Investigation of *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) Function in Flowering Time Control of *Arabidopsis thaliana*

CHEN HONGYAN

NATIONAL UNIVERSITY OF SINGAPORE

2007

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CHEN HONGYAN

(M.S., NUS)

A THESIS SUBMITTED

FOR THE DEGREE OF MASTER

DEPARTMENT OF BIOLOGICAL SCIENCES

NATIONAL UNIVERSITY OF SINGAPORE

2007

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and meristem tissues of developing wild-type seedlings.

CHAPTER 1

Summary

Recent studies have shown that *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) is an important flowering integrator in *Arabidopsis thaliana*. The main objective of this study is to clarify the *SOC1*-mediated regulatory network and to find out its upstream regulators and downstream targets.

Two homologous genes, *SVP* (*SHORT VEGETATIVE PHASE*) and *AGL24* (*AGAMOUS LIKE 24*) have been identified as *SOC1* regulators, though they have opposite effects. Quantitative real-time RT-PCR results indicated that *SVP* constantly and dominantly represses *SOC1* early at the vegetative phase, while *AGL24* promotes *SOC1* expression in a temporally restricted manner - only during the floral transition. Furthermore, chromatin immunoprecipitation (ChIP) assays showed that SVP-6HA and AGL24-6HA fusion proteins can bind to different regions of *SOC1* genomic sequence *in vivo*. Since both *SVP* and *AGL24* encode MADS-box transcription factors, this set of data suggests that they can directly regulate *SOC1* at the transcriptional level. On the other hand, ChIP assays using a *35S:SOC1-9myc* tagging system demonstrated that SOC1-9myc fusion protein reciprocally binds to *AGL24* and *SVP* promoters, implying the existence of a feedback regulation of *AGL24* and *SVP* by *SOC1*. This was further supported by expression analyses showing that a change in *SOC1* mRNA level affects *AGL24* and *SVP* expression in young seedlings

ChIP assays further revealed that SOC1-9myc fusion protein binds to the

genomic sequence of *LEAFY* (*LFY*), a key floral meristem identity gene in *Arabidopsis thaliana*. This is the first set of biochemical evidence supporting a direct flowering signal transduction from *SOC1* to *LFY*, which has been proposed in recent years. However, genetic crossing results in another lab showed that constitutive expression of *LFY* and *SOC1* has additive effects on flowering time, implying that *LFY* is not the only output of *SOC1*. Therefore, microarray analysis would be necessary for screening other *SOC1* targets on the whole-genome scale.

CHAPTER 2

Literature Review

2.1 The genetic network controlling floral transition in *Arabidopsis thaliana*

The floral transition is a crucial developmental step for plants because it determines when plants enter the reproductive stage from the vegetative stage. Comprehensive studies on this field have been conducted in the past decade. It is widely believed that the floral induction is controlled by an intricate regulatory network affected by both external and internal signals. With well established genetic tools, *Arabidopsis* has proved to be an excellent model system for studying floral transition. Generally, four major pathways have been found to control flowering time in *Arabidopsis*, including, the photoperiod, autonomous, vernalization and gibberellin (GA) pathways.

2.1.1 Photoperiod pathway

Arabidopsis is a facultative long-day plant, flowering more rapidly under long days (LDs) than short days (SDs). This phenomenon suggests that some genes in *Arabidopsis* are involved in recognizing the light signal. Photoreceptors in *Arabidopsis* comprise five phytochromes (*PHYA* to *PHYE*) and two cryptochromes

(*CRY1* and *CRY2*) (Thomas and Vince-Prue, 1997). Specifically, red and far-red light are perceived by *PHYs* (Briggs et al., 2001; Quail et al.,1994), while blue light and ultraviolet-A are perceived by CRY1 and CRY2 (Briggs et al., 2001; Ahmad et al., 1993; Lin et al., 1998). The mechanism of ultraviolet-B perception is still unknown. After initiation by *PHYA*, *CRY1* and *CRY2*, the photoperiod pathway signal enters a circadian cycle. If the length of the dark period decreases below a critical level, downstream genes are activated (Levy and Dean. 1998). Interestingly, light quality also affects flowering time, with far-red and blue light promoting flowering while red light inhibiting it (Martinez-Zapater et al., 1994; Guo et al., 1998).

Among the genes located downstream of photoreceptors, *CONSTANS* (*CO*) and *GIGANTEA* (*GI*) have been thoroughly investigated. Mutations of these two genes cause delayed flowering under LDs but have little effect under SDs. *CO* is probably the most important target of *PHYs* and *CRYs*. The *CO* gene has homology to the Zinc-finger domain transcription factor (Putterill et al., 1995). Photoreceptors regulate *CO* precisely through the light cycle. The circadian rhythm of *CO* mRNA was shown to be critical for control of flowering via the photoperiod pathway (Valverde et al., 2004), while flowering activation through *CO* is a dosage-dependent process (Putterill et al., 1995). On the other hand, *GIGANTEA* (*GI*) encodes a membrane located protein with six putative membrane-spanning domains, and its expression is also regulated by the circadian clock. Previous studies have shown that GI is essential for the maintenance of circadian rhythm. The *gi* mutant is defective for the expression of *LHY* and *CCA1*, which are two other circadian clock-associated genes (Fowler et al.,

1999; Park et al., 1999).

2.1.2 Autonomous pathway

Plants require not only external environmental factors but also internal developmental factors to promote flowering. The mutations of some genes, such as *FVE*, *FCA* and *LUMINIDEPENDENS* (*LD*), cause delay flowering under both LDs and SDs, hence they are placed in the autonomous pathway. The *LD* gene encodes a protein of 953 amino acids with two bipartite nuclear localization motifs. The LD protein contains a glutamine-rich domain, which is homologous to certain transcription factors in other species. In addition, *LD* seems to be involved in light quality perception, because *ld* mutant plants are insensitive to light with a high red/far-red ratio (Lee et al., 1994). The FCA protein includes two RNA-recognizing motifs and a WW (two conserved tryptophan [W]) protein interaction domain. This structure strongly suggests that *FCA* may function in the posttranscriptional process (Macknight et al., 1997). The *FCA* transcript itself can be alternatively spliced as α, β, γ and δ products. However, only γ mRNA encodes functional FCA protein, which is consistent with the fact that only the constitutive expression of γ mRNA causes early flowering in transgenic plants (Macknight et al., 2002). FVE is a putative retinoblastoma-associated protein. It has bee reported that FVE is part of a protein complex performing histone deacetylation function in order to repress *FLOWERING LOCUS C* (*FLC*), a key factor integrating autonomous and vernalization signals

(Israel et al., 2004). Additionally, *FPA* and *FY* genes act redundantly to repress *FLC*, through which plants ensure the developmental switch-on of flowering (Yushibumi, 2004; Schomburg et al., 2001).

2.1.3 Vernalization pathway

Vernalization (extreme cold treatment) promotes flowering of *Arabidopsis* winter annual ecotypes in response to extended exposure to low temperature, which helps plants flower in time after prolonged cold in winter. This pathway performs redundantly with the autonomous pathway. Both of them activate flowering mainly through the repression of *FLOWERING LOCUS C* (*FLC*), a member of MADS-domain protein family. *FLC* is expressed predominantly in shoot and root apices but is also detectable in leaf tissues (He et al., 2003; Michaels and Amasino, 1999). An expression study of *FLC* with tissue-specific promoters demonstrated that *FLC* expression is required in both leaf and shoot apical meristem tissues for the full repression of flowering (Searle et al., 2006). The abundance of *FLC* mRNA is reduced by vernalization (Michaels et al., 1999), whereas *FLC* is not necessary for vernalization response since other *FLC*-independent vernalization pathways that may regulate *AGL24* and *AGL19* have been reported in recent years (Michaels et al., 2003; Schonrock et al., 2006). The *FRIGIDA* (*FRI*) gene is a powerful positive regulator of *FLC*. The coiled-coil domains of FRI protein may be the regulatory component. Allelic variation at the *FRI* locus confers the flowering differences among

Arabidopsis ecotypes (Johanson et al., 2000). Moreover, mutation of *FLC* is epistatic to dominant alleles of *FRI*. Similarly, overexpression of *FLC* showed late flowering phenotype in the absence of an active *FRI* allele (Michaels et al., 1999).

There are several genes which have been specially located in the vernalization pathway, namely *VRN1*, *VRN2* and *VRN3* (Chandler et al., 1996). VRN1 protein may bind DNA in a non–sequence-specific manner and functions in constant repression of *FLC*. Overexpression of *VRN1* also reveals a vernalization-independent function for *VRN1*, mediated mainly through the floral pathway integrator *FT* (Levy et al., 2002). *VRN2* encodes a nuclear-localized zinc finger protein with homology to Polycomb Group (PcG) proteins in *Drosophila* and maintains *FLC* repression after vernalization (Gendall et al., 2001). In addition, another PcG protein, *VIP4* has been found to be an activator of *FLC* (Zhang et al., 2002).

The observation that *FLC* repression is maintained through mitotic cell divisions in plants experiencing the cold treatment suggests an epigenetic mechanism of vernalization. Many components in vernalization pathways have been found to cause remodeling of *FLC* chromatin structure and histone modifications related to heterochromatin formation. These regulators includes *VRN2*, *LIKE HP1* (*LHP1*) and *VERNALIZATION INDEPENDENTS3 (VIN3)*. *LHP1* encodes a protein showing high homology to HETEROCHROMATIN PROTEIN1 (HP1) in animals, which is able to stabilize the histone repressive methylation and recruit other complexes for heterochromatin formation (Bannister et al., 2001; Mylne et al., 2006). VIN3 is a plant-specific DNA-binding protein involved in histone deacetylation at *FLC*.

However, *VIN3* itself is not sufficient to initiated the vernalization response since it is expressed only after an extended cold treatment (Sung and Amasino 2004).

2.1.4 Gibberellin (GA) pathway

Gibberellin (GA) is a major flowering promoter for *Arabidopsis* under SDs. Besides flowering, this class of plant hormones participates in many other processes during plant development, including seed germination and cell elongation (Finkelstein and Zeevaart, 1994). The *ga1-3* mutant, which is severely defective in gibberellin synthesis, never flowers under SDs, while it only slightly delays flowering under LDs (Wilson et al., 1992). GA promotes flowering partly through the activation of *LFY* because the constitutive expression of *LFY* is able to restore flowering of *ga1-3* mutants in SDs (Blazquez et al., 1998). Meanwhile, *Arabidopsis* has several negative regulators for the GA signal transduction, including *RGA* and *GAI*, which are highly homologous and may function redundantly. While the *gai* and *rga* single mutant have limited effect on suppressing the flowering defects in the GA-deficiency mutant *ga1-3*, the *rga gai* double mutant can completely rescue these defects in *ga1-3*, indicating that *RGA* and *GAI* are repressors of the GA pathway in the control of flowering time. These genes also participate in feedback-control of GA biosynthesis. *SPY* is another repressor of the GA pathway, acting upstream of *RGA* and *GAI*. *SPY* activates these two genes probably through the GlcNAc modification because *SPY* is predicted to encode an *O*-linked N-acetylglucosamine (GlcNAc) transferase.

2.2 Floral integrators

Previous research work provides evidence that the above mentioned four genetic pathways converge on some key genes in order to integrate inputs from the different flowering cascades. *LFY*, *FLOWERING LOCUS T* (*FT*) and *SOC1* have been identified as such floral pathway integrators in *Arabidopsis* (Simpson and Dean, 2002).

2.2.1 *LEAFY* (*LFY*)

LFY has dual roles in flower development, as a flowering time promoter and a floral meristem identity gene. The *LFY* gene encodes a plant specific transcription factor, which is localized primarily in the nucleus (Parcy et al., 1998; Weigel et al., 1992). The LFY protein can be transferred to different layers of floral meristem through plasmodesmata. The cell-cell movement provides a potential mechanism to ensure complete conversion of a meristem into a flower (Sessions et al., 2000). The confirmed functions of *LFY* protein are positive regulation of *APETALA1* (*AP1*) and *AGAMOUS* (*AG*) through *cis*-elements binding (Busch et al., 1999; Lohmann et al., 2001). Constitutive expression of *LFY* causes early flowering while *lfy* mutants slightly delay flowering and produce a flower-like shoot structure, which is related with the role of *LFY* on floral meristem specification (Weigel et al., 1992). The overexpression of *LFY* partially rescues the *co* mutant phenotype suggests that *LFY*

might be the downstream target of *CO*-mediated photoperiod pathway. This has been further proven by the finding that the increase of *CO* (using inducible CO-GR transgenic plants) promotes *LFY* mRNA expression. Moreover, *CO* may not be a direct activator of *LFY* because the *LFY* induction by *CO* takes more than 24 hours (Samach et al., 2000). As mentioned in part 2.1.4, *LFY* expression is dramatically reduced in *ga1* mutant under SD condition. GA signals upregulate *LFY* possibly through a *cis*-element in the *LFY* promoter (Blazquez and Weigel 2002). It is noteworthy that this regulatory region does not affect *LFY* induction by the photoperiod pathway. Therefore, GA and photoperiod pathway signals are independently integrated at *LFY* (Blazquez and Weigel 2002; Parcy 2005).

2.2.2 *FLOWERING LOCUS T* (*FT*)

The *FT* gene has been simultaneously isolated by activation-tagging and T-DNA insertion screening. *FT* transcripts are detectable in seedlings before floral transition, increasing gradually with vegetative growth. The mRNA expression patterns under LD and SD conditions are subtly different, though both reach a maximum around the period of floral transition. *FT* encodes a 20KDa protein with similarities to phosphatidylethanolamine binding protein (PEBP) and Raf kinase inhibitor protein (RKIP) in animals (Kardailsky et al.; 1999; Kobayashi et al., 1999). FT protein is not able to regulate transcription process unless assembled with FD, a bZIP transcription factor (Abe et al., 2005; Kardailsky et al., 1999). The *FT::GUS* reporter gene shows

that *FT* is primarily expressed is the vasculature, while *FD* is found at the shoot apex, suggesting that *FT* mRNA or protein need move from leaf to shoot apical meristem, where it interacts with *FD* to activate *AP1* (Abe et al., 2005; Baurle and Dean, 2006; Takada and Goto, 2003). This assumption has been partly proven by a recent paper that *FT* fusion protein can move from phloem cells to the apex, acting as a florigen (Corbesier et al., 2007).

FT constitutive expression causes extremely early flowering under both LD and SD conditions, while the *ft* mutant is late flowering under LDs and has slight effect under SDs, implying that *FT* is regulated by the photoperiod pathway. *CO* seems to directly upregulate *FT* expression (Samach et al., 2000). The early flowering of overexpression *CO* transgenic plants can be repressed by mutations in the *FT* gene. The interaction between *CO* and *FT* is also validated by expression of *CO* with different localized promoters. *CO* triggers early flowering in the leaf phloem but not in the shoot apex, indicating that the activation signals of *CO* in leaf need to be transmitted into the apex through a florigen factor, which is possibly *FT* (An et al., 2004; Ayre and Trugeon, 2004). Another well-known regulator of *FT* is *FLC*, which is the convergence point of the autonomous and vernalization pathways. Elevated *FT* expression is found in *flc* mutant. *FLC* represses *FT* transcription mainly in the leaf phloem and delays *FD* upregulation in shoot apex. Chromatin immunoprecipitation demonstrates that *FLC* protein physically binds to the first intron of *FT* and to the promoter region of *FD* (Baurle and Dean, 2006; Searle et al., 2006). GA might also play a role in *FT* induction since the GA-dependent *ebs* mutant derepresses *FT* to

promote early flowering phenotype (Gomez-Mena et al., 2001; Pineiro et al., 2003).

2.2.3 *SUPPRESSOR OF CO OVEREXPRESSION 1* (*SOC1*)

Previous research reveals that *SOC1* integrates all the four pathways signals through the actions of *CO*, *FLC* etc. The details will be discussed later in this thesis.

2.3 Interaction between floral integrators

2.3.1 *LFY* and *FT*

There is some evidence showing that *LFY* expression is regulated by *FT*, although this regulation might be indirect. *LFY* is ectopically expressed in the apical meristem of transgenic plants overexpressing *FT*, and its expression is reduced in *ft* mutant under LD and SD conditions (Schmid et al., 2003; Kardailsky et al., 1999). However, the *LFY::GUS* reporter gene is normally expressed in leaf primordia of the *ft* mutant (Nillson et al., 1998). The relation between *FT* and *LFY* therefore requires further investigation. In wild-type plants, *LFY* mRNA is not detectable in the shoot apical meristem due to repression by *TERMINAL FLOWER1* (*TFL1*) (Ratcliffe et al., 1998). TFL1 protein is highly homologous to *FT*, but performs the opposite function in flowering time control. These two proteins are functionally exchangeable with a single amino acid conversion (Hanzawa et al., 2005).

2.3.2 *LFY* and *SOC1*

The direct interaction between *LFY* and *SOC1* has been proposed in recent years. It is believed that *LFY* may act at least partially downstream from *SOC1*. The constitutive expression of *SOC1* activates *LFY* in the shoot meristem, producing solitary flowers from axillary inflorescences (Lee et al., 2000; Mouradov et al., 2002; Parcy 2005). Nevertheless, *LFY* expression is not abolished in the *soc1* mutant, indicating that there are other upstream factors activating *LFY*. Consistent with this hypothesis, overexpression of *SOC1* and *LFY* have additive effects on flowering (Lee et al., 2000). Because *AGL24* also affects *LFY* expression, it has been suggested that *AGL24* is another upstream regulator of *LFY* (Yu et al., 2002). Since *AGL24* and *SOC1* mutually regulate each other's expression (Yu et al., 2002; Michaels et al., 2003), they may function together to control *LFY* expression.

2.3.3 *FT* and *SOC1*

Currently it is widely accepted that *FT* and *SOC1* acts on independent pathways. Although *SOC1* upregulation after a shift from SD to LD conditions is decreased in the *ft* mutant, this difference could be a side effect of the whole flowering-regulatory network (Schmid et al., 2003). Nevertheless, a recently published paper mentioned that FT may recruit FD in order to promote *SOC1* expression at the shoot apical meristem during floral transition (Searle et al., 2006). Moreover, some evidence also

implies that *FT* may perform as an intermediate factor between *SOC1* and *CO* (Yoo et al., 2005).

2.4 Floral meristem identity (FMI) genes

Once floral integrators are activated, they regulate downstream floral meristem identity (FMI) genes, which determine the apical meristem fate to produce floral meristems that further develop into flowers with four whorls of floral organs. The appearance of floral meristem identity genes in floral primordia symbolizes the completion of floral transition. In *Arabidopsis*, *APETALA1* (*AP1*), *LFY* and *CAULIFLOWER* (*CAL*) are well studied FMI genes.

2.4.1 *APETALA1* (*AP1*)

Like *LFY*, *AP1* has dual functions during floral development, namely the determinations of floral meristem identity and floral organ identity. *AP1* encodes a MADS-domain transcription factor, which specifies the identity of floral meristem and determines sepal and petal development as a class A gene in *Arabidopsis* (Gustafson-Brown et al., 1994). The *ap1* mutant shows the defects in the floral meristem specification, and constitutive expression of *AP1* results in early flowering (Bowman et al., 1993). *LFY* has been confirmed to act as a direct transcriptional regulator of *AP1* (Wagner et al., 1999). *In situ* data shows that *AP1* is expressed in a

sub-domain of the region expressing *LFY* (Mandel et al., 1992). Overexpression of *LFY* significantly promotes *AP1* expression, and *AP1* mRNA can be found in leaf primordia, which is the expression region of *LFY* in wild-type plants. Correspondingly, *AP1* expression is delayed in the *lfy* mutant (Liljegren et al., 1999; Parcy et al., 1998; Ruiz-Garcia et al., 1997; Weigel and Meyerowitz, 1993). In addition, the *LFY:GR* inducible system and chromatin immunoprecipitation (ChIP) have been applied to demonstrate that *LFY* activates *AP1* through protein binding to the *AP1* promoter region, and this regulation does not require any intermediate translational process (Wagner et al., 1999; William et al., 2004). However, *LFY* is not the only upstream regulator of *AP1* as *FT* is also able to activate *AP1* as mentioned in Section 2.2.2. The *ft lfy* double mutant abolishes *AP1* expression as seen in the *lfy* mutant, suggesting that *FT* and *LFY* controls *AP1* in parallel pathways (Ruiz-Garcia et al., 1997).

