floral transitional stage (3, 6 or 9 days after germination) accelerated flowering.

germination) after they were transferred into Murashige and Skoog (MS) liquid medium supplemented with 10 μM β-estradiol. The XVE system proved to be a potent and reliable inducible system, as pER22-AGL24 plants demonstrated consistent induction of AGL24 expression irrespective of the developmental stage of the tested seedlings (data not shown). Fig. 1A shows an example of induction of AGL24 expression in transgenic pER22-AGL24 seedlings at 9 days after germination, in which AGL24 induction nearly reached a maximal level after 8 hours of β-estradiol treatment and remained saturated thereafter.

We further applied continuous β-estradiol treatment on pER22-AGL24 seedlings at different developmental stages to test the biological effects of AGL24 induction (Fig. 1B,C). The pER22-AGL24 seedlings initially treated with β-estradiol at the vegetative stage (3 and 6 days after germination) showed comparable flowering time to those initially treated at the floral transitional stage (9 days after germination). They flowered much earlier than the mock-treated transformants and wild-type seedlings (Fig. 1C). However, pER22-AGL24 seedlings initially treated with β-estradiol at 12 and 15 days after germination did not flower significantly earlier than other seedlings (Fig. 1C). Thus, the selected pER22-AGL24 line is biologically functional, and upregulation of AGL24 to a certain threshold level during floral transition is responsible for promoting flowering.

**SOC1 is induced by AGL24**

We then chose 9-day-old pER22-AGL24 seedlings at the floral transitional stage to investigate the change in transcriptomes responding to the induced AGL24 expression. As AGL24 induction reached a steady maximal level 8 hours after β-estradiol treatment (Fig. 1A), we collected seedlings at this time point for microarray analyses. Statistical analysis of the microarray data revealed 97 AGL24-downregulated genes and 87 AGL24-upregulated genes (see Table S1 in the supplementary material), among which SOC1, a flowering pathway integrator, was one of the genes activated by AGL24.

In pER22-AGL24 seedlings treated with estradiol, AGL24 expression was continuously induced, whereas SOC1 expression was gradually upregulated up to 12 hours of induction, after which it was dramatically increased (Fig. 2A). This result, together with a previous observation that overexpression of AGL24 affected SOC1 expression in FLC-dependent and late flowering backgrounds (Michaels et al., 2003), indicates that AGL24 affects SOC1 expression under certain conditions. In wild-type plants grown in soil, AGL24 expression was increased at 7 days after germination and was dramatically upregulated during floral transition, which was marked by significantly increased AP1 expression from 9 days after germination (Fig. 2C, Fig. 5A). SOC1 expression was gradually elevated in wild-type seedlings after germination and significantly increased from 9 days after germination, whereas its upregulation was delayed in agl24-1 during floral transition (Fig. 2B). SOC1 expression was much more elevated in 35S:AGL24 than in wild-type seedlings after 9 days post-germination (Fig. 2B). We further dissected developing agl24-1 and wild-type seedlings to detect the change in SOC1 expression in the leaf (cotyledon and rosette leaf) and the aerial part without leaf, including the shoot apex and young leaf primordia (Fig. 2D). SOC1 expression was slightly altered in the leaf of agl24-1, whereas its expression in the aerial part without leaf of agl24-1 was significantly reduced. In situ hybridization further revealed the reduced SOC1 expression mainly at the shoot apex of agl24-1 during floral transition (Fig. 2E). Thus, AGL24 mainly upregulates SOC1 at the shoot apex during floral transition, which is in accordance with the observation that upregulation of AGL24 in floral transition is responsible for accelerating flowering (Fig. 1C).