2.4.2 *CAULIFLOWER* (*CAL*)

CAL also encodes a putative MADS-domain transcription factor. Phylogenetic analysis indicates that *CAL* and *AP1* are paralogous to each other (Purugganan and Suddith, 1998). The expression patterns of these two genes are quite similar. As expected, the activity of *CAL* appears to be redundant to that of *AP1*. The *CAL* promoter also contains a *LFY* protein binding site (William et al., 2004). However, the meristem identity functions of *CAL* and *AP1* are not entirely equivalent, because *ap1* mutants show signficant flower meristem defects even in the presence of *CAL* while

cal mutants have no obvious floral phenotypes (references?).. Some studies support that *CAL* and *AP1* act redundantly to upregulate *LFY* to control inflorescence architecture, implying the reciprocal interactions among all the three FMI genes (Ferrandiz et al., 2000).

2.5 Overview of the clarified regulatory network controlling floral transition in *Arabidopsis thaliana*

As mentioned above, four genetic pathways have been found in mediating floral transition in *Arabidopsis*. These flowering signals would converge to several floral integrators, which further activate floral meristem identity genes and finally determine flower formation in the shoot apex (Figure 1).
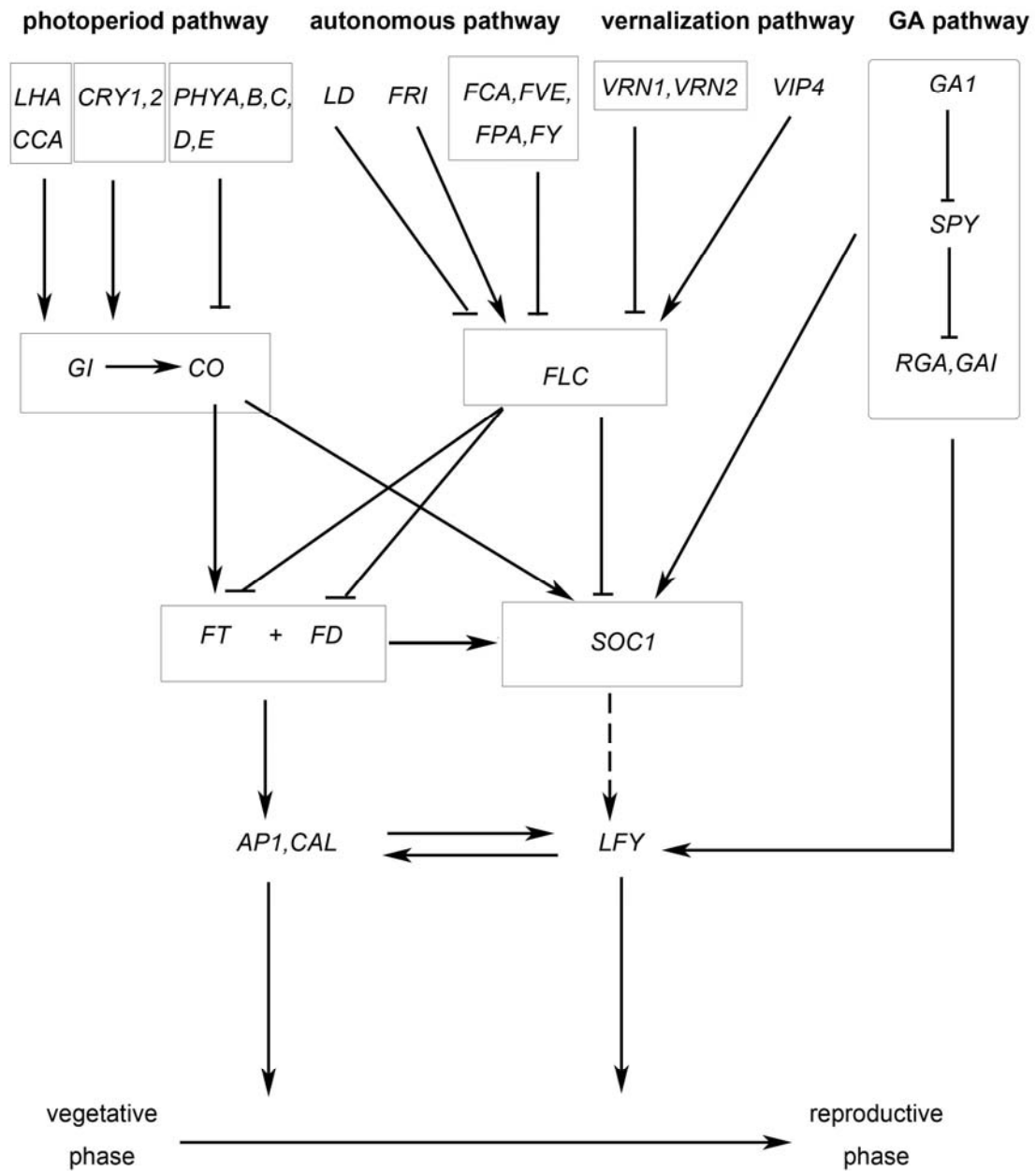
Figure 1. The schematic flowering pathways in *Arabidopsis thaliana*. Arrows and T-lines indicate positive and negative regulations, respectively. Dotted line is a possible interaction.

2.6 Previous research on *SUPPRESSOR OF CO OVEREXPRESSION 1* (*SOC1*)

SOC1, formerly named *AGAMOUS-LIKE 20* (*AGL20*), consists of seven exons and six introns. It encodes a typical MADS-domain transcription factor of 214 amino acids, showing 96% identity to a mustard ortholog *MADSA*, which responds to inductive long-day signals (Borner et al., 2000; Lee et al., 2000). Phylogenetic analysis indicates that the most homologous proteins of SOC1 in *Arabidopsis* are AGAMOUS-LIKE 14 (AGL14) and FLC (Lee et al., 2000). *SOC1* was contemporaneously identified as a floral activator using activation tagging and cDNA screening for suppressors of *CO* overexpression (Lee H et al., 2000; Samach et al., 2000). Expression studies showed that *SOC1* transcripts are present in most tissues of *Arabidopsis* seedlings, including root, leaf, shoot apex, etc. The mRNA abundance is temporally upregulated after seed germination. During floral transition, there is a sharp increase of *SOC1* mRNA and strong *SOC1* expression could be found in the inflorescence meristem, after which it was absent from the stage 1 floral meristem, then reappeared in the center of older floral meristem, overlapping the spatial expression pattern of *AG* (Borner et al., 2000; Samach et al., 2000). However, floral organs of the *soc1* mutant normally develop, suggesting that *SOC1* might be a redundant co-factor in floral organogenesis (Borner et al., 2000).

2.6.1 *SOC1* is a flowering promoter in *Arabidopsis*

It has been suggested that *SOC1* is a major factor in determination of flowering time. Overexpression of *SOC1* causes extremely early flowering under both LD and SD conditions. Similarly, constitutive expression of the orthologous gene *MADSA* in the short-day tobacco (*Nicotiana tabacum* Maryland Mammoth) can overcome the photoperiodic barrier of floral induction (Borner et al., 2000). On the other hand, the *soc1* mutant demonstrates significantly delayed flowering, especially under LDs. In the *soc1* mutant without any detectable *SOC1* transcripts, the leaf number is twice as that of wild-type. Whereas, the *soc1* mutant is still responsive to the photoperiod pathway since the mutant flowers earlier under LDs than under SDs (Borner et al., 2000; Lee et al., 2000).

2.6.2 *SOC1* integrates all the four flowering pathways in *Arabidopsis thaliana*

Expression studies confirm that *SOC1* mRNA level is promoted after a shift from SDs to LDs, mainly in the shoot apical meristem and leaf primordia (Borner et al., 2000; Samach et al., 2000). *CO* seems to play an essential role in the photoperiodic response of *SOC1*. *SOC1* expression has been examined in a 35S::CO:GR inducible system. *SOC1* appears to be one of the immediate targets of *CO*. The translational inhibitor cycloheximide (*CYC*) was also applied to demonstrate that regulation from *CO* to *SOC1* does not require any intermediate protein synthesis (Samach et al., 2000). This result is consistent with the study of the *SOC1::GUS*

reporter gene, showing that a 351bp fragment in *SOC1* promoter region is necessary for activation by CO. Although the CO protein might not directly bind to the *SOC1* promoter, CO could recruit other DNA binding co-factors to perform transcriptional regulation through its zinc fingers and CCT domain, which mediate protein-protein interaction (Hepworth et al., 2002). Genetic data are helpful to further clarify the relation between *CO* and *SOC1*. The *soc1* and *ft* mutants partially suppress the early flowering of overexpression of *CO*, while *ft soc1* double mutations completely eliminate the phenotype, and cause *ft soc1 35S::CO* plants to flower as late as the *co* mutant. Therefore, *SOC1* and *FT* are two major outputs of *CO*-mediated signals and partly independently perform their functions (Hepworth et al., 2002). However, some researchers proposed that *SOC1* may be regulated by *CO* through *FT* since *FT* has a positive effect on *SOC1* expression (Yoo et al., 2005). Additionally, a separate experiment indicates that *FT* is required in phloem for the early activation of *SOC1* at meristem under LDs, possibly in a *FD*-dependent manner (Searle et al., 2006). This interesting idea still needs to be further validated.

One expression study also suggested that *SOC1* expression is more dependent on the autonomous pathway since the autonomous pathway mutants, *fca-1*, *fve-1*, and *fpa-1*, show more reduction of *SOC1* transcripts compared with the photoperiod pathway mutants, *co-2*, *gi-3*, and *ft-1* (Lee et al., 2000). Nevertheless, there is no evidence supporting the direct interaction among *SOC1* and these autonomous pathway factors. As previously mentioned, *FLC* is a key gene which integrates vernalization and autonomous pathways in *Arabidopsis*. It seems that *FLC* acts as an

intermediate factor involved in *SOC1* regulation by these two pathways. The investigation of *FLC* and *SOC*1 mRNA levels in each other's loss-of-function mutants show that *FLC* is an upstream repressor of *SOC1* (Lee et al., 2000). *SOC1* is quantitatively induced by vernalization in a *FLC*-dependent manner (Sheldon et al., 2006). Independent ChIP analysis using different tagging systems indicate that *in vivo* binding of *FLC* protein to the *SOC1* genomic sequence occurs through a CArG box motif, which is recognized specifically by MADS-domain transcription factors (Helliwell et al., 2006; Searle et al., 2006). This result is consistent with the *SOC1::GUS* study and in vitro assay (Hepworth et al., 2002). Moreover, it is believed that *FLC* participants in a protein complex to perform its function as more than one *FLC* polypeptide can be detected in the complex *in vivo*. In support of this finding, *in vitro* gel shift experiments indicate that *FLC* needs to form a homodimer to interact with the CArG box motif in the *SOC1* promoter (Helliwell et al., 2006; Hepworth et al., 2002).

GA treatment accelerates *Arabidopsis* flowering under SDs, and this process is correlated with the increase of *SOC1* expression, implying that GA might be another upstream signal of *SOC1* (Borner et al., 2000; Moon et al., 2003). This regulation is not mediated by *FLC* since GA treatment does not affect *FLC* expression under SDs. In the GA-biosynthetic defective mutant *ga1-3*, *SOC1* expression is lower than in wild-type plants, and exogenous GA treatment promotes both *SOC1* expression and flowering. On the contrary, although *SOC1* expression is reduced in GA-insensitive mutant *gai-1*, exogenous GA treatment can recover neither *SOC1* expression nor the

normal flowering phenotype (Moon et al., 2003). In addition, overexpression of *SOC1* is able to bypass the block to flowering in *ga1-3* mutant and the *soc1* mutant is less sensitive to *GA*, suggesting that *SOC1* integrates the *GA* pathway signals although other additional downstream factors may exist (Moon et al., 2003).

2.7 *AGL24* and *SVP*

2.7.1 *AGL24*

Like *SOC1*, *AGL24* also encodes a putative MADS-domain transcription factor. *AGL24* protein is translocated from the cytoplasm to the nucleus to perform its transcriptional function through phosphorylation by a meristematic receptor-like kinase (MRLK) (Fujita et al., 2003). In the past few years, studies on *AGL24* have mainly focused on two stages of plant growth: flowering time control and flower development.

2.7.1.1 *AGL24* is an activator of flowering

Constitutive expression of *AGL24* causes early flowering while *agl24* mutant and RNAi transgenic plants delay flowering. Further studies demonstrated that *AGL24* is a dosage-dependent flowering promoter (Michaels et al., 2003; Yu et al.,

2002), and suggests that *SOC1* is involved in the floral activation of *AGL24*. Both

SOC1 and *AGL24* transcripts are found in the shoot apical meristem an the vegetative

growth phase and highly accumulated in the inflorescence during floral transition.

When overexpressed, *SOC1* and *AGL24* significantly upregulate each other's

expression especially in autonomous pathway mutant or *FRI*-dominant plants

(Michaels et al., 2003). Furthermore, expression results showing that *AGL24* is

downregulated in most late flowering mutants (except *ft -1*) can be explained by the

hypothesis that *AGL24* acts partially downstream of *SOC1*, which is a key floral

signal integrator in *Arabidopsis*. This opinion is also supported by the genetic data.

Overexpression of *AGL24* is able to partially rescue the late flowering phenotype of

the *soc1* mutant and the mutation of *AGL24* suppresses the early flowering of

overexpression of *SOC1*, indicating that *AGL24* is one of the downstream target genes

of *SOC1* (Yu et al., 2002). Nevertheless, *SOC1* and *AGL24* act differently in the

vernalization pathway. Although both of them are activated through vernalization,

AGL24 is regulated in a *FLC*-independent manner while *SOC1* is predominantly

affected by *FLC* (Michaels et al., 2003).

2.7.1.2 *AGL24* regulates floral meristem formation

AGL24 overexpression plants also display some floral alterations, including the

reversion of floral meristem to inflorescence meristem, which is similar to the *ap1*

mutant. Besides, *AGL24* is found to be repressed in both *AP1* and *LFY* inducible

systems, implying that *AGL24* might determine the inflorescence identity and it is regulated by floral meristem identity genes, including *AP1*, *LFY*, etc. In accordance with that, *in situ* studies show that *AGL24* is ectopically expressed in the whole zone of floral meristems in *ap1-1* and *lfy-6* mutants while in wild-type, *AGL24* is expressed mainly in the inflorescence meristem and downregulated in young floral meristems. Moreover, the mutation of *AGL24* is able to reduce the excess inflorescence apices of *ap1-1* and *lfy-6* mutants (Yu et al., 2004). In conclusion, *AGL24* maintains the inflorescence fate in *Arabidopsis* and repression of *AGL24* is required for normal floral meristem development.

2.7.2 *SVP*

SVP was first identified from early flowering mutants with the En-1 transposon (Baumann et al., 1998). *SVP* encodes a typical MADS-box protein, which has high sequence homology to AGL24 except for the C-terminal region. However, it has an antagonistic effect on flowering compared with *AGL24*. The *svp* mutant plants accelerate flowering under both LDs and SDs and the plants are still photoperiod-sensitive. Obvious morphological alterations are not observed in the *svp* mutant, although the potential effect of *SVP* overexpression on floral organogenesis needs further investigation. In accordance with its physiological functions, *SVP* expression is repressed in the apical meristem during floral transition, while the expression is maintained in young floral meristems at stages 1 and 2. Additionally,

SVP represses flowering in a dosage-dependent manner because of the different flowering time between homozygous and heterozygous *svp* mutants (Hartmann et al., 2000). Another interesting finding is that the *SVP* genomic sequence produces several transcripts with different molecular size. It seems that the longer transcript is able to produce the entire protein while the function of the shorter ones remains to be clarified (Hartmann et al., 2000). It is possible that *SVP* is regulated via a post-transcriptional process, which is regulated by the function of *FCA*, another floral regulator in the autonomous pathway.

2.8 MADS-domain proteins

The majority of flowering time genes in *Arabidopsis* encodes transcription factors, such as *LFY*, *FLC*, *SOC1*, *SVP*, etc. A lot of them belong to the MADS-box protein family, which is recognized with a highly conserved DNA-binding motif in the N terminal. This conserved motif would specifically recognize the "CArG box" sequence (CC (A/T)$_6$GG) as a binding site. The name of "MADS" comes from the initials of the first four members of this protein family: *MCM1*, *AGAMOUS*, *DEFICIENS* and *SRF*. A large number of MADS-domain proteins have been identified in species from all eukaryotic kingdoms, including *Arabidopsis thaliana, Drosophila, Oryza sativa,* etc. The MADS-domain proteins can be divided into three different types, the MEF2 type, the ARG80 type and the plant type. The plant type has been further subdivided into class I and class II groups. Class I consists of the MIKC

and Mδ subtypes and class II is composed of Mα, Mβ and Mγ subtypes (Alvarez-Buylla et al., 2000; Parenicova et al., 2003). Most of the known plant MADS-box proteins belong to the MIKC-type and their typical structure is shown in Figure 2.

Figure 2. **The schematic structure of a MADS-domain protein.** MADS: the conserved DNA-binding motif; I: the intermediate region between MADS-domain and K-box; K-box: relative less conserved region; C terminal: the most diverse sequence accounting for the protein-protein interactions and various functions.

CHAPTER 3

Materials and Methods

3.1 Plants growth conditions

Wild-type and transgenic *Arabidopsis* plants of the Columbia ecotype were grown on soil at 22°C under long days (16 h light/8 h dark) or short days (16 h dark/8 h light). Seeds were stratified on soil at 4°C for 4-5 days before being transferred to a growth room in order to ensure synchronized germination. Basta selection was conducted twice within 10-20 days after seed germination to screen transgenic plants.

For plant growth on Murashige and Skoog (MS) agar medium, sterilization of seeds was first performed: Seeds were initially incubated in sterile water for 20-30 min until precipitation. Then they were washed with 70% ethanol and rinsed with sterile water three times. After incubation in 15% Clorox® for 20 min, seeds were rinsed with sterile water again and sequentially sowed in Petri dishes containing autoclaved MS agar medium, which was adjusted to pH 5.8. The plates were maintained in a tissue culture room under LDs (16 h light/ 8 h dark). In addition, the successful *pER22-SVP* transgenic plants in MS agar medium were obtained with hygromycin selection (15μg/ml).

3.2 Vernalization treatment

Seeds were first sown on MS agar plates and germinated in the tissue culture room. 2-3 day-old seedlings were then transferred to a 4°C cold room for 6-week vernalization treatment (without light), after which plants were transferred to soil growth conditions under a short-day photoperiod.

3.3 Plasmid construction and plant transformation

To construct the *35S:SOC1-9myc* plasmid, the *SOC1* cDNA was amplified with the primers SOC1-F1-*Xho*I (5'-CCG<u>CTCGAG</u>TAGCCAATCGGGAAATTAACTA-3') and SOC1-R1-*Xma*I (5'-CG<u>CCCGGG</u>CTTTCTTGAAGAACAAGGTAAC-3'). The resulting PCR products were digested with *Xho*I and *Xma*I and cloned into the corresponding sites of a pGreen-35S-9myc vector to obtain an in-frame fusion of *SOC1-9myc* under the control of a 35S promoter. The pGreen-35S-9myc vector was generated by cloning 9 repetitive myc epitopes into the *Spe*I site of pGreen-35S (Yu et al., 2004).

To construct the *35S:AGL24-6HA* plasmid, the *AGL24* cDNA was amplified with primers AGL24-F1-*Xho*I (5'-CCG<u>CTCGAG</u>GTAGTGTAAGGAGAGATCTGG-3') and AGL24-R1-*Apa*I (5'-AT<u>GGGCCC</u>TTCCCAAGATGGAAGCCCAA-3'). The resulting PCR products were digested with *Xho*I and *Bsp*120I and cloned into the corresponding sites of the pGreen-35S-6HA vector to obtain an in-frame fusion of *AGL24-6HA* under the control of a 35S promoter. The pGreen-35S-6HA vector was generated by cloning 6 repetitive HA epitopes into the *Spe*I site of pGreen-35S (Yu et

al., 2004).