**AGL24-6HA binds directly to the SOC1 promoter**

To examine whether AGL24 directly controls SOC1 transcription, we performed ChIP assays using a functional transgenic line expressing an AGL24-6HA fusion protein driven by the CaMV 35S promoter. By examining the phenotypes and genetic segregation ratios, we isolated one transgenic line containing a single insertion of the 35S:AGL24-6HA transgene, which showed comparable flowering time to 35S:AGL24 (Fig. 3A,D). A notable floral phenotype relevant to AGL24 function in promoting inflorescence identity is the generation of secondary flowers from a primary floral meristem when AGL24 is overexpressed (Yu et al., 2004), a phenotype which was also observed in the selected 35S:AGL24-6HA plant (Fig. 3C). These observations suggest that the fusion protein of AGL24-6HA retains the same biological function as AGL24.
We scanned the SOC1 genomic sequence for CArG motifs with a maximum one nucleotide mismatch, and designed ten pairs of primers near the identified motifs for measurement of DNA enrichment by quantitative real-time PCR (Fig. 4A). The number 6 genomic fragment (–1260 to –1133, relative to the translation start site) containing one CArG motif showed the strongest enrichment of around 6-fold (Fig. 4B), suggesting that AGL24-6HA binds directly to this site in vivo.

Effect of mutagenesis of the AGL24 binding site in the SOC1 promoter

To evaluate whether the CArG motif within the number 6 fragment is responsible for the upregulation of SOC1 during floral transition, we transcriptionally fused a SOC1 5' upstream sequence to the GUS reporter gene (Fig. 4C). This upstream sequence included a 1.4 kb SOC1 promoter upstream of the SOC1 transcription start site, because a SOC1 genomic fragment including this promoter is sufficient to complement soc1 mutation (Samach et al., 2000). Based on this construct, we created another reporter gene cassette in which the putative AGL24 binding site was mutated (Fig. 4C). Among 24 independent lines of transformants harboring ProSOC1:GUS, 20 lines displayed strong GUS staining during floral transition (Fig. 4D,E), whereas among 18 lines of the transformants harboring the construct with the mutated AGL24 binding site, 11 lines displayed intermediate GUS staining (Fig. 4D,E). It is noteworthy that the difference in GUS staining conferred by ProSOC1:GUS and its mutated form was most apparent at the shoot apex.
Development 135 (8)

Fig. 4. AGL24 directly regulates SOC1. (A) Schematic of the Arabidopsis SOC1 genomic region. Black boxes, exons; white boxes, introns and upstream regions. Bent arrows denote translation start sites and stop codons. Arrowheads indicate the sites containing either single mismatch or perfect match with the consensus binding sequence (CArG box) of MADS-domain proteins. Ten PCR fragments corresponding to the DNA sequences near these CArG boxes were designated for ChIP analysis. (B) ChIP enrichment test by quantitative real-time PCR shows the binding of AGL24-6HA to the region near the number 6 fragment. (C) Schematic of the ProSOC1:GUS construct. The native CArG box within the number 6 fragment identified in 8 was mutated as indicated. (D) GUS staining of ProSOC1:GUS plants. Representative GUS staining of 12-day-old transformants containing ProSOC1:GUS and its mutated form is shown in the upper panels. Representative lines were crossed with 35S:AGL24, and GUS staining of 10-day-old F1 plants is shown in the lower panels. (E) Distribution of relative GUS staining intensity in the transformants containing ProSOC1:GUS and its mutated construct. (F) Distribution of flowering time in T1 transgenic plants carrying the wild-type SOC1 gene and its mutated form in the soc1-2 mutant background.

observations, which are consistent with the different expression of SOC1 at the shoot apex of wild-type and agl24-1 seedlings, demonstrate that the tested AGL24 binding site is responsible for upregulating SOC1 expression at the shoot apex during floral transition. We further crossed the transformants harboring ProSOC1:GUS and its mutated construct with 35S:AGL24, and examined the change in GUS staining in response to the increased AGL24 activity. In the 35S:AGL24 background, GUS staining of both ProSOC1:GUS and its mutated form slightly increased in the leaf compared with that in wild-type plants (Fig. 4D). By contrast, GUS staining of ProSOC1:GUS at the shoot apex of 35S:AGL24 during floral transition increased compared with that in the wild-type background (Fig. 4D), whereas staining of the mutated construct remained lower at the shoot apex of 35S:AGL24 than in wild type (Fig. 4D). Thus, mutation of the AGL24 binding site almost completely abolishes upregulation of SOC1 by AGL24 at the shoot apex, corroborating that AGL24 specifically binds to this site to promote SOC1 expression at the shoot apex during floral transition.

To confirm that the AGL24 binding site is essential for SOC1 function in flowering, soc1-2 was transformed with either a genomic SOC1 construct or its derived construct with the mutated AGL24 binding site. The average flowering time of soc1-2 mutants transformed with the SOC1 genomic construct, which comprised 1.97 kb of 5′ upstream sequence (Fig. 4C) and the full gene coding region plus introns, was around 11.1 rosette leaves (Fig. 4F). This was comparable with the average flowering time of wild-type plants (10.3 rosette leaves), but was earlier than that of soc1-2 mutants transformed with the mutated SOC1 construct (14.5 rosette leaves) (Fig. 4F). These results substantiate that the AGL24 binding site is important for SOC1 function in promoting flowering.

SOC1-9myc binds directly to the AGL24 and LFY promoters

Since AGL24 expression is also affected by SOC1 (Michaels et al., 2003; Yu et al., 2002), we quantitatively examined the effect of SOC1 on AGL24 expression. AGL24 expression was increased in wild-type seedlings from 5 days after germination, whereas its upregulation was delayed in soc1-2 (Fig. 5A). In 35S:SOC1, AGL24 expression was high in seedlings 3 and 5 days after germination, and reduced thereafter (Fig. 5A). AP1 expression was notably higher in 35S:SOC1 than in wild-type seedlings and its expression in 35S:SOC1 5 days after germination was almost comparable with that in wild-type seedlings 11 days after germination (Fig. 5B). As AGL24 expression is repressed by induced AP1 activity (Yu et al., 2004), AGL24 expression in 35S:SOC1 may reflect a combined effect of repression of AGL24 by AP1 and promotion of AGL24 by overexpression of SOC1.

We also dissected developing soc1-2 and wild-type seedlings to detect the change in AGL24 expression in the leaf and aerial part without leaf (Fig. 5C). In wild-type seedlings, AGL24 expression in the leaf was much lower than that in the aerial part without leaf (data
not shown). Compared with its expression in wild-type tissues, AGL24 expression only slightly decreased in the leaf of soc1-2, whereas its expression in the aerial part without leaf of soc1-2 was significantly reduced during floral transition. Thus, SOC1 upregulates AGL24 mainly at the shoot apex during floral transition.

We further tested whether SOC1 could directly regulate AGL24 by ChIP assays using a functional line harboring a SOC1-9myc fusion transgene driven by the CaMV 35S promoter (Fig. 3B,D).

The number 1 genomic fragment (~2125 to ~1987, relative to the translation start site) that lies near two CArG motifs, each with one nucleotide mismatch, was enriched by about 5-fold (Fig. 5D,E), suggesting that SOC1-9myc binds directly to the AGL24 genomic region in vivo.

Using the same ChIP approach, we tested whether SOC1-9myc and AGL24-6HA could bind directly to the genomic sequences of two floral meristem identity genes, AP1 and LFY. Our results

Fig. 5. SOC1 directly regulates AGL24. (A,B) Relative temporal expression of AGL24 (A) and AP1 (B) in developing Arabidopsis seedlings of different genetic background under long-day conditions. (C) Relative temporal expression of AGL24 in the aerial part without leaf and leaf of soc1-2 and wild-type seedlings. Transcript levels in A-C were determined by quantitative real-time PCR analyses of three independently collected samples. Results were normalized against the expression of TUB2. Error bars indicate s.d. (D) Schematic of the AGL24 genomic region. Arrowheads indicate the sites containing either single mismatch or perfect match with the consensus binding sequence (CArG box) of MADS-domain proteins. Four PCR fragments corresponding to the DNA sequences near these CArG boxes were designed for ChIP analysis. (E) ChIP enrichment test shows the binding of SOC1-9myc to the region near the number 1 fragment indicated in D. (F) Schematic of the ProAGL24:GUS construct. Two native CArG boxes within the number 1 fragment identified in D and E were mutated as indicated. (G) Representative GUS staining in 12-day-old transformants containing ProAGL24:GUS and its derived constructs with the mutated CArG boxes (M-2003 and M-2039). (H) Distribution of relative GUS staining intensity in the transformants containing M-2003 and M-2039. (I) GUS staining of ProAGL24:GUS and M-2039 in the wild-type (left) and 35S:SOC1 (right) background. Representative lines of transformants containing ProAGL24:GUS and M-2039 were crossed with 35S:SOC1, and GUS staining of 4-day-old F1 plants is shown on the right. (J) Distribution of flowering time in T1 transgenic plants carrying the wild-type AGL24 gene and its mutated forms (M-2003 and M-2039) in the agl24-1 mutant background.
showed that only one fragment near a CArG motif in the LFY promoter was enriched by anti-myc antibody in SOCI-9myc plants (Fig. 6), suggesting that SOCI-9myc binds directly to the LFY promoter in vivo. In addition, we found that SOCI-9myc and AGL24-6HA did not bind directly to their own genomic sequences (see Fig. S1 in the supplementary material).