The *35S:SVP-6HA* and *pER22-SVP* constructs were constructed by co-workers in the lab (Li et al., unpublished data). Single insertion transgenic lines were used in our experiments.

For the construction of *Prosoc₁:GUS*, the *SOC1* genomic sequence of 4.4 kb was amplified with the primers SOC1-P4-*Xma*I and SOC1-R1-*Xma*I (Table 1). The amplified products were digested by *Xma*I and cloned into the corresponding site of pHY107. This construct was further mutagenized to produce the mutated AGL24 and SVP binding sites by using the QuikChange® II XL-Site-Directed Mutagenesis Kit (Stratagene). For generation of the mutated CArG box for AGL24 binding, the primers SOC1-M1-F2 and SOC1-M1-R2 (Table 1) were used. For generation of two mutated CArG boxes for *SVP* binding, two pairs of primers, SOC1PM3-F and SOC1PM3-R and SOC1PM4-F and SOC1PM4-R (Table 1), were used, respectively.

A derivative pGreen-35S vector (Yu et al., 2004) was cut by *Kpn*I and *Xho*I to remove the 35S promoter, filled in the ends by T4 DNA polymerase, and self-ligated to generate a promoterless pGreen vector pHY105. A GUS fragment was then amplified from pBI101 and cloned into the *Xba*I site of pHY105 to generate pHY107. These constructs were introduced into wild-type Columbia plants using the *Agrobacterium*-mediated floral dip method (Clough and Bent, 1998). The T3 homozygous lines with single insertion of transgenes were isolated for most studies, while both T2 and T3 plants were used for GUS expression analysis.

Table 1. Primers for GUS constructs

Primer name	Sequence
SOC1-P4-*Xma*I	5'-AACCCGGGATCGTATTTACTAGTGGTATACG-3'
SOC1-R1-*Xma*I	5'-GTCCCGGGCTTTCTTGAAGAACAAGGTAA-3'
SOC1-M1-F2	5'-GGGATGGAAAGATATTATAAAAATTGATTAAAAGGAATATACCTGTATTACTCACAGGTAAG-3'
SOC1-M1-R2	5'-CTTACCTGTGAGTAATACAGGTATATTCCTTTTAATCAATTTTTATAATATCTTTCCATCCC-3'
SOC1PM3-F	5'-GTCCATATGTATCAAAATATGGGATTTTTCCTCTTTCTTAAGGCTTTTTTCC-3'
SOC1PM3-R	5'-GGAAAAAAGCCTTAAGAAAGAGGAAAAATCCCATATTTTGATACATATGGAC-3'
SOC1PM4-F	5'-GGTCTTTCTTAAGGCTTTTTTGGAAAATACCTAAAGGATGAGGTTTCAGACGTCCATC-3'
SOC1PM4-R	5'-GATGGACGCTTGAAACCTCATCCTTTAGGTATTTTCCAAAAAAGCCTTAAGAAAGACC-3'

3.4 Chromatin Immunoprecipitation (ChIP) Assay

Nine-day old *35S:AGL24-6HA, 35S:SOC1-9myc* and *35S:SVP-6HA* seedlings were fixed at 4°C for 40 mins in 1% formaldehyde under vacuum. Fixed tissues were homogenized, and chromatin was isolated and sonicated to produce DNA fragments below 500 bp. The solubilized chromatin was incubated with anti-HA agarose beads (Sigma) or anti-myc agarose beads (Sigma) for 90 min at 4°C. Beads were washed 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) for 5 times and incubated with elution buffer (50 mM Tris, pH 8.0, 1% SDS, and 10 mM EDTA) for 30 min at 65 °C. The supernatant was collected, and co-immunoprecipitated DNA was recovered according to a published protocol (Wang et al., 2002). The enrichment of an unrelated DNA sequence from the *ACTIN2/7* (*ACTIN*) gene that is constitutively expressed in *Arabidopsis* was used as an internal reference for the DNAs co-immunoprecipitated by anti-HA or anti-myc antibodies (Johnson et al., 2002). For *35S:AGL24-6HA* and *35S:SVP-6HA*, a DNA eluted sample pulled down by the anti-myc antibody was applied as a negative control to measure the enrichment fold. Similarly, a DNA eluted sample pulled down by the anti-HA antibody was used in ChIP assays with *35S:SOC1-9myc*. DNA enrichment was evaluated by real-time quantitative PCR in triplicates. All primer sequences used for the ChIP enrichment test are listed in Table 2.

Table 2. Primers for ChIP assay

Primer pairs	Sequence
ACTIN	5'-CGTTTCGCTTTCCTTAGTGTTAGCT-3' 5'-AGCGAACGGATCTAGAGACTCACCTTG-3'
SOC1-1	5'-TGAAAAGTCTTGTACTTTTTCCCC-3' 5'-AATAAAATGTGCTCTTTCGTAGCC-3'
SOC1-2	5'-GCTAAATAGTCAGTCATATGTGTCGC-3' 5'-GGATTAATGGTCACTTAGGTAATGAGG-3'
SOC1-CHIP-1	5'-GGATGCAACCTCCTTTCATGAG-3 5'-ATATGGGTTTGGTTTCATTTGG-3'
SOC1-CHIP-2	5'-AAAAACCTAACCAGGAGGAAGC-3' 5'-CTTCTTCTCCCTCCAGTAATGC-3'
SOC1-CHIP-3	5'-GCAAAAGAAGTAGCTTTCCTCG-3' 5'-AGCAGAGAGAGAAGAGACGAGTG-3'
SOC1-CHIP-4	5'-TGGACGCTTGAAACCTCATCCT-3' 5'-GGGAGGGAAAAAGATGTGTATG-3'
SOC1-CHIP-5	5'-AGTTGGATGGAAATGCCTGTCA-3' 5'-TTACAAGTGGGGGCATATAGGT-3'
SOC1-CHIP-6	5'-TCTCGTACCTATATGCCCCCACT-3' 5'-TTTATCTGTTGGGATGGAAAGA-3'
SOC1-CHIP-7	5'-GAGGCTAGTACAGAGACAATGG-3' 5'-GACCAAAAATAGCAAATGCCTC-3'
SOC1-CHIP-8	5'-TATATCGGGAGGAGGACCACAC-3' 5'-ATCCATACAGATTTTCGGACCT-3'
SOC1-CHIP-9	5'-ATCACATCTCTTTGACGTTTGCTT-3' 5'-GCCCTAATTTTGCAGAAACCAA-3'
SOC1-CHIP-10	5'-CTTTTGGTTTGAACTAATCTTTGTCTTG-3'

	5'-AATGAGCATGAAATGAAGCATGA-3'
SOC1-CHIP-11	5'-TGTTTCAGACATTTGGTCCATTTG-3'
	5'-AGTCTTGTACTTTTTCCCCCTATTTTAG-3'
AGL24-CHIP-1	5'-ACAAGTTCGAAATTTGGGCCA-5'
	5'-TTCACGTTTTACCATTTGCCGT-3'
AGL24-CHIP-2	5'-TGCTGTTCATCAGTTCATCTACC-3'
	5'-CTTATCAGGTGTCGCATCTAG-3'
AGL24-CHIP-3	5'-ATCCCCAATCATACCAAGTGAC-3'
	5'-GTACTGGGAAATAAGAGAGCAG-3'
AGL24-CHIP-4	5'-AGTTCAATCCATCAAGATCCTCTC-3'
	5'-TCTTTGGTAGACCTACTGAACA-3'
SVP-CHIP-1	5'-ATGGGTTTGTAGTAGTTGCGTGGAGTA-3'
	5'-TTGGGACACGATCCATTGTCCGTACAG-3'
SVP-CHIP-2	5'-TTCAGTGATGATTGATACCCCC-3'
	5'-CACTAATTTGGAAAGTTTGTCATGC-3'
SVP-CHIP-3	5'-AAAAAGAAATTCCCCACTGACC-3'
	5'-GATATTGGTCTGTGTGTGGTGG-3'
SVP-CHIP-4	5'-TCCATTTCAGTCGTCTTGTCAC-3'
	5'-GAAGAGATGGAGGAGGAGGAAG-3'
SVP-CHIP-5	5'-CTGATACATAGGAGTTTACTGTATC-3'
	5'-GAATATTACCGTAGTTAGATACC-3'
SVP-CHIP-6	5'-TGTTGGGAAAAGTCATTTAGAGAG-3'
	5'-TGTATGTCCCCGAAGATAGGAAC-3'
SVP-CHIP-7	5'-GAGAACAAGCGGTTGAGGCAGCAAGT-3'
	5'-CAACTGGGGATTAAATTTAGGGGT-3'
LFY-CHIP-1	5'-CTATACGACGTCGTTTGAAAGGGATCC-3'
	5'-GCGTTTATATCTTCTCGGTCAGCCCA-3'
LFY-CHIP-2	5'-TATCTTCCCCTAACAATACTTCCAAAGC-3'
	5'-TCTTTGCAGAAGCCCGATAAGTTACT-3'

AP1-CHIP-1	5'-CAAGTATCTTCTCCATACTGATC-3'
	5'-TTATAAAGGTATCATAGAGATCGG-3'
AP1-CHIP-2	5'-GAGTTAATTCTTTTTATGGATCCC-3'
	5'-CATGTTTTGCAAATCTAAGCAAAG-3'
AP1-CHIP-3	5'-GCAGTAGTGAATAATTAGGGCAA-3'
	5'-AAGTTGCTCTTGTTGTCTTCTCCC-3'
AP1-CHIP-4	5'-GTACGCAGGATTTAAGGAAAGAG-3'
	5'-GATTTTTGTCCTGATCATCTACAAC-3'
AP1-CHIP-5	5'-TTTGGTGTTTTCCACGTGTCTTC-3'
	5'-ATAATACCGTAAGCAATAGTTGC-3'

3.5 Quantitative Real-time PCR

Only the aerial part of seedlings was collected for RNA extraction. Total RNAs were extracted by RNeasy Plant Mini Kit (Qiagen) and reverse-transcribed by the ThermoScript RT-PCR system (Invitrogen). Quantitative Real-time PCR was performed in triplicates on 7900HT Fast Real-Time PCR system (Applied Biosystems) with SYBR Green PCR Master Mix (Applied Biosystems). Efficiency of each pair of primers was determined based on its standard curve that was obtained from a series of 10-fold diluted template DNAs. The difference between the cycle threshold (Ct) of target genes and the Ct of control primers ($\Delta Ct = Ct_{target\ gene} - Ct_{control}$) was used to obtain the normalized expression of target genes. The relative fold change was eventually calculated based on both Ct value and primer efficiency according to a published protocol (Pfaffl, 2001). A constitutively expressed gene, *β-TUBLIN* was used as an internal control. Primer sequences used for gene expression analyses are listed in Table 3

Table 3. Primers for real-time PCR

Primer name	Sequence
TUB-P1	5'-ATCCGTGAAGAGTACCAGAT-3'
TUB-P2	5'-AAGAACCATGCACTCATCAGC-3'
SOC1-P1	5'-AGCTGCAGAAAACGAGAAGCTCTCTG-3'
SOC1-P2	5'-GGGCTACTCTCTTCATCACCTCTTCC-3'
SVP-P1	5'-CAAGGACTTGACATTGAAGAGCTTCA-3'
SVP-P2	5'-CTGATCTCACTCATAATCTTGTCAC-3'
AGL24-P1	5'-GAGGCTTTGGAGACAGAGTCGGTGA-3'
AGL24-P2	5'-AGATGGAAGCCCAAGCTTCAGGGAA-3'
AP1-P1	5'-CATGGGTGGTCTGTATCAAGAAGAT-3'
AP1-P2	5'-CATGCGGCGAAGCAGCCAAGGTT-3'
FT-P1	5'-CTTGGCAGGCAAACAGTGTATGCAC-3'
FT-P2	5'-GCCACTCTCCCTCTGACAATTGTAGA-3'
FLC-P1	5'-CTAGCCAGATGGAGAATAATCATCATG-3'
FLC-P1	5'-TTAAGGTGGCTAATTAAGTAGTGGGAG-3'
AG-P1	5'-ATCGGACAATTCTAACACCGGATCG-3'
AG-P2	5'-CCCATCAATTGCCTGTTGGAGTTTTG-3'
GUS-P1	5'-CCATCGCAGCGTAATGCTCTACA-3'
GUS-P2	5'-GCAGTTCAACGCTGACATCACCATT-3'

3.6 GUS histochemical assay and expression analysis

GUS staining was performed as previously described (Jefferson et al., 1987). For the examination of *GUS* transcription, RNA was extracted from transgenic plants harboring different reporter genes, reverse-transcribed into cDNA, and further detected for *GUS* gene expression with a specific primer pair (Table 3) by quantitative real-time PCR.

3.7 Western blot analysis

Western blot analyses were performed to ensure recovery of AGL24-HA, SOC1-9myc and SVP-6HA fusion proteins during ChIP assays. Proteins were separated on 12% SDS-polyacrylamide resolving gel and blotted onto PVDF membrane (Bio-Rad). The membrane was blocked with 5% non-fat dry milk powder dissolved in PBS buffer for 1 hour, and incubated with 1:10000 (v/v) anti-HA or 1:8000 anti-myc alkaline phosphatase conjugate antibody (Sigma) that was diluted with PBS buffer supplemented with 0.05% Tween 20 at room temperature for 45 min-60min. The membrane was subsequently washed three times with PBS buffer containing 0.05% Tween 20 for 15 min each. Finally, the membrane was incubated with CDP-Star (Roche) and exposed to CL-X Posure X-ray film (Pierce).

3.8 β-Estradiol induction of pER22-*SVP*

For continuous treatment, 10 μM β-estradiol was applied and replaced every two days once treatment started. To examine the induced *SVP* expression, the seedlings at different developmental stages grown on solid MS medium were transferred into MS liquid medium supplemented with 10 μM β-estradiol for a single treatment. 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 by replacing the β-estradiol with the same amount of dimethyl sulfoxide (DMSO) that was used to dissolve β-estradiol.

CHAPTER 4

Results

4.1 Direct interaction between *SOC1* and *AGL24*

4.1.1 Temporal expression of *SOC1* and *AGL24* in seedlings

It has been suggested that *AGL24* might be an upstream regulator of *SOC1* (Michaels et al., 2003). In order to further elucidate the relationship between *AGL24* and *SOC1*, the temporal expression patterns of *SOC1* and *AGL24* in wild-type Columbia seedlings (aerial part) were examined (Figure 3). The expression of *AP1* was used as an indicator for floral transition (Hempel FD et al., 1997), which was significantly increased during floral transition starting from day 9 (Figure 3B). Under LD conditions, the expression of both *SOC1* and *AGL24* steadily showed similar increasing trend after seed germination (Figure 3A). However, the expression of *SOC1* and *AGL24* did not continuously increase 13 days after germination, which could be a result of floral meristem formation as *AGL24* and *SOC1* have to be repressed by *AP1* in emerging floral meristems (Yu et al., 2004; Liu et al., 2007).

4.1.2 *AGL24* promotes *SOC1* expression

To examine in detail how *AGL24* regulates *SOC1*, we quantitatively measured
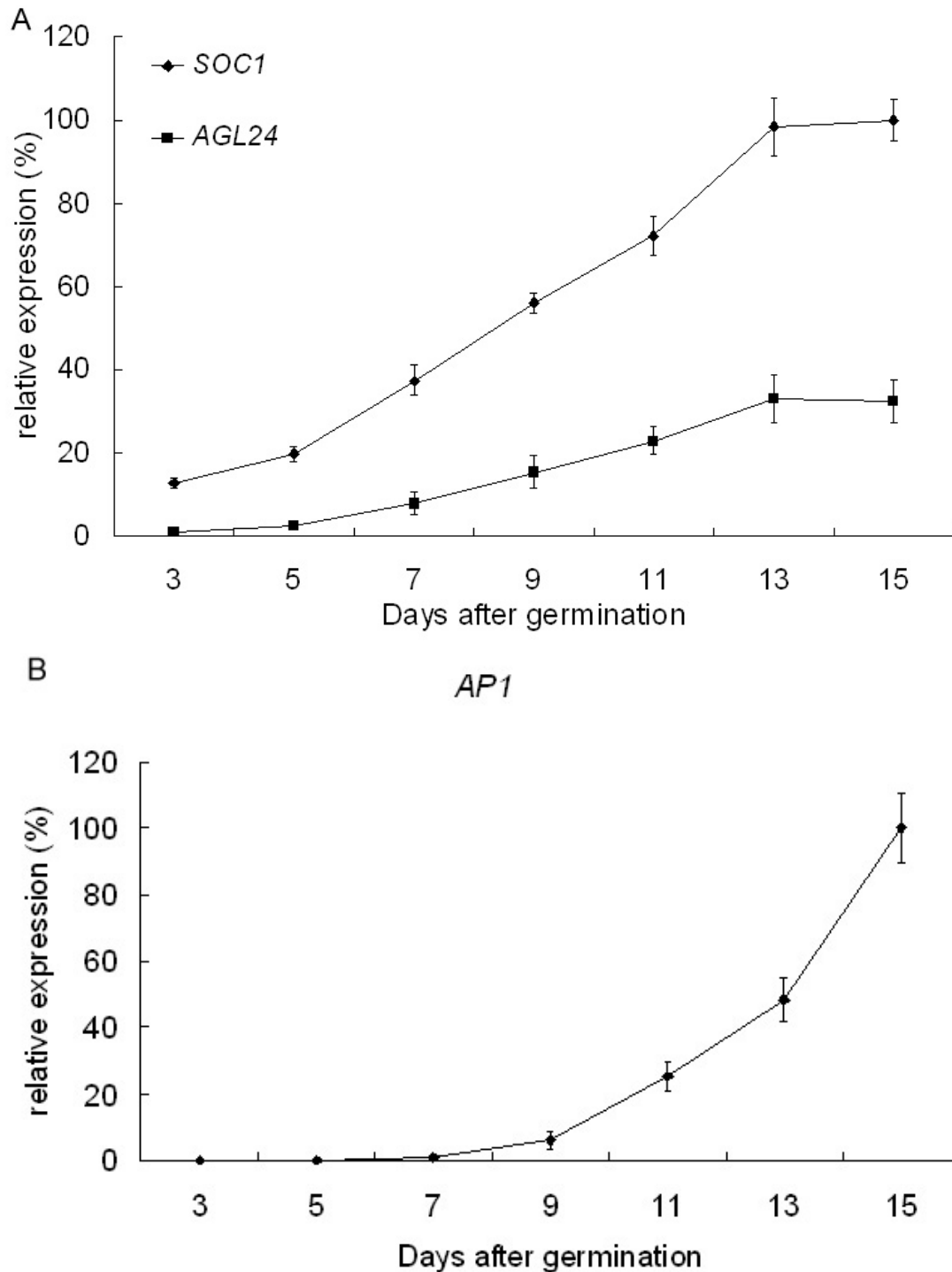
Figure 3. Temporal expression patterns of *SOC1* and *AGL24* in wild-type seedlings grown under long days. Transcript levels were determined by quantitative real-time PCR analyses of three independently collected samples. Results were normalized against the expression of *TUB2*. Error bars indicate the standard deviation. (A) Expression of *SOC1* and *AGL24* in developing seedlings. (B) Expression of *AP1* in developing seedlings. During floral transition starting from day 9, *AP1* expression is significantly upregulated.

SOC1 mRNA abundance in wild-type, *35S:AGL24* and *agl24-1* plants under long days. As shown in Figure 4, *SOC1* mRNA level in *35S:AGL24* plants was elevated compared to that in wild-type, especially at the floral transition stage (9 to 11 days after germination). Conversely, *SOC1* expression was significantly delayed in *agl24-1* mutants. From 3 days to 7 days after seed germination, there was no obvious difference in *SOC1* expression between *agl24-1* and wild-type plants. However, when plants began to enter the floral transitional stage from 7 days after germination, *SOC1* was repressed in *agl24-1* mutants. Consistent with the above changes, alteration of *AP1* expression was also observed in *35S:AGL24* and *agl24-1*.

4.1.3 *AGL24* directly promotes *SOC1* transcription

Since *AGL24* encodes a MADS domain transcription factor, its protein product should bind to the *SOC1* promoter through the CArG motif if *AGL24* directly controls *SOC1* transcription. To examine this possibility, we performed chromatin immunoprecipitation (ChIP) assay with a functional transgenic line expressing an AGL24-6HA fusion protein driven by the *CaMV* 35S promoter. The *35S:AGL24-6HA* transgenic line exhibited a similar early flowering phenotype as *35S:AGL24* plants (Figure 5). *35S:AGL24-6HA* also showed the generation of secondary flowers from a primary floral meristem, which is characteristic of *35S:AGL24* plants (Yu et al., 2004). These observations suggest that the AGL24-6HA fusion protein retained the biological functions of the native AGL24 protein, making the *35S:AGL24-6HA* plants
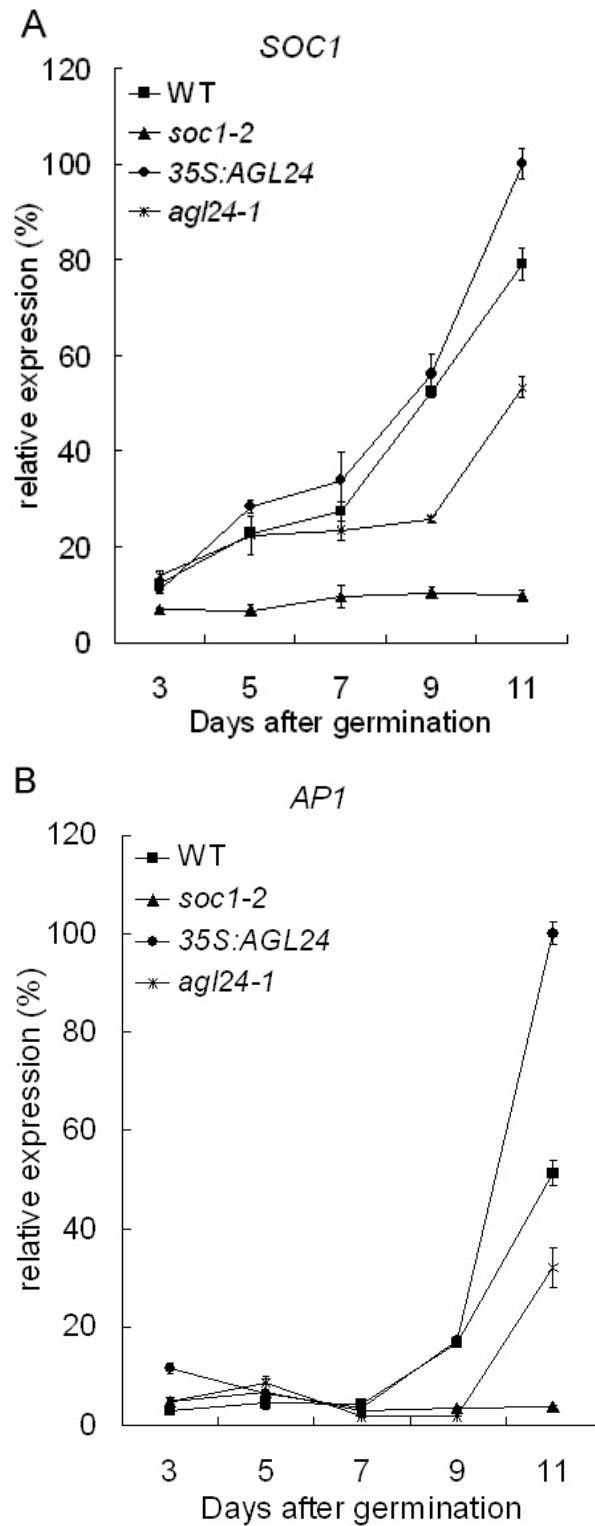
Figure 4. *SOC1* expression is upregulated by *AGL24* during floral transition. Real-time PCR data were normalized against the constitutive expression of *TUB2*. Error bars indicate SD. Plants were grown under long days condition. (A) Temporal expression of *SOC1* in different genetic backgrounds. (B) Temporal expression of *AP1* in different genetic backgrounds, showing when floral transition occurred.
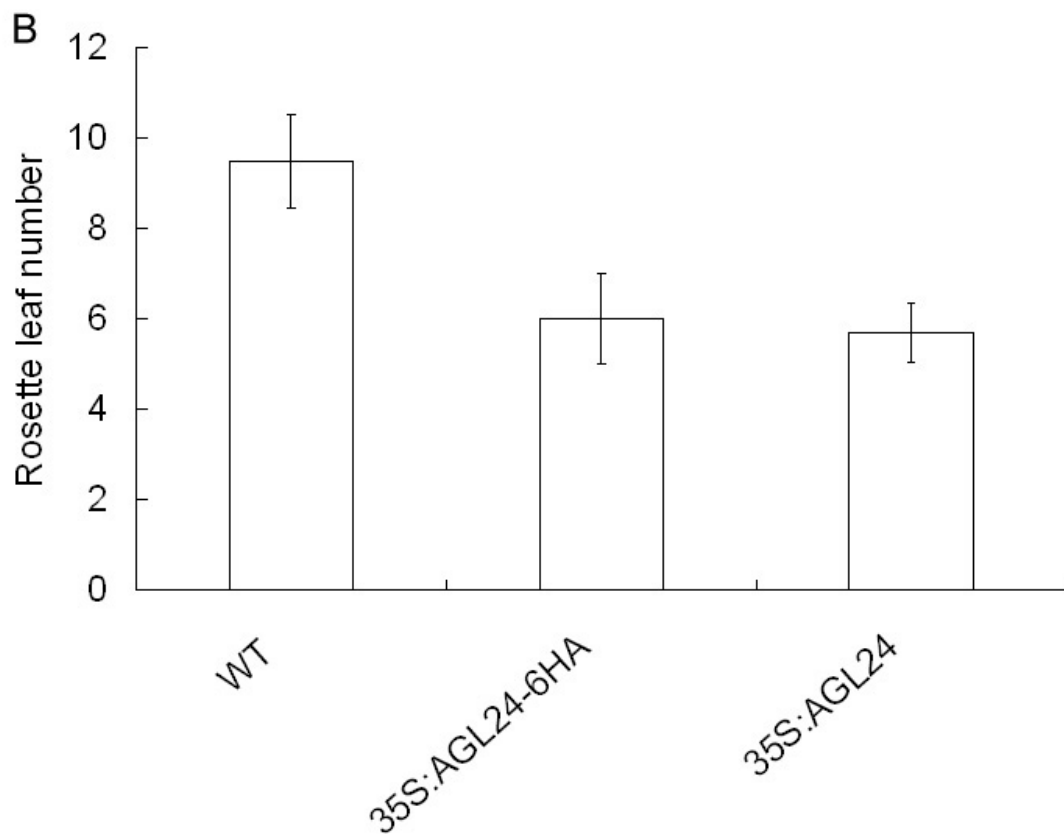
Figure 5. Generation of functional *35S:AGL24-6HA* transgenic line. (A) *35S:AGL24-6HA* (middle) and *35S:AGL24* (right) plants exhibit early flowering compared with a wild-type plant (left) under long days. (B) Flowering time comparison. Number of rosette leaves is used for flowering time calculation.

a suitable transgenic line for ChIP assay.

We analyzed the *SOC1* genomic sequence to identify putative CArG motifs with a maximum of one nucleotide mismatch and designed two pairs of primers (SOC1-1 & SOC1-2) located near these motifs for the measurement of DNA enrichment (Figure 6). The SOC1-2 fragment was found to be significantly enriched when *35S:AGL24-6HA* DNA was precipitated by an anti-HA antibody in ChIP assays (Figure 6B). To identify the precise binding site of AGL24, we dissected the 1.6 kb *SOC1* promoter region centered by the SOC1-2 fragment with 8 pairs of primers and performed the quantitative real-time PCR. Genomic fragment 6 (-1260 to -1133 nt from the translational start site) containing a CArG motif with one nucleotide mismatch showed the strongest enrichment (Figure 6C), implying that it is the AGL24-6HA protein binding site *in vivo*.

To further validate that this CArG motif is responsible for the upregulation of *SOC1 in vivo*, GUS histochemical assay was performed. We made a translational fusion of the *SOC1* genomic region and GUS reporter gene (Figure 7A). 2 kb native 5' upstream sequence from *SOC1* translational start site was included in this construct, because it has been suggested that a *SOC1* genomic fragment containing 1.4 kb upstream sequence is at least sufficient to complement the *soc1* mutation (Samach et al., 2000). According to ChIP results, the corresponding AGL24 binding site was mutated in this construct in order to generate another reporter gene cassette (Figure 7A). As shown in Figure 7B, GUS staining pattern in the transformants with mutated CArG motif was weaker than that in the transformants with native CArG motif, which
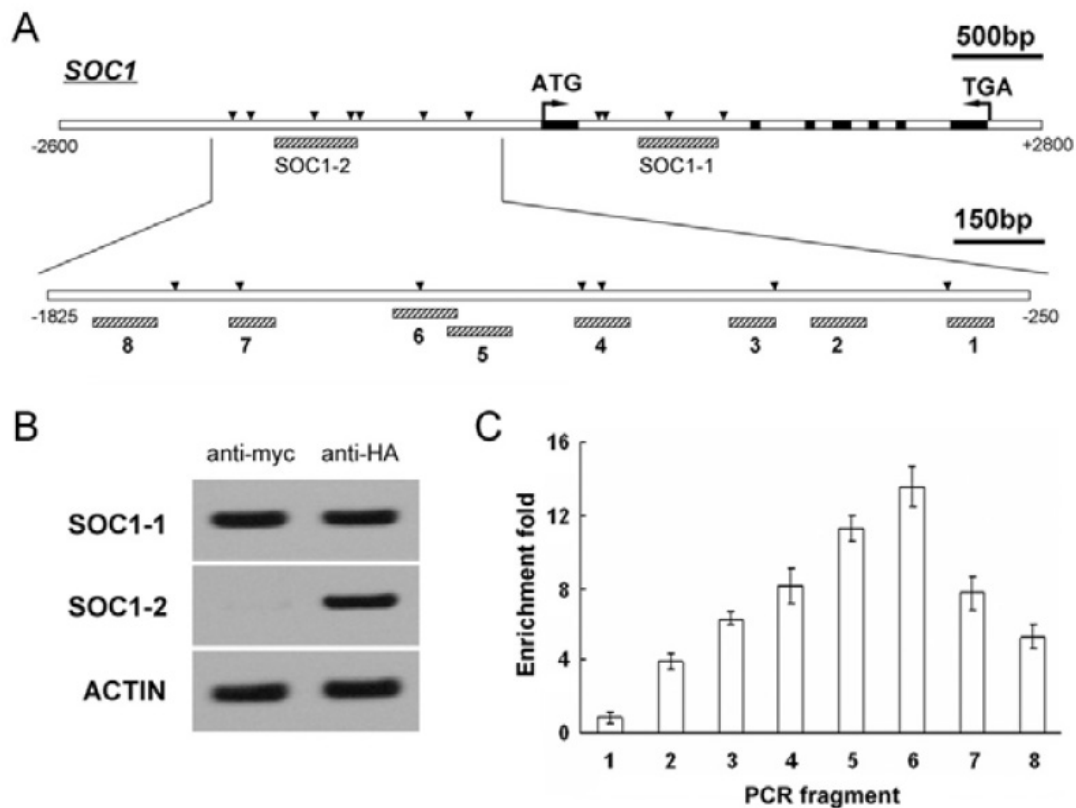
Figure 6. *AGL24* directly regulates *SOC1*. (A) Schematic diagram of the *SOC1* genomic sequence. Exons and introns are represented by black boxes and white boxes, respectively. Arrows indicate translation start site and stop condon. 11 arrowheads show putative MADS-domain protein binding sites with either perfect match or single mismatch of CArG box. Two fragments SOC1-1 and SOC1-2 were used for ChIP enrichment analysis. Eight pairs of primers spanning the SOC1-2 region were designed for precise AGL24 binding motif examination. (B) Gel electrophoresis result shows that SOC1-2 DNA fragment was enriched in *35S:AGL24-6HA* sonicated chromatin with anti-HA beads. Anti-myc beads were applied as nonspecific binding controls. (C) Quantitative real-time PCR reveals that the accurate binding site of AGL24-6HA fusion protein is located near the number 6 fragment. Enrichment fold was first calculated against *ACTIN*, and then this value from anti-HA eluted sample is normalized against the value from anti-myc eluted sample. Error bars show SD.
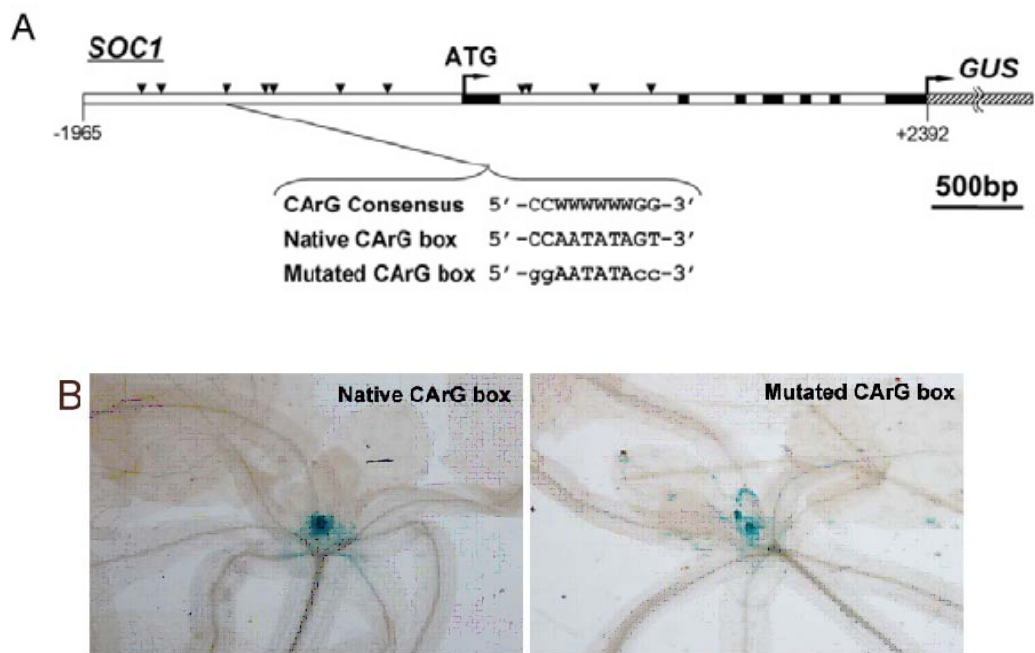
Figure 7. Validation of AGL24-6HA binding site to *SOC1* with GUS expression analysis. (A) Schematic diagram of the *Pro$_{SOC1}$:GUS* construct. The native one-mismatch CArG box (5'-CCAATATAGT-3') was mutated into (5'-ggAATATAcc-3'). (B) GUS staining patterns in 16-day-old transformants containing *Pro$_{SOC1}$:GUS* and its derived construct with the mutated CArG box.

is further supported by real-time PCR detecting *GUS* mRNA level (unpublished data). These results confirm that AGL24 binds to the *SOC1* promoter to promote its expression.

4.1.4 *SOC1* reciprocally affects *AGL24* expression

As *AGL24* was also downregulated in *soc1-2* mutant (Yu et al., 2002), we further quantitatively analyzed the effect of *SOC1* on *AGL24* expression. With the same strategy to investigate *SOC1* expression, *AGL24* mRNA levels in wild-type, *35S:SOC1* and *soc1-2* developing seedlings were compared under LD conditions. In *soc1-2* mutants, *AGL24* expression increased from 5-day old seedlings in a trend similar to that shown in wild-type plants. However, accumulation of *AGL24* mRNA was significantly lower in *soc1-2* mutants as compared to that in wild-type plants (Figure 8A). On the other hand, *AGL24* was significantly up-regulated in *35S:SOC1* plants before 5 days after germination, although it decreased afterwards. These opposite effects could be due to extremely early flowering of *35S:SOC1* plants as the basal *AP1* mRNA level in *35S:SOC1* plants was notably higher than that in wild-type plants. As it has been reported that *AGL24* is repressed by *AP1* in emerging floral meristems (Yu et al., 2004), down-regulation of *AGL24* in *35S:SOC1* from 5 days after germination may occur because of the elevated *AP1* expression in *35S:SOC1*.
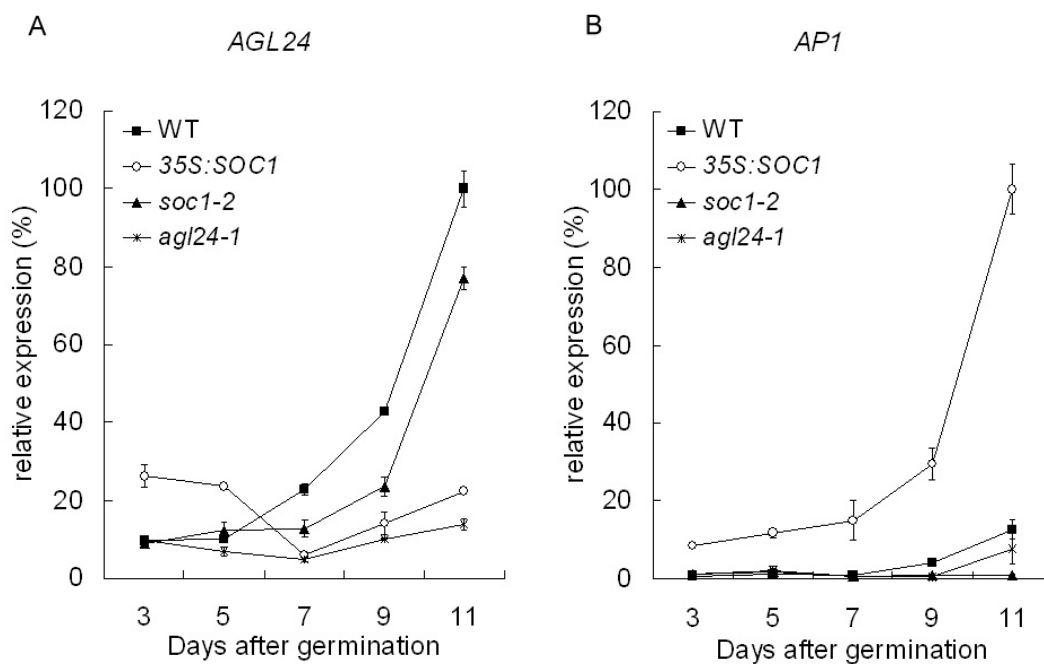
Figure 8. *SOC1* **regulates** *AGL24* **expression in developing seedlings.** Real-time PCR data were normalized against the constitutive expression of *TUB2*. Error bars indicate SD. Plants were grown under long days condition.   (A) Temporal expression of *AGL24* under different genetic backgrounds. (B) Temporal expression of *AP1* under different genetic backgrounds, revealing the floral transition phase. *35S:SOC1* plants exhibited much higher *AP1* expression level, compared with other transgenic and wild-type plants.

4.1.5 *SOC1* directly controls *AGL24* expression

Since *SOC1* also encodes a MADS-domain transcriptional factor, we performed similar ChIP assays to test whether *AGL24* is a direct target of *SOC1*. We established a functional *35S:SOC1-9myc* system (Figure 9A and 9B) that shows similar early flowering as *35S:SOC1*. *AGL24* genomic region was scanned to identify putative CArG motifs. Four pairs of primers were designed to cover the entire promoter region of *AGL24* in ChIP assays (Figure 9C). Using an anti-myc antibody, *35S:SOC1-9myc* precipitated DNA fragments showed around 9-fold enrichment in genomic region 1 (-2125 to -1987 nt from the translational start site), where two CArG motifs each with one nucleotide mismatch are located (Figure 9D).