Effect of mutagenesis of the SOCI binding site in the AGL24 promoter
To identify the precise CArG motif that is responsible for the upregulation of AGL24 by SOCI, we used an established ProAGL24::GUS reporter line in which an AGL24 genomic fragment containing 4.7 kb of sequence upstream of the stop codon was translationally fused with the GUS reporter gene (Liu et al., 2007). The GUS expression in this line is similar to that of endogenous AGL24 expression. Based on this ProAGL24::GUS construct, we generated two reporter gene cassettes, M-2003 and M-2039, in which one or other of two CArG motifs within the number 1 genomic fragment were mutated (Fig. 5F). Among 17 independent lines of the transformants bearing the M-2003 mutation (Fig. 5G,H), 13 lines exhibited strong GUS staining, which was comparable with that conferred by the ProAGL24::GUS construct. By contrast, the majority of 20 independent lines of the transformants bearing the M-2039 mutation exhibited intermediate or weak GUS staining (Fig. 5G,H). The difference in the GUS staining of wild-type, M-2003 and 2039 mutation exhibited intermediate or weak GUS staining (Fig. 5G,H). The difference in the GUS staining of wild-type, M-2003 and M-2039 plants was most apparent at the shoot apex. These results, together with differential expression of AGL24 in soc1-2 and wild-type plants, strongly suggest that SOCI1 mainly binds to the CArG motif of M-2039 to upregulate AGL24 expression at the shoot apex during floral transition.

We further crossed the transformants harboring ProAGL24::GUS and its mutated construct M-2039 with 35S::SOC1, and examined the change in GUS staining in response to the increased SOCI activity. As 35S::SOC1 showed very early flowering (Lee et al., 2000; Samach et al., 2000) and AGL24 was only upregulated at early developmental stages of 35S::SOC1 (Fig. 5A), we compared GUS staining in 4-day-old seedlings. GUS staining of 4-day-old ProAGL24::GUS and M-2039 seedlings did not reveal any difference in wild-type background (Fig. 5I), which was consistent with unaltered AGL24 expression in soc1-2 and wild-type seedlings at a similar developmental stage (Fig. 5A). However, GUS staining of ProAGL24::GUS at the shoot apex and hypocotyl of 35S::SOC1 was increased compared with that in the wild-type background, whereas staining of M-2039 remained the same in 35S::SOC1 as in wild type (Fig. 5I). Thus, mutation of the SOCI binding site indeed compromises upregulation of AGL24 in young seedlings.

To confirm that the revealed SOCI binding site is essential for AGL24 function in flowering, agl24-1 was transformed with either a genomic AGL24 construct or its derived construct with the M-2003 or M-2039 mutation. The average flowering time of agl24-1 mutants transformed with the AGL24 genomic construct, which comprised 2.23 kb of 5′ upstream sequence (Fig. 5F) and the full gene coding region plus introns, was around 11.9 rosette leaves (Fig. 5I). This was comparable to the average flowering time of agl24-1 mutants transformed with the M-2003 mutation (12.2 rosette leaves), but was earlier than that of agl24-1 mutants transformed with the M-2039 mutation (14.4 rosette leaves) (Fig. 5I). These results substantiate that the SOCI binding site at M-2039 is important for AGL24 function in promoting flowering.

Interaction of AGL24 and SOCI mediates the effect of gibberellins on flowering
Previous studies have revealed that the expression of AGL24 and SOCI is differently controlled by the photoperiod, autonomous and vernalization pathways (Michaels et al., 2003; Yu et al., 2002). Although it has been shown that GA could affect the expression of AGL24 and SOCI (Lee et al., 2000; Moon et al., 2003; Yu et al., 2002), it remains elusive how the GA pathway regulates their expression. We examined the expression of both genes in the wild-type and mutant seedlings grown under short-day conditions. In the wild-type seedlings, the expression of AGL24 and SOCI gradually increased under mock treatment and their expression was upregulated upon GA treatment (Fig. 7A,B), confirming that both genes are targets of the GA pathway (Lee et al., 2000; Moon et al., 2003; Yu et al., 2002). In agl24-1 and soc1-2, the respective upregulation of SOCI and AGL24 was nearly abolished upon GA treatment (Fig. 7A,B). This suggests that upregulation of SOCI and AGL24 in response to GA is mediated by AGL24 and SOCI, respectively. Under long-day conditions, GA treatment did not promote flowering in wild type or mutants, indicating that signals from other flowering genetic pathways play major roles in regulating flowering time (Fig. 7C). During our experimental period, soc1-2 agl24-1 did not flower under short-day conditions without GA treatment, which was significantly different from the flowering phenotype exhibited by either of the single mutants (Fig. 7D). Upon GA treatment, flowering of wild type, soc1-2 and agl24-1 was accelerated, whereas soc1-2 agl24-1 still flowered extremely late
DISCUSSION

The transition to flowering involves multiple genetic pathways in response to developmental and environmental signals. Several global expression analyses have been performed to discover genes or pathways affecting floral induction (Schmid et al., 2003; Wigge et al., 2005; Wilson et al., 2005). In this study, we used an estradiol-inducible gene expression system in combination with microarray analysis to identify genes induced by the flowering promoter AGL24 and identified SOC1 as one of these induced genes. At the vegetative phase, SOC1 expression remains low and is almost unaffected by altered AGl24 activity, whereas upregulation of SOC1 expression at the shoot apex during floral transition is highly dependent on AGl24 activity (Fig. 2). ChiP assay revealed that AGl24-6HA can bind to the regulatory sequence of SOC1, and mutagenesis of the AGl24-6HA binding site reduces SOC1 expression at the shoot apex (Fig. 4), demonstrating that AGl24 directly regulates SOC1 transcription specifically at the shoot apex during floral transition. These results, together with the observations that AGl24 is significantly upregulated during floral transition and that induced AGl24 expression during floral transition is sufficient to promote flowering (Fig. 1C, Fig. 5A), suggest that direct upregulation of SOC1 by increased AGl24 expression is an important molecular event during floral transition. On the other hand, several pieces of evidence have also shown that AGl24 expression at the shoot apex is directly upregulated by SOC1 (Fig. 5), suggesting that AGl24 and SOC1 regulate each other to provide positive-feedback control of their expression at the shoot apex during floral transition.

In soc1 and agl24 mutants, changes in AGl24 and SOC1 expression, respectively, still affect flowering time, implying that they might regulate different genes involved in flowering (Michaels et al., 2003; Yu et al., 2002). Our ChiP assay revealed that the LFY genomic sequence is only bound by SOC1-9myc, and not by AGl24-6HA (Fig. 6). This confirms that AGl24 and SOC1 control distinct genes, while they directly regulate each other.

A significant aspect of the mutual interaction between AGl24 and SOC1 is the integration of flowering signals from several genetic pathways (Fig. 8). The vernalization pathway regulates flowering through at least several different regulators. In a FLC-independent pathway, vernalization regulates the expression of at least two genes, AGl24 (Michaels et al., 2003; Yu et al., 2002) and AGL19 (Schonrock et al., 2006). In a FLC-dependent pathway, FLC plays a dual rule in directly repressing SOC1 transcription in the meristem and indirectly delaying SOC1 expression by repression of FT, a systemic signal required for the activation of SOC1, in the leaf (Hepworth et al., 2002; Searle et al., 2006). Several recent studies have provided in vitro and in vivo data showing that FLC binds to a CArG box at the SOC1 5' promoter (Hellwell et al., 2006; Hepworth et al., 2002; Searle et al., 2006). Nevertheless, vernalization can still upregulate SOC1 expression in fca mutants under short-day conditions, indicating that SOC1 is also regulated in a FLC-independent way (Moon et al., 2003). This can be partly explained by direct regulation of SOC1 by AGl24.