4.1.6 Investigation of combined effect of *SOC1* and *AGL24* in the vernalization pathway

The direct interaction between *SOC1* and *AGL24* raises the question how they act together to receive floral signals, such as those in the vernalization pathway. Since *SOC1* is one of the main targets of *FLC*, which responds to vernalization treatment, while *AGL24* has been identified in the *FLC*-independent vernalization pathway, we proceeded to examine the integrated effect of *SOC1* and *AGL24* in the vernalization pathway (Hepworth et al., 2002; Helliwell et al., 2006; Michaels et al., 2003; Searle et al., 2006; Yu et al., 2002). As shown in Figure 10, *agl24 soc1* double mutants were
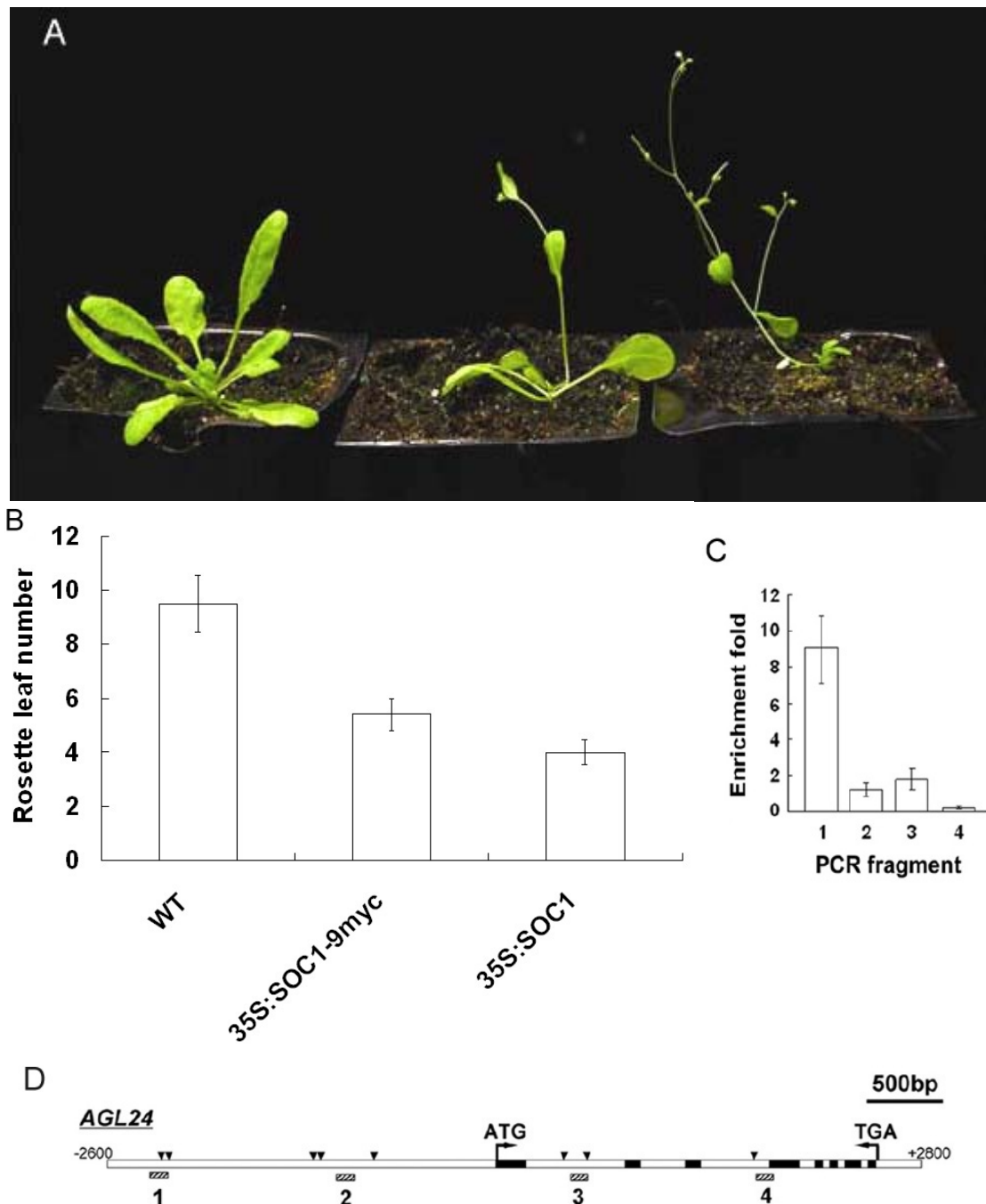
Figure 9. *SOC1* directly controls *AGL24*. (A) *35S:SOC1-9myc* (middle) and *35S:SOC1* (right) plants exhibit early flowering compared with a wild-type plants (left) under long days. (B) Flowering time comparison among wild-type, *35S:SOC1-9myc, 35S:SOC1*. Number of rosette leaves is used for flowering time calculation. (C) Quantitative real-time PCR result shows significant enrichment of the number 1 DNA fragment. Fold enrichment was calculated as shown in Figure 6. (D) Schematic diagram of *AGL24* genomic sequence. Exons and introns are represented by black and white boxes, respectively. The arrowheads denote putative MADS-domain protein binding sites with either perfect match or single mismatch of CArG box.

still responsive to vernalization under SDs, implying that other *FLC* irrelevant factors such as *AGL19* or *FLC*-related factors such as *FT* can be independently activated by the vernalization pathway to promote the transition from the vegetative to reproductive growth (Schonrock et al., 2006).
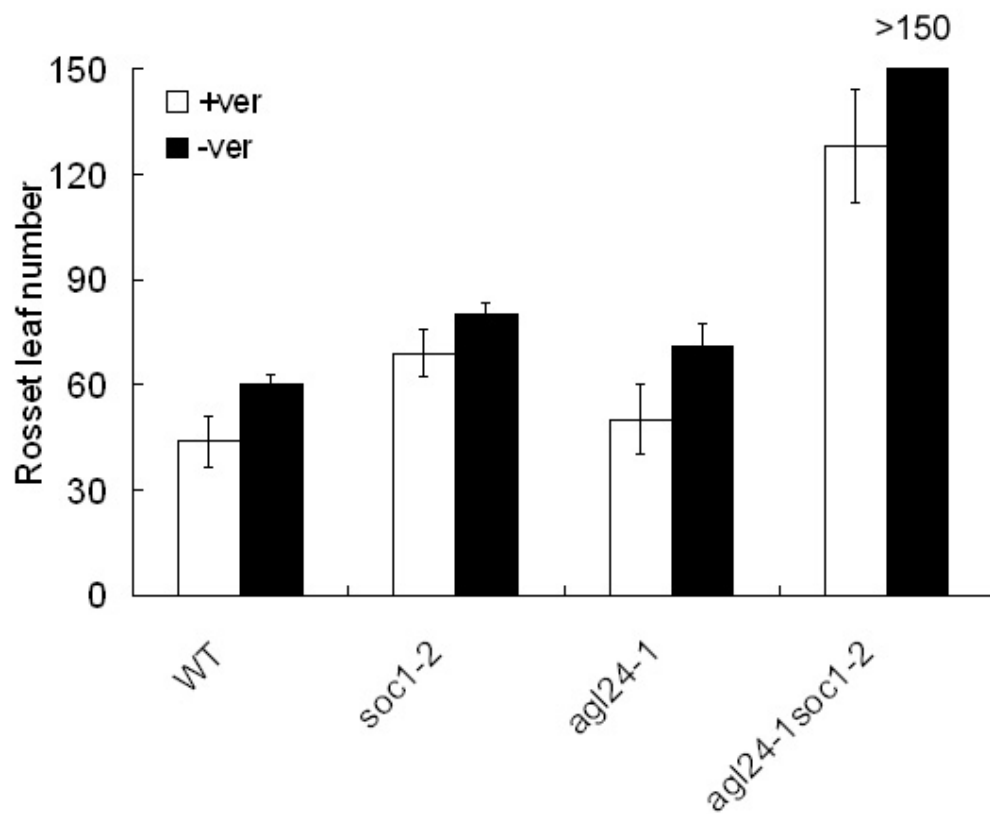
Figure 10. Comparison of flowering time of wild-type, *soc1-2*, *agl24-1* and *agl24-1soc1-2* plants under short days after vernalization treatment. All plants are Columbia ecotype. Number of rosette leaves represents flowering time. SD were scored from at least 10 plants of each genotype

4.2 *SVP* controls flowering time through repression of *SOC1*

SVP encodes another MADS domain protein and its mutants exhibited early flowering but no floral organ defects under both LD and SD conditions (Hartmann et al., 2000), suggesting that *SVP* is involved in flowering time control and may perform its function through the floral signaling integrators, including *SOC1*.

4.2.1 *SVP* constantly suppresses *SOC1* expression

To investigate whether *SVP* is relevant with *SOC1*, firstly we quantitatively measured the *SOC1* expression in wild-type, *svp-41* mutant and *SVP* overexpression transgenic lines. As shown in Figure 11, *SOC1* expression was dramatically upregulated in *svp-41* mutants background and repressed in *35S:SVP* plants. The expression level of *SOC1* in *35S:SVP* was as low as that in *soc1-2* mutants. Furthermore, unlike *AGL24,* the repressive effect of *SVP* on *SOC1* expression sustained from the vegetative to reproductive stage. In 3-day old seedlings, *SOC1* expression in *svp-41* mutants was already significantly elevated.(Figure 11A). Consistent with the change of *SOC1* expression, alteration of *AP1* expression was observed in *svp-41* and *35S:SVP* plants, especially during the floral transitional phase (Figure 11B), which should be one of the major reasons causing the altered flowering time in these plants (Figure 11C).
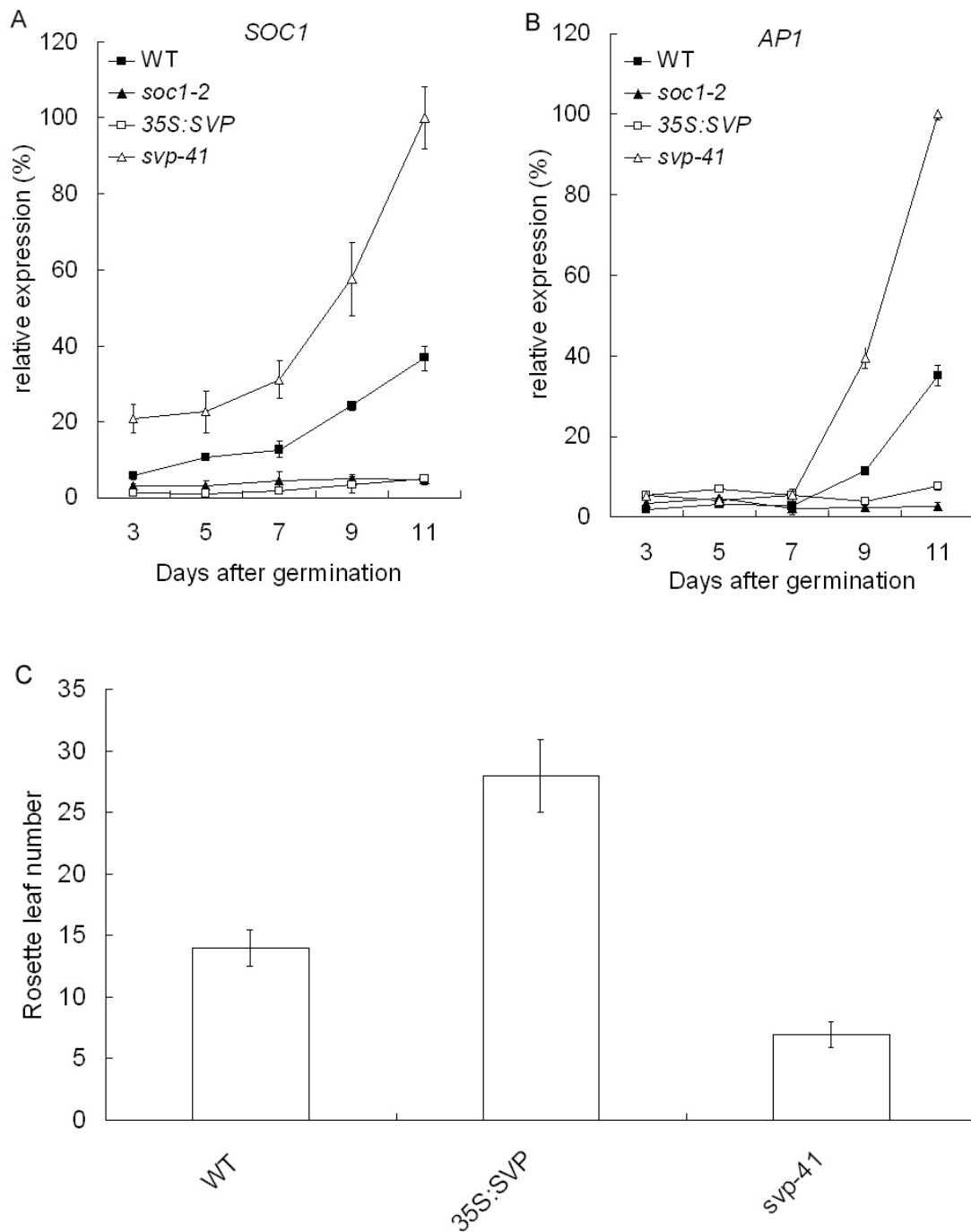
Figure 11. *SVP* constantly represses *SOC1* expression in developing seedlings. *TUB2* was used to normalize real-time PCR results of three independently collected samples. Error bars indicate SD. (A) Temporal expression of *SOC1* under different genetic backgrounds. (B) Temporal expression of *AP1* under different genetic backgrounds. *AP1* expression was greatly upregulated in *svp-41* mutants during floral transition phase (7 d to 9 d). (C) Flowering time of different transgenic lines and wild-type (Col) under long days. Number of rosette leaves represents flowering time. Values representing the mean ±SD were scored from at least 15 plants of each genotype.

4.2.2 *SVP* represses *SOC1* expression mainly in the shoot apex

SOC1 and *SVP* transcripts are detectable in both the leaf and shoot apical meristem of developing seedlings (Figure 12). As flowering promoters, *SOC1* and *AGL24* showed different expression patterns in the leaf and shoot apical meristem. *SOC1* expression was continuously increased in both tissues of developing seedlings (Figure 12A), while *AGL24* expression was elusively increased in the shoot apical meristem (Figure 12C). Interestingly, *SVP* expression in the whole seedlings did not demonstrate a steady increasing or decreasing pattern (Figure 12B and 12E). This could be due to the opposite expression pattern of *SVP* in the leaf and shoot apical meristem. Since *SVP* has been proposed as a flowering repressor, it is possible that *SVP* needs to be suppressed only in the meristem region in order to activate *SOC1*, thus promoting floral transition. To examine this possibility, we collected tissue-specific materials for reverse transcription PCR to test whether *SVP* can repress *SOC1* in a spatially specific manner. We found that *SOC1* expression in the shoot apical meristem of *svp-41* mutants was greatly upregulated (nearly 45 fold change) compared with that in wild-type plants, while the increase of *SOC1* expression was relatively low in the leaf tissue (Figure 13). This suggests that *SVP* regulates the transcription of *SOC1* mainly in the shoot apex. Nevertheless, the upregulation of *SOC1* in leaf may be partially responsible for promoting flowering since *SOC1* was also notably expressed in the leaf tissues of wild-type plants.
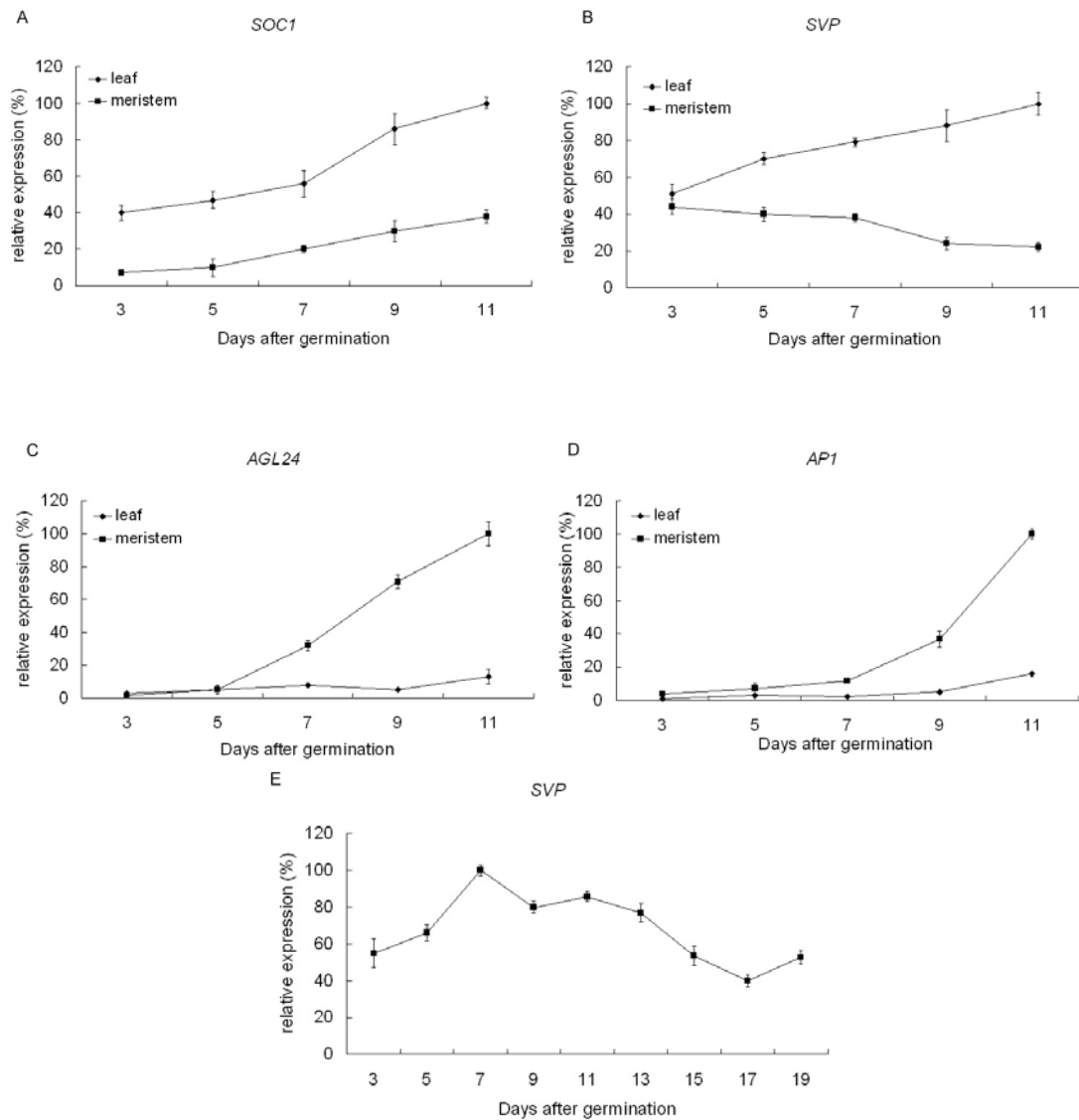
Figure 12. Temporal expression of *SOC1*, *SVP*, *AGL24* and *AP1* in leaf and meristem tissues of developing wild-type seedlings. Transcript levels were determined by quantitative real-time PCR. Results were normalized against the expression of *TUB2*. Error bars stand for SD. (A) Temporal expression of *SOC1* in leaf and meristem tissues. (B) Temporal expression of *SVP* in leaf and meristem tissues. (C) Temporal expression of *AGL24* in leaf and meristem tissues. (D) Temporal expression of *AP1* in leaf and meristem tissues. (E) Temproal expression of *SVP* in the whole developing seedlings from 3-day old to 19-day old.
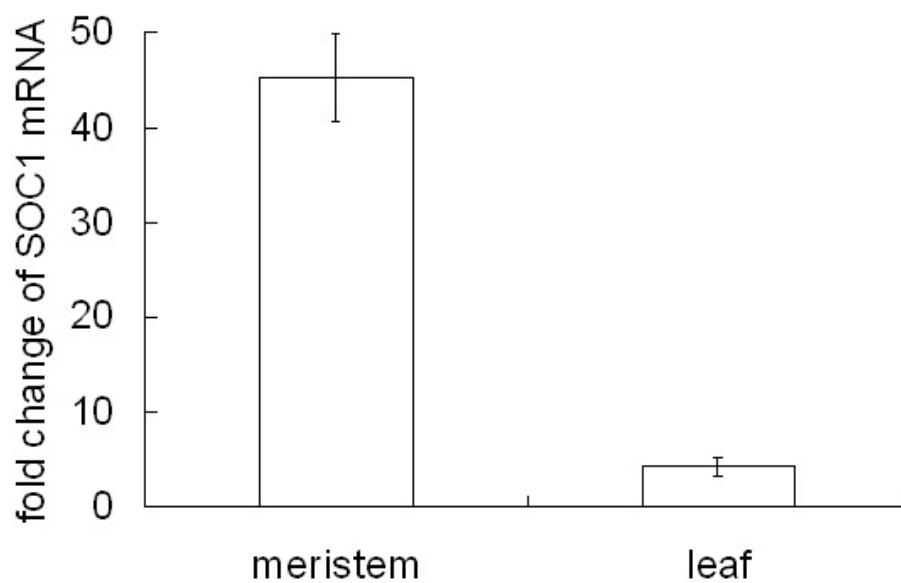
**Figure 13. Comparison of *SOC1* expression in the shoot apical meristem and leaf of *svp-41* and wild-type mutants.** Transcript levels were calculated by quantitative real-time PCR. Results were normalized against the expression of *TUB2*. Error bars indicate SD. Fold change represents the ratio of *SOC1* mRNA abundance in *svp-41* leaf/meristem against that in wild-type corresponding materials.