The autonomous pathway promotes flowering by repressing FLC (Michaels and Amasino, 2001) and thus affecting SOC1 expression. Although AGl24 expression is not affected by FLC, its expression is significantly reduced in several mutants in the autonomous pathway, such as fve, fpa and fca (Michaels et al., 2003; Yu et al., 2002), suggesting that the autonomous pathway also upregulates AGl24 in a FLC-independent way. Since FLC and AGl24 bind to distinct sites of the SOC1 promoter region, it will be interesting to further elucidate the SOC1 transcription complex, in which AGl24 may compete with FLC in response to the signals from vernalization and autonomous pathways.

In the photoperiod pathway, SOC1 is mainly regulated by FT and indirectly by CO via other unknown DNA-binding factors(s) (Hepworth et al., 2002; Lee et al., 2000; Samach et al., 2000; Yoo et al., 2005), whereas AGl24 is affected by the activity of CO, but not...
of FT (Yu et al., 2002). Although FT has been suggested as a major output of CO (Samach et al., 2000; Wigge et al., 2005; Yoo et al., 2005), FT integrates other floral signals irrespective of CO. For example, FLC directly represses FT in the leaf, thus affecting its activation of SOC1 (Helliwell et al., 2006; Searle et al., 2006). In addition, thermal induction of flowering by elevated growth temperature is also mediated by FT (Balasubramanian et al., 2006). Thus, positive regulation of SOC1 by FT is only partially controlled by the photoperiod pathway. It is likely that direct regulation of SOC1 by AGL24, which is regulated by CO, provides an alternative channel to enhance the effect of the photoperiod pathway on SOC1 expression.

Under short-day conditions, the GA pathway is a major flowering pathway that mainly affects AGL24, but not FLC and FT (Moon et al., 2003). Removal of FLC repression only derepresses SOC1 expression and is not sufficient to activate SOC1 under short-day conditions, suggesting that GA activation of SOC1 needs positive regulator(s) (Moon et al., 2003). AGL24 is a possible regulator of SOC1 in the GA pathway because AGL24 upregulates each other in response to GA, and loss of either gene compromises the effect of GA on the promotion of another gene (Fig. 7). In addition, flowering of overexpression of SOC1 under short-day conditions is partially delayed in the GA-deficient mutant ga1-3 (Moon et al., 2003), indicating that GA regulates other target(s) in addition to SOC1. Our results have identified that AGL24 is another major target of the GA pathway as soc1-2 ago2-1 double mutants do not flower under short-day conditions without GA treatment (Fig. 7D). Taken together, direct interaction of AGL24 and SOC1 allows a synergistic integration of environmental and endogenous signals from several upstream genetic pathways to promote flowering (Fig. 8).

Overall, the results presented here show that AGL24 and SOC1 directly upregulate each other at the shoot apex during floral transition. This integrates flowering signals perceived by these two regulators and provides positive-feedback regulation of their own expression to a quantitative threshold required for the transition of the shoot apical meristem from a vegetative to a reproductive state. Direct cross-regulation between AGL24 and SOC1 represents a novel regulatory mode for the transcription factors involved in the control of flowering time and further investigation of their target genes would provide a better understanding of the subtle regulatory hierarchy of floral transition.

We thank Nam-Hai Chua for providing the vector pER8; Dr Ilha Lee for the seeds of soc1-2 and 355-SOC1; Drs Rick Amasino and Marty Yanofsky for agl24-1; and Drs Toshiro Ito, Frederic Berger and Yuehui He for critical reading of the manuscript. This work was supported by Academic Research Funds R-154-000-282-112 and R-154-000-337-112 from the National University of Singapore and R-154-000-263-112 from the Ministry of Education, Singapore, and the intramural research funds from Temasek Life Sciences Laboratory.

Supplementary material
Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/8/1481/DC1

References