4.2.3 *SVP* directly controls *SOC1* expression

The significant effect of *SVP* on *SOC1* transcription at the vegetative phase implies a potential direct interaction between these two genes, which was further explored by our following experiments using a functional pER22-*SVP* transgenic line. In this transgenic line, the overexpression of *SVP* is controlled by an estradiol-induced XVE system (Zuo et al., 2000). In this system, an intermediate transcriptional activator consisting of a DNA-binding domain of the bacterial repressor LexA, a VP16 acidic transcription activation domain and the regulatory region of the human estrogen receptor is transported into the nucleus upon estradiol treatment and drives *SVP* expression by a specific promoter that contains eight copies of the LexA operator. Continuous β-estradiol treatment on pER22-*SVP* seedlings before floral transition caused a delay in flowering compared with those mock-treated with DMSO, demonstrating that this line was biologically functional (Figure 14A). We further applied this system to examine whether *SOC1* expression is regulated by *SVP in vivo*. pER22-*SVP* seedlings were subjected to β–estradiol treatment five days after germination, when the plants were still at the vegetative stage. Quantitative real-time PCR results showed that *SOC1* expression decreased in correspondence to elevated expression of *SVP* (Figure 14B and 14C).

Since *SVP* encodes another MADS domain protein, ChIP assay was performed to test whether SVP protein directly binds to the *SOC1* genomic region. We used a *35S:SVP-6HA* single-insertion transgenic line in ChIP assays. The SVP-6HA fusion
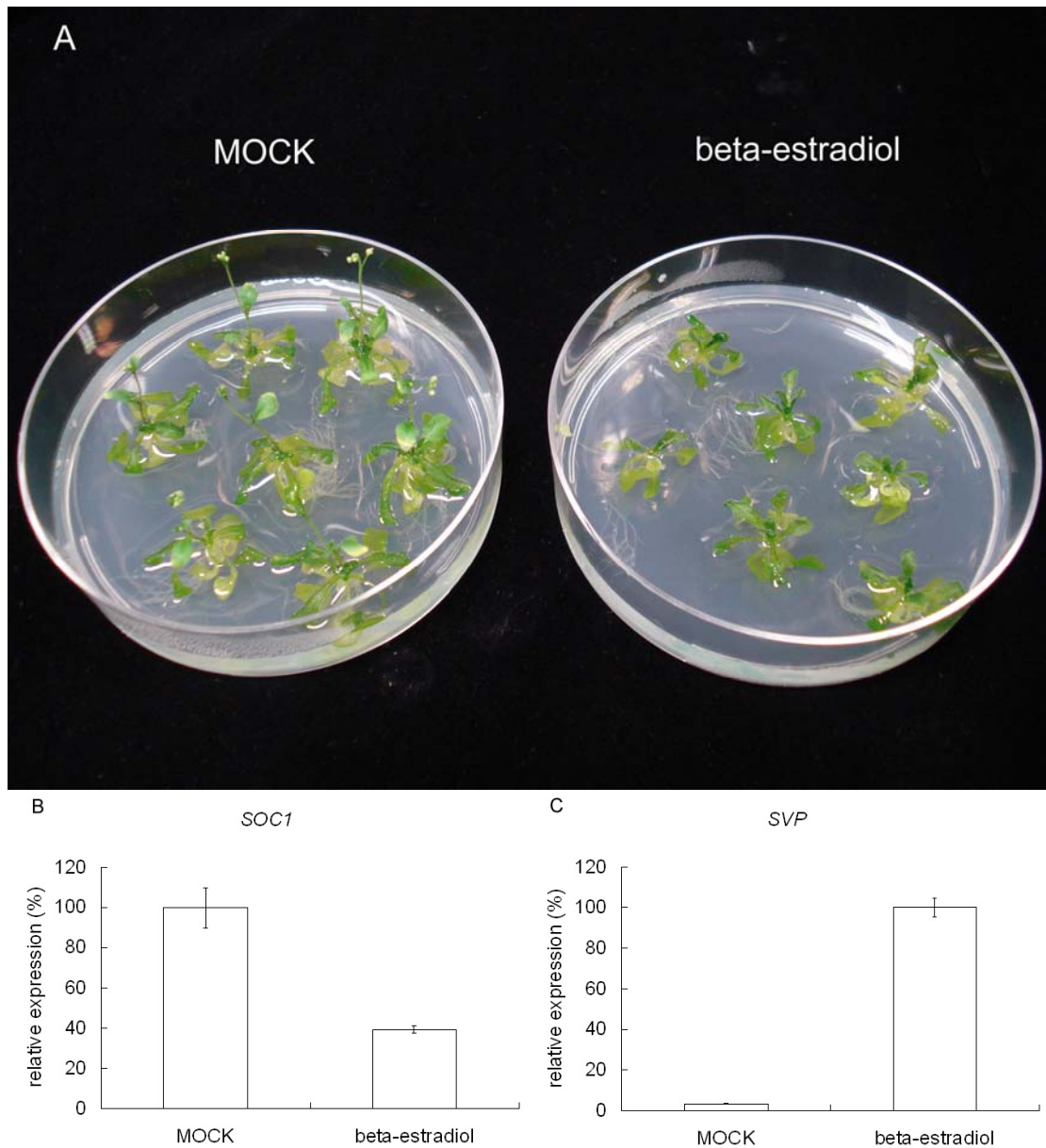
Figure 14. *SVP* directly represses *SOC1* expression. (A) pER22-*SVP* seedlings were treated with 10 μM β-estradiol or mock-treated every two days. Late flowering phenotype was observed in β-estradiol treated plants. (B) β-estradiol treatment induced repression of *SOC1* in 5-day-old pER22-*SVP* seedlings. *TUB2* expression was used for normalization. (C) *SVP* was upregulated in β-estradiol treated plants.

protein in this line is at least partially functional since this transgenic line exhibited delayed flowering compared with wild-type plants (Figure 15A). The genomic fragments bound to SVP-6HA was precipitated with an anti-HA antibody and further examined by designated PCR primers. As explained in previous experiments on the study of AGL24 binding site to *SOC1* genomic sequence, the same strategy was applied to locate the precise SVP-6HA binding site on the *SOC1* promoter. Eleven pairs of primers covering the entire *SOC1* promoter sequence were designed for real-time PCR and the number 5 fragment showed the largest enrichment, demonstrating that the two CArG motifs with one nucleotide mismatch located in this fragment may be the potential SVP-6HA binding site *in vivo* (Figure 15).

Subsequently, we conducted GUS histochemical assays to detect the regulatory role of these two CArG motifs in regulating *SOC1* expression. We mutated these CArG boxes (Figure 16A) individually in the native *SOC1* promoter construct, which was used in previous experiments testing the *AGL24* binding to the *SOC1* promoter. The intensity of GUS staining in M1 was significantly elevated than that in native transgenic plants (Figure 16B). Additionally, preliminary experiments show the GUS signal in M1 is also stronger than that in M2 plants. Taken together, these data suggest that the CArG motif 1 could be predominantly responsible for the repression of *SOC1* expression by SVP.
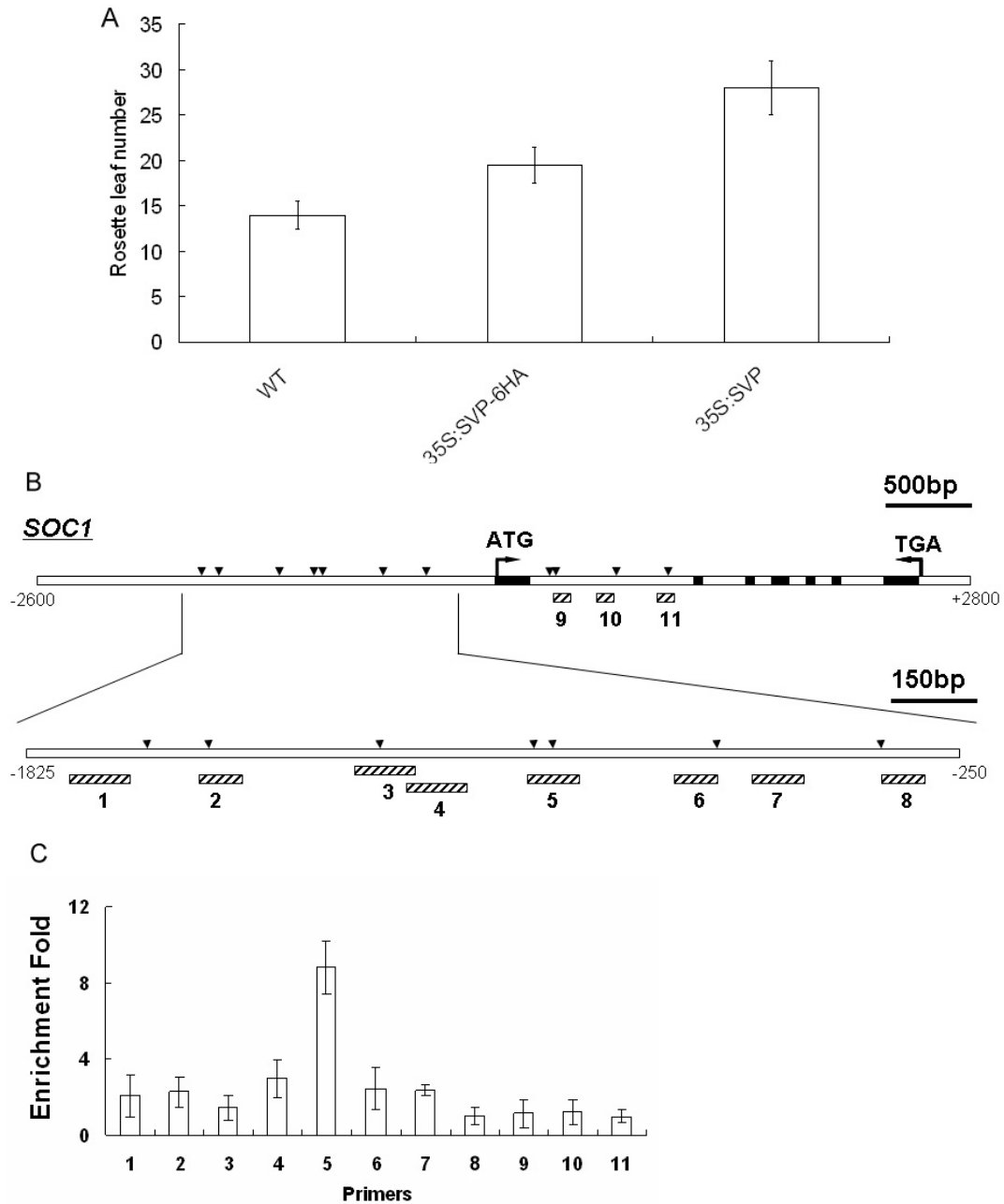
Figure 15. SVP-6HA protein directly binds to the *SOC1* genomic region. (A) Flowering time of different transgenic lines and wild-type (Col) under long days. Number of rosette leaves represents flowering time. Values representing the mean ±SD were scored from at least 15 plants of each genotype. (B) Schematic diagram of the *SOC1* genomic sequence. Exons and introns are represented by black boxes and white boxes, respectively. Arrows indicate the translation start site and stop codon. 11 arrowheads show putative MADS-domain protein binding sites with either perfect match or single mismatch of CArG box. Eleven pairs of primers spanning the whole genomic region were designed to locate the precise SVP binding site. (C) Quantitative real-time PCR result shows that the binding site of SVP-6HA fusion protein is located near the number 5 DNA fragment. Enrichment fold is calculated as described in Figure 6.
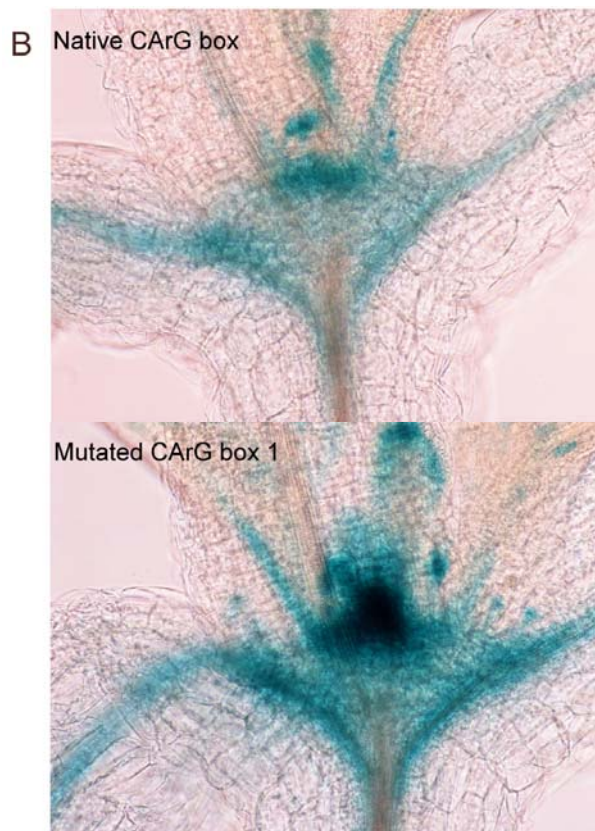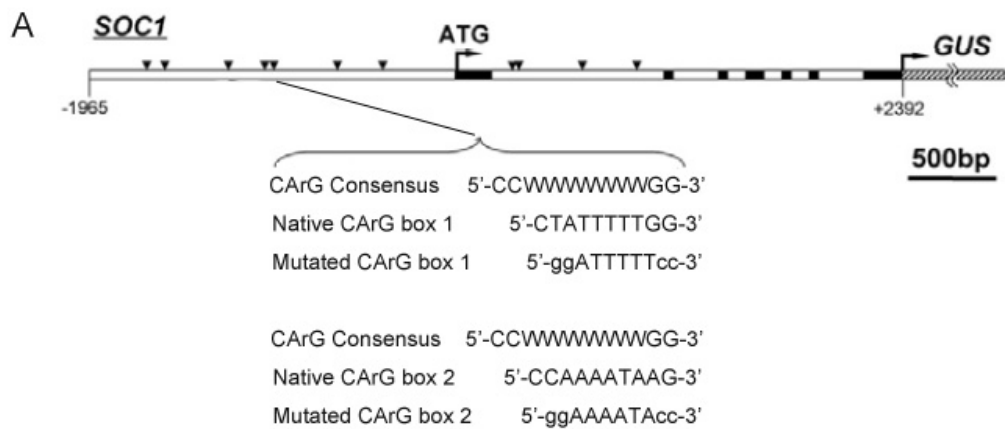
Figure 16. Validation of SVP-6HA binding site to *SOC1* with GUS reporter gene.
(A) Schematic diagram of the *Pro_SOC1:GUS* construct. The native one-mismatch
CArG boxes (5'-CTATTTTTGG-3'), (5'-CCAAAATAAG-3') were mutated into
(5'-ggATTTTTcc-3'), (5'-ggAAAATAcc-3'), respectively. (B) GUS staining pattern
comparison in 9-day-old transformants containing *Pro_SOC1:GUS* and its derived
construct with the mutated CArG box 1.

4.2.4 *SVP* dominantly represses *SOC1* expression

Since *SOC1* is an floral signal integrator in *Arabidopsis* (Lee et al., 2000) and several upstream regulators such as *FLC*, *AGL24* and *FT* have been identified (Lee et al., 2000; Samach et al., 2000; Michaels et al., 2005; Searle et al., 2006 ; Helliwell et al., 2006), we further investigated the role of *SVP* in the whole *SOC1* regulatory network through expression studies and genetic crossing experiments. Our results showed that *SVP* has a dominant effect on *SOC1* expression in developing seedlings.

4.2.4.1 The antagonistic effect of *SVP* and *AGL24* on *SOC1*

SVP and AGL24 proteins showed remarkably high similarity (Figure 17). The major difference of amino acid sequence lies in the C terminal region, which could account for their opposite effects on *SOC1* expression. 9-day old seedlings of wild-type and *agl24-1 svp-41* double mutant were collected for quantitative real-time PCR. Figure 18A shows that elevated expression of *SOC1* was maintained in *agl24-1 svp-41* mutants, resembling the *SOC1* expression pattern in *svp-41* mutants (Figure 11A), while *SOC1* expression was notably downregulated in *agl24-1* mutants at this stage (Figure 4A). Consistent with real-time PCR data, *agl24-1svp-41* plants exhibited early flowering phenotype under long days (Figure 18B), suggesting that *SVP* has a more dominant effect than *AGL24* on *SOC1* expression in the control of flowering time.

```
AGL24   MAREKIRIKKIDNITARQVTFSKRRRGIFKKADELSVLCDADVALIIFSATGKLFEFSSS 60
SVP     MAREKIQIRKIDNATARQVTFSKRRRGLFKKAEELSVLCDADVALIIFSSTGKLFEFCSS 60
        ******:*:**** *************:****:*****************:*******.**

AGL24   RMRDILGRYSLHASNINKLMDPPSTHLRLENCNLSRLSKEVEDKTKQLRKLRGEDLDGLN 120
SVP     SMKEVLERHNLQSKNLEKLDQPSLELQLVENSDHARMSKEIADKSHRLRQMRGEELQGLD 120
         *:::* *:.*::.*::** :*.       :**.: :*:***: **::**::***:*:**:

AGL24   LEELQRLEKLLESGLSRVSEKKGECVMSQIFSLEKRGSELVDENKRLR-------DKLET 173
SVP     IEELQQLEKALETGLTRVIETKSDKIMSEISELQKKGMQLMDENKRLRQQGTQLTEENER 180
        :****:*** **:**:** *.*.: :**:* .*:*:* :*:*******       :: *

AGL24   L--------------ERAKLTTLKEALETESVTTNVSSYDSGTPLEDDS-DTSLKLGLPS 218
SVP     LGMQICNNVHAHGGAESENAAVYEEGQSSESITN--AGNSTGAPVDSESSDTSLRLGLPY 238
        *             *   : :. :*. .:**:*.  :.  .:*:*::.:* ****:****

AGL24   WE 220
SVP     GG 240
```

Figure 17. Amino acid sequence comparison between SVP and AGL24. Identical residues are marked with asterisks. Conserved and semi-conserved substitutions are denoted by ":" and ".", respectively.
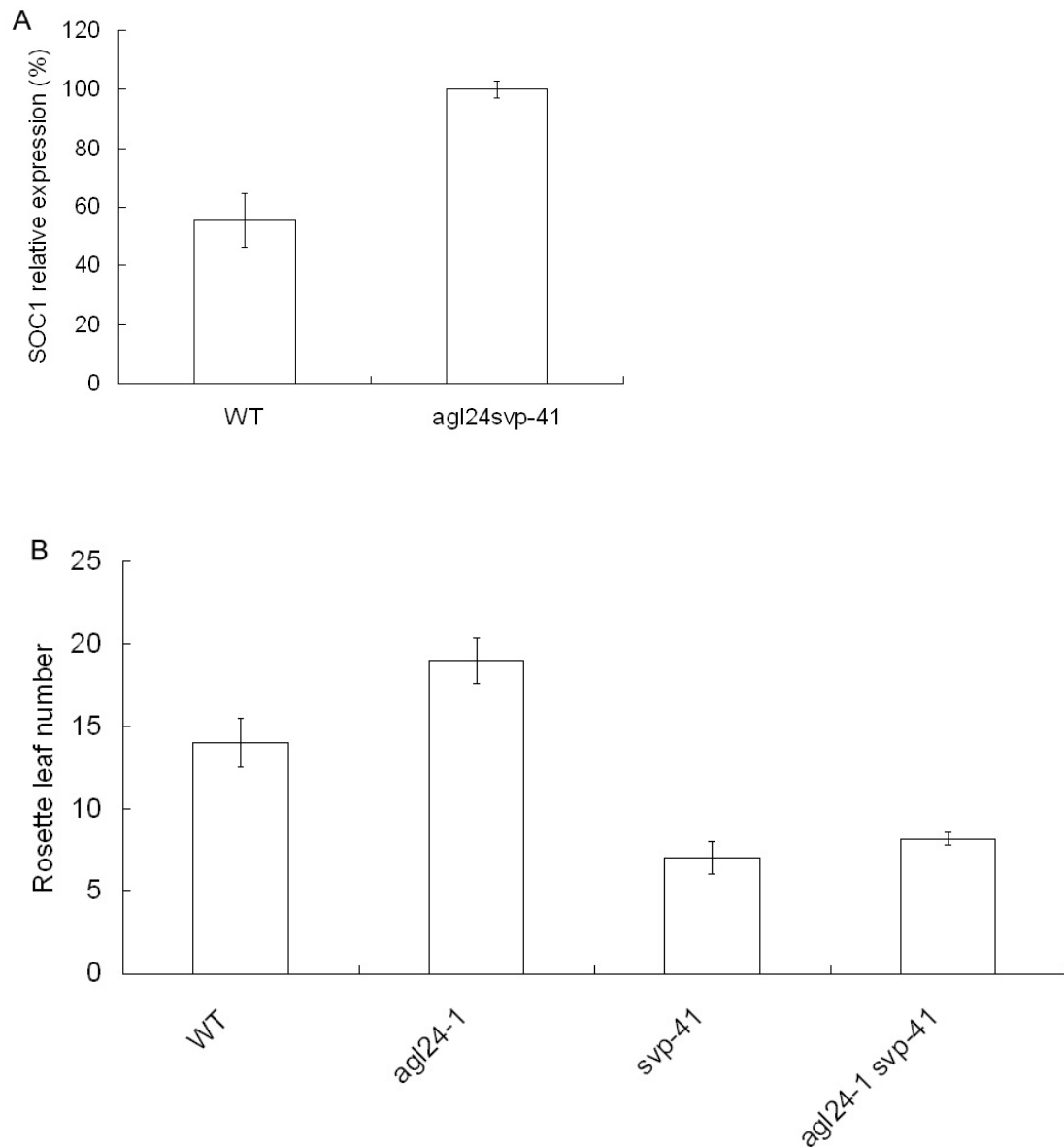
Figure18. *SVP* has a dominant effect on *SOC1* transcription compared with *AGL24*. (A) *SOC1* transcript levels in 9-day old seedlings were determined by quantitative real-time PCR. *TUB2* expression was used for normalization. Error bars stand for SD. (B) Flowering time of different mutants and wild-type plants under long days. Number of rosette leaves represents flowering time. Values representing the mean ± SD were scored from at least 15 plants of each genotype.

4.2.4.2 The antagonistic effect of *SVP* and *FT* on *SOC1*

It has been reported that FT recruits FD to activate *SOC1* in the shoot apical meristem (Searle et al., 2006), where *SVP* greatly suppresses *SOC1* expression (Figure 13). In addition, it has also been proposed that *FT* mediates the regulation of *SOC1* by *CO* (Yoo et al., 2005). To clarify the combined effect of *SVP* and *FT* on *SOC1*, a temporal expression study was conducted. As revealed in Figure 19A, *SOC1* expression still increased in *svp-41ft-1* background, although it was relatively lower than that in *svp-41* mutants. On the other hand, *svp-41 ft-1* mutants showed intermediate flowering time compared with single mutants (Figure 19B). These results suggest that *SVP* and *FT* act at least partially independently to regulate *SOC1* expression and that *SVP* appears to be a more dominant regulator of *SOC1* as compared with *FT*.

We further examined the possibility that *SVP* regulates *SOC1* through *FT* by measuring *FT* mRNA abundance in wild-type and *svp-41* mutants. Consistent with previous studies (Abe et al., 2005; Baurle and Dean, 2006; Takada and Goto, 2003), *FT* was primarily found in the leaf tissues of both wild-type and *svp-41* seedlings, while its expression level was low in the shoot apex before floral transition (Figure 20A). We did not observe significant difference in *FT* transcripts levels between wild-type and *svp-41*, implying that *FT* might not be the major target of *SVP* under our growth conditions (Figure 20B and 20C).
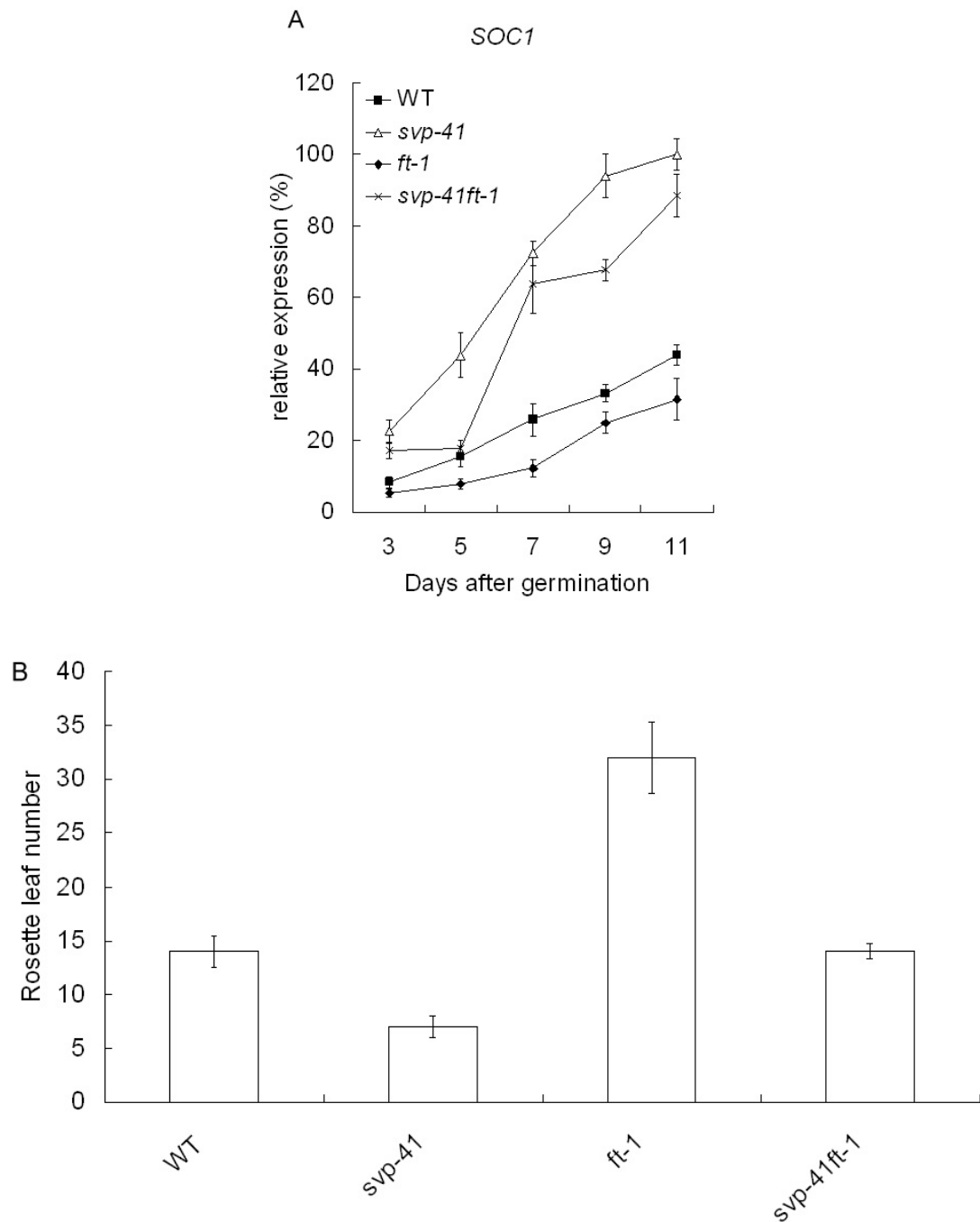
Figure 19. *SVP* has a dominant effect on *SOC1* transcription compared with *FT*.
(A) Temporal expression of *SOC1* in developing seedlings of different genetic backgrounds. Plants were grown under long days. Transcript levels were caculated by quantitative real-time PCR. *TUB2* was used for normalization. Error bars indicate SD. (B) Comparison of flowering time of wild-type, *svp-41*, *ft-1* and *svp-41ft-1* under long days. Flowering time was determined by the number of rosette leaves. Values representing the mean ±SD were scored from at least 15 plants of each genotype.

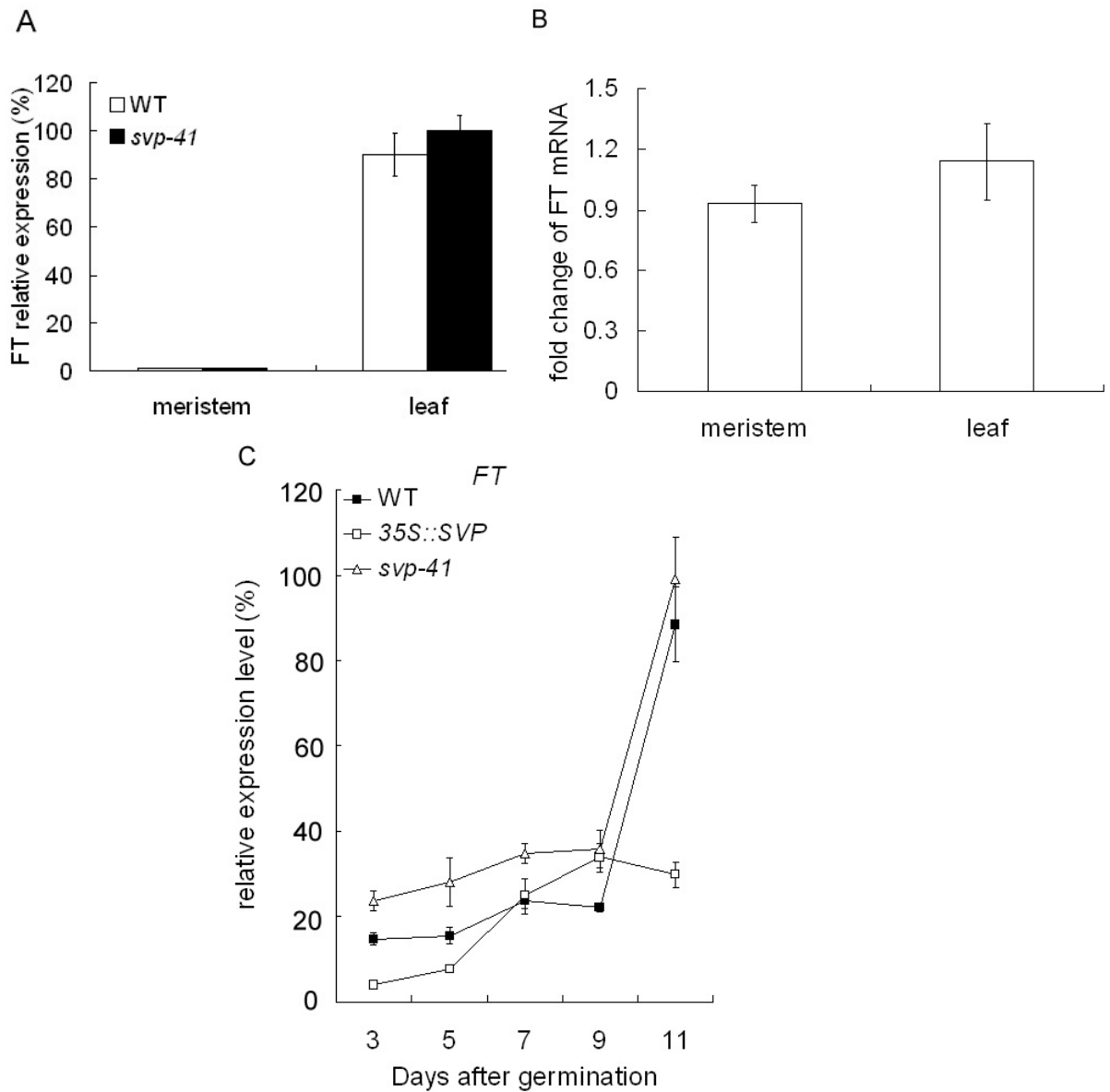**Figure 20. Comparison of *FT* expression levels in wild-type and *svp-41* plants.** Error bars mean SD. (A) Real-time PCR assay of *FT* expression in different tissues of 7-day old seedlings. The expression level of *FT* in leaf tissue of *svp-41* mutants was manually set to be 100%. (B) Fold change indicates the ratio of *FT* mRNA abundance in *svp-41* leaf/meristem against that in wild-type corresponding materials. (C) Temporal investigation of *FT* in wild-type, *35S:SVP* and *svp-41* transgenic plants. Only aerial part of seedlings was collected. *TUB2* expression was used for normalization.

4.2.4.3 The possible interaction between *SVP* and *FLC*

Previous research has suggested that *FLC* is a central flowering repressor in *Arabidopsis* (Michaels and Amasino, 1999). *FLC* in both shoot apex and vasculature has been shown to reduce *SOC1* expression (Helliwell et al., 2006; Lee et al., 2000; Searle et al., 2006; Sheldon et al., 2006). Since *SVP* has also been found to repress *SOC1* in our study, we proceeded to examine the interaction between *SVP* and *FLC*.

FLC transcript levels were measured in the shoot meristem and leaf of wild-type and *svp-41* plants. As shown in Figure 21A, *FLC* expression was significantly reduced in *svp-41* background, especially in the leaf tissue. This result implies that the early flowering phenotype of *svp-41* could be partially caused by a diminished effect of *FLC* on *SOC1* and *FT*. Nevertheless, overexpression of *SVP* could not promote *FLC* (Figure 21B), suggesting that *SVP* may be required for the maintenance of *FLC* expression and that the suppression of *SOC1* in *35S:SVP* plants is mediated directly by *SVP* rather than *FLC*. Furthermore, dramatic upregulation of *SOC1* in the shoot apex of *svp-41* mutants can not be explained solely by the relatively slight change of *FLC* expression in this region (Figure 13 & Figure 21A). Since ChIP assays have shown that *SVP* and *FLC* bind to different CArG motif of *SOC1* promoter (Figure 14; Searle et al., 2006), we conclude that *SVP* is able to repress *SOC1* in a *FLC*-independent manner.
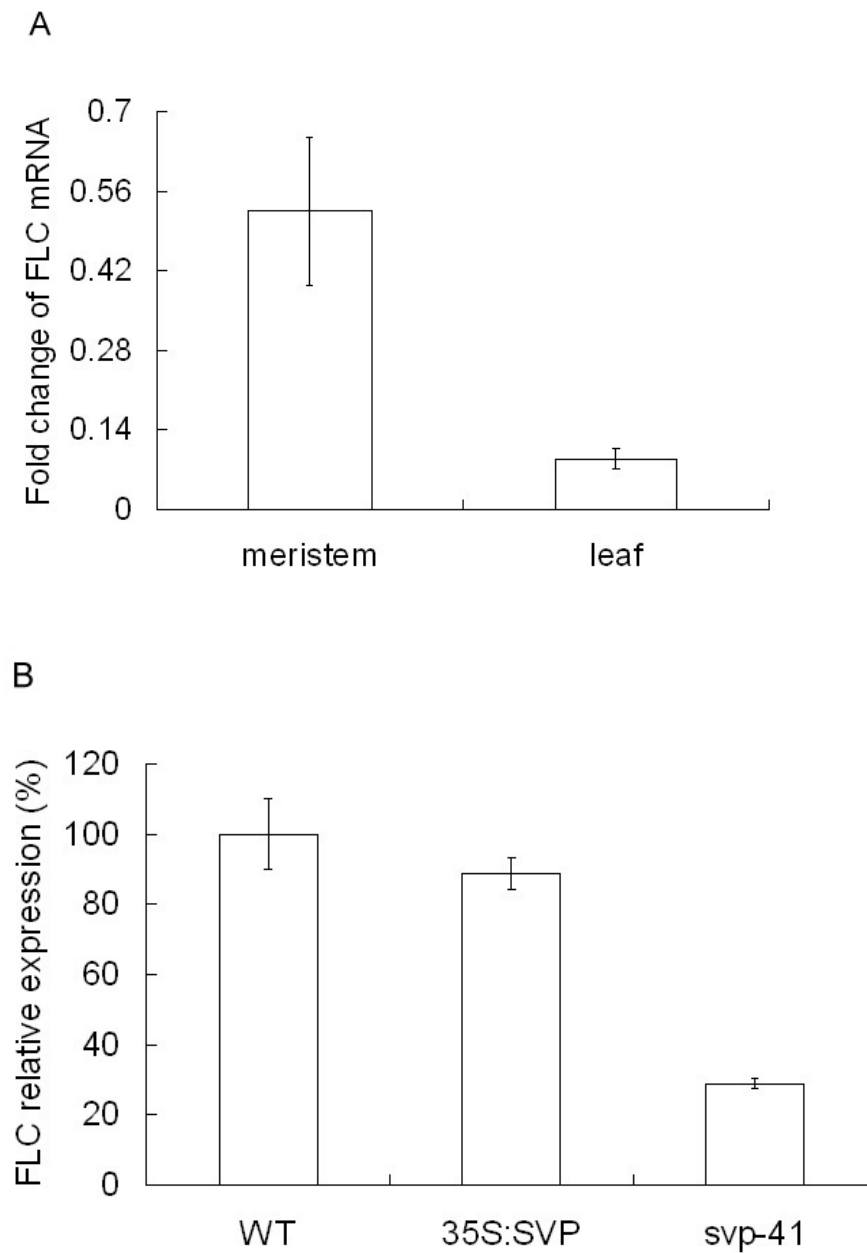
Figure 21. Expression study to investigate the interaction between *SVP* and *FLC.*
Transcript levels were determined by quantitative real-time PCR analyses of three independently collected samples. *TUB2* expression was used for normalization. Error bars indicate the standard deviation. (A) Fold change means the ratio of *FLC* transcription level in 7-day old *svp-41* leaf/meristem against that in wild-type corresponding materials. (B) Comparison of *FLC* mRNA abundance in 7-day old wild-type, *35S:SVP* and *svp-41* transgenic plants.

4.2.5 Feedback regulation of *SVP* by *SOC1*

Resembling the interaction pattern between *SOC1* and *AGL24*, *SOC1* and *SVP* may form a feedback regulation loop. Our further experiments clearly showed that *SVP* was also directly regulated by *SOC1*.

4.2.5.1 *SOC1* affects *SVP* expression

The *SVP* mRNA levels in wild-type, *35S:SOC1* and *soc1-2* mutant were quantitatively measured. As shown in Figure 22, *SVP* expression in wild-type plants remained at a relatively high level during the entire vegetative stage and the floral transition phase. It seemed that *SVP* expression could be changed due to different expression levels of *SOC1*. However, low expression of *SVP* in *35S:SOC1* may be caused by its early entry into the floral transitional stage. It is also worthy to mention that the relatively slight change of *SVP* expression level in *soc1-2* mutants can not explain the extremely late flowering phenotype of *soc1-2* under LD conditions.

4.2.5.2 SOC1 directly binds to the *SVP* promoter

To further investigate the possible direct interaction between *SOC1* and *SVP,* we applied the ChIP assays to detect the potential binding of SOC1-9myc fusion protein to the *SVP* genomic sequence using the functional *35S:SOC1-9myc* transgenic line
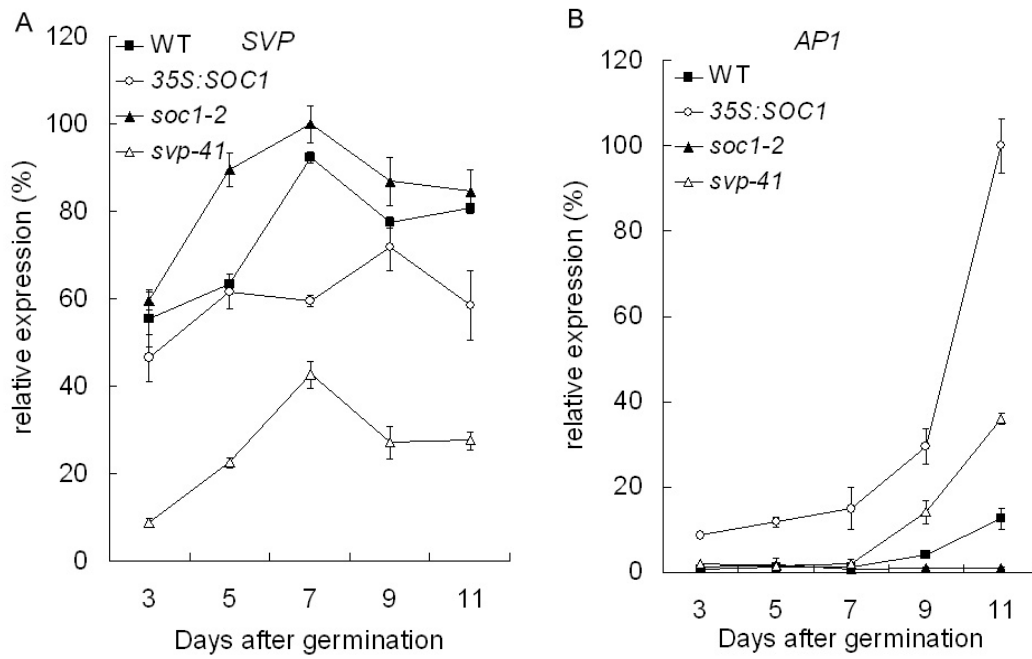
Figure 22. *SOC1* **affects** *SVP* **expression in developing seedlings under long days.**
The *TUB2* expression was used to normalize real-time PCR results of three independently collected samples. Error bars indicate SD. (A) Temporal expression of *SVP* under different genetic backgrounds. (B) Temporal expression of *AP1* under different genetic backgrounds.

(Figure 9). The *SVP* genomic sequence was examined based on putative CArG boxes with a maximum of one nucleotide mismatch (Figure 23A). Seven pairs of primers covering the *SVP* genomic sequence were designed for real-time PCR and DNA fragment 3 showed the most significant enrichment (Figure 23B). Sequence analysis of the genomic region near the fragment 3 found a two-nucleotide mismatch CArG motif, which could be the target of SOC1-9myc fusion protein *in vivo*. These results suggest that SOC1 may directly regulate *SVP* expression. Further promoter mutagenesis experiments could further verify this possibility.

4.2.6 *SVP* has other target genes in addition to *SOC1*

Since *svp-41* can also significantly rescue late flowering of *soc1-2*, it is possible that *SVP* could repress other target genes in addition to *SOC1*(Figure 24). A possible candidate is *AGAMOUS* (*AG*), whose overexpression results in early flowering. As revealed in Figure 25, the early onset of *AG* has been observed in *svp-41* mutants, and it was delayed in plants overexpressing *SVP*. Nevertheless, it has been also reported that the ectopic expression of *AG* would generate floral organ defects (Mizukami and Ma, 1992), which is absent in *svp-41* mutants. Early flowering of *svp-41* mutants may cause early formation of inflorescence and floral meristems and thus indirectly induce upregulation of *AG*. Thus, weather or not *SVP* regulates *AG* needs further investigation. Other possible candidates of *SVP* target genes include floral meristem identity genes *AP1* and *LFY*. However, our preliminary ChIP assays did not show the
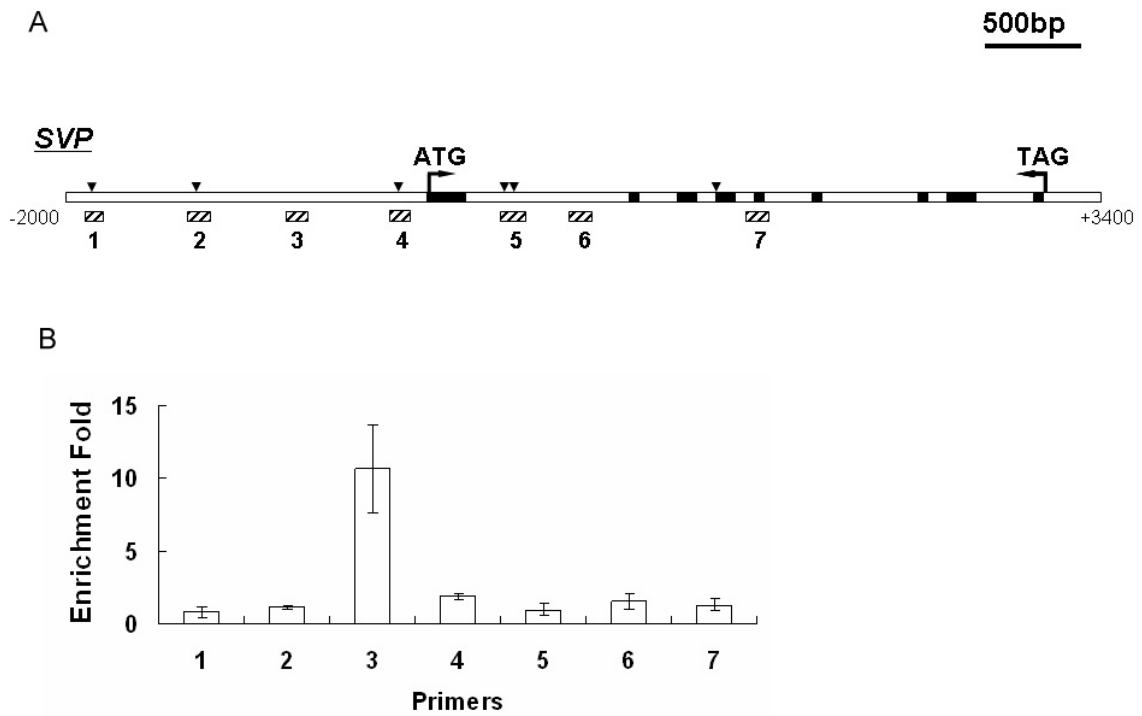
Figure 23. SOC1 directly binds to the *SVP* genomic sequence. (A) Schematic diagram of the *SVP* genomic sequence. Exons and introns are represented by black boxes and white boxes, respectively. Arrows indicate translation start site and stop condon. 6 arrowheads show putative MADS-domain protein binding sites with either perfect match or single mismatch of CArG box. Seven pairs of primers spanning the whole genomic region were designed to find the precise SOC1-9myc binding site. (B) Quantitative real-time PCR reveals that the potential binding site of SOC1-9myc fusion protein is located near the number 3 DNA fragment. Enrichment fold was calculated as described in Figure 6.
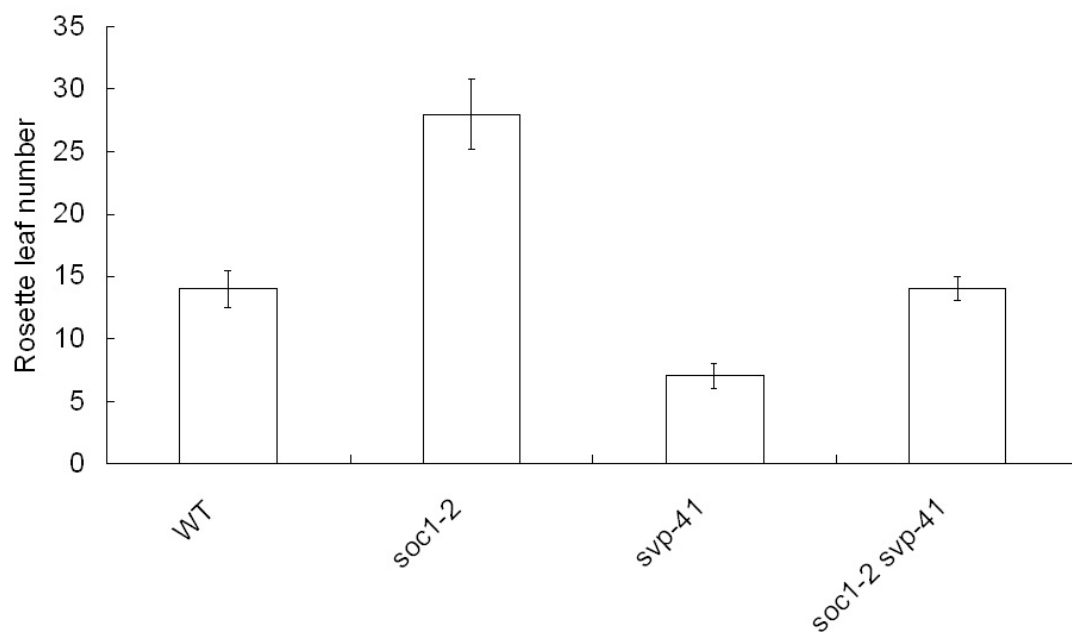
Figure24. Flowering time comparison among wild-type, *soc1-2*, *svp-41* and *soc1-2svp-41* plants under LDs. Number of rosette leaves represents flowering time. Values representing the mean ± SD were scored from at least 15 plants of each genotype.
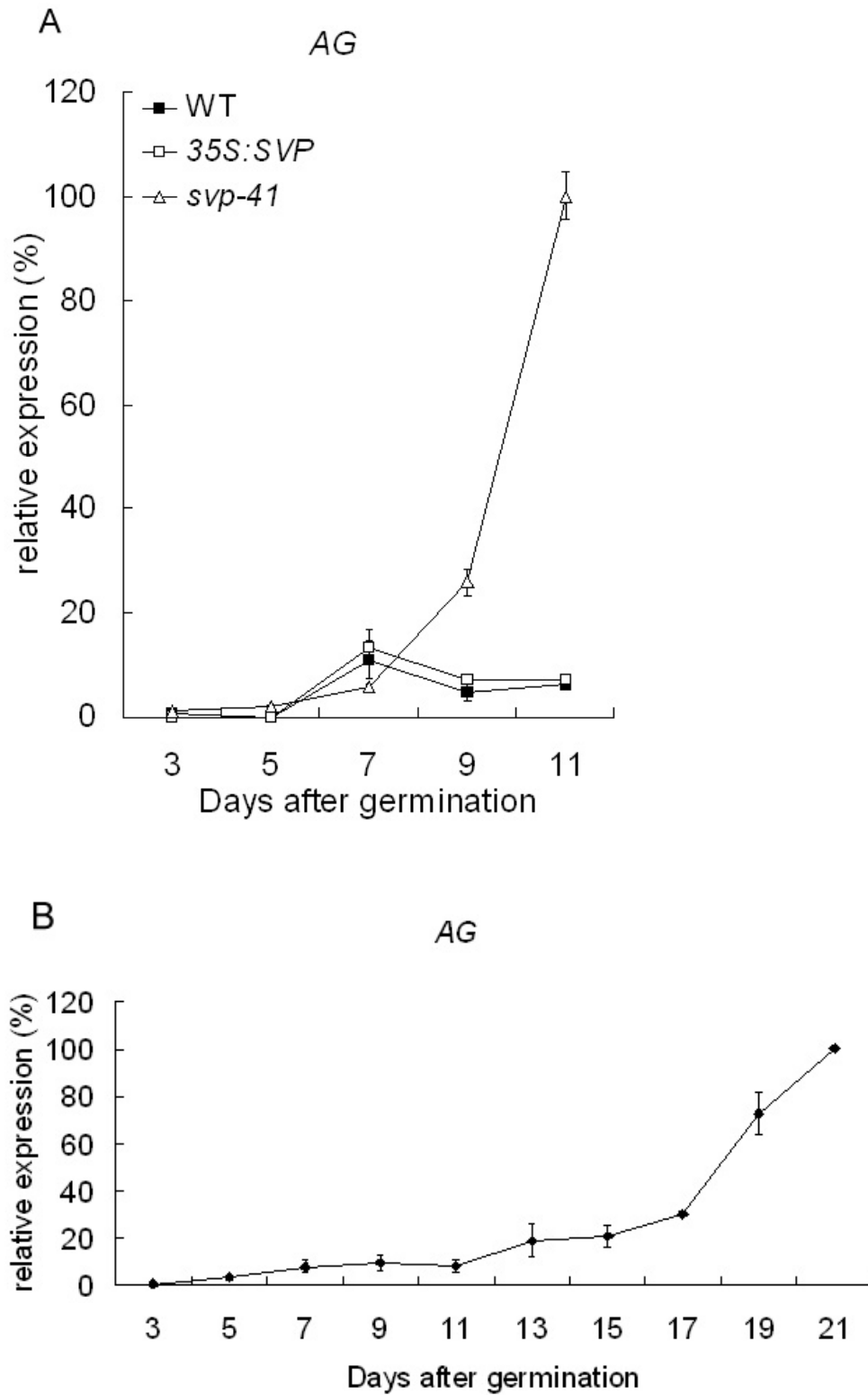
Figure 25. The potential effect of *SVP* on *AG* expression. (A) *AG* expression in wild-type, *35S:SVP* and *svp-41* plants. (B) Temporal expression pattern of *AG* in wild-type (Col) plants.

direct binding of SVP to their promoter regions, indicating that *AP1* and *LFY* may not

be the direct targests of SVP (data not shown).

4.3 Investigation of downstream targets of *SOC1*

Since *SOC1* has been identified as a key flowering time integrator in *Arabidopsis,* it is

proposed that *SOC1* should transmit flowering signals to downstream factors,

especially floral meristem identity genes (Lee et al., 2000; Parcy 2005). With our

established *35S:SOC1-9myc* system, we were able to examine this hypothesis using

ChIP assays. We focused on two well-studied floral meristem identity genes in

Arabidopsis, *AP1* and *LFY*. The promoter sequences of these two genes were first

analyzed based on the consensus binding motif (CArG box) of MADS domain

proteins with perfect match or single nucleotide mismatch. Five and two pairs of

primers were used to measure the enrichment fold of SOC1-9myc fusion protein to

AP1 and *LFY* genomic fragments, respectively. As revealed in Figure 25, no

significant enrichment was found along the *AP1* genomic region, while the *LFY-1*

fragment showed around 10-fold enrichment. These results strongly suggest that *LFY*

is a direct target of *SOC1*. However, *LFY* is not the only target gene of *SOC1* since

plants overexpressing both *SOC1* and *LFY* flowers earlier that either single

overexpression transgenic line (Lee et al., 2000). In order to thoroughly clarify the

role of *SOC1* in flowering time control, the global investigation of downstream target

genes using microarray analysis would be necessary.
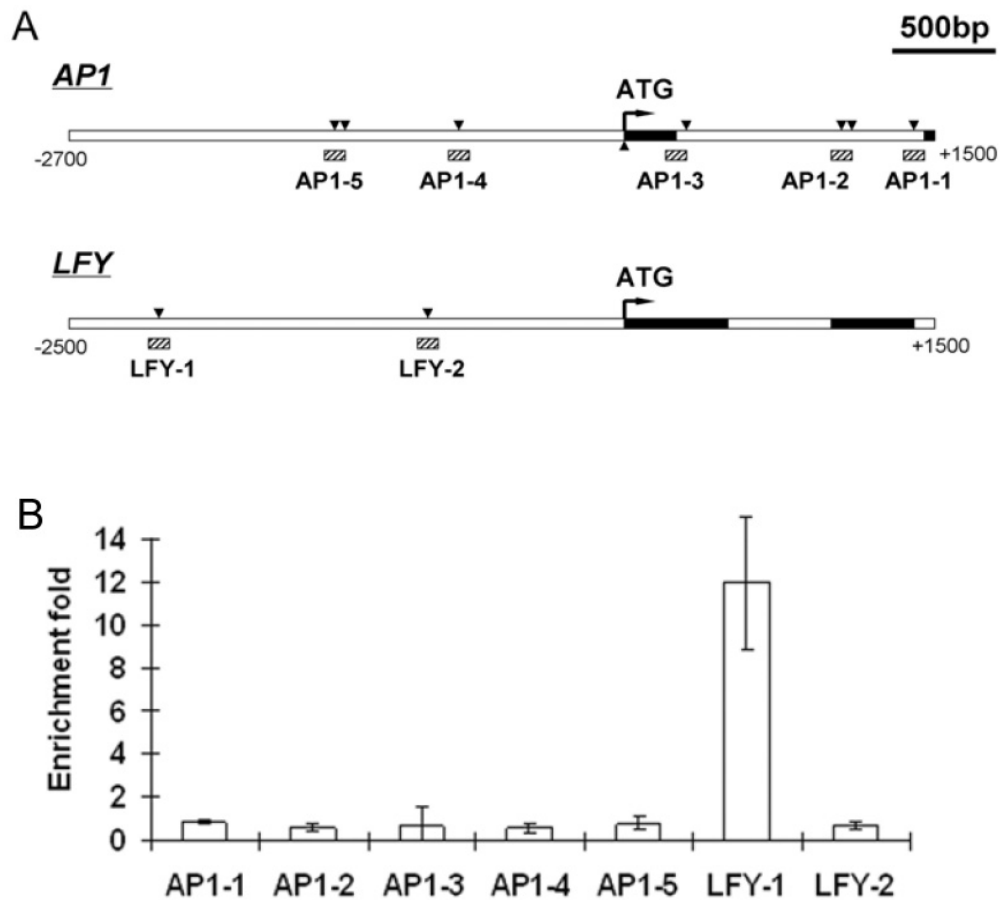
Figure 26. ChIP analysis to test the binding of SOC1-9myc to the *AP1* and *LFY* promoters. (A) Schematic diagram of the *AP1* and *LFY* genomic sequences. Exons and introns are represented by black and white boxes, respectively. Arrows indicate translation start sites and stop condons. The arrowheads denote putative MADS-domain protein binding sites with either perfect match or single mismatch of CArG box. The hatched boxes show DNA fragments designed for ChIP assays. (B) ChIP enrichment test by quantitative real-time PCR reveals the binding of SOC1-9myc to the *LFY* genomic region near the number 1 DNA fragment. Enrichment was calculated as described in Figure 6.

CHAPTER 5

Discussion and conclusion

5.1 *SOC*1 and *AGL24*

Expression analysis revealed that the upregulation of *SOC1* during floral transition is highly dependent on *AGL24* activity (Figure 4). ChIP assays further proved that the AGL24-6HA fusion protein is able to bind to the regulatory elements of *SOC1* in developing seedlings (Figure 6). Moreover, mutagenesis of the AGL24-6HA binding motif in the *SOC1* promoter can reduce *SOC1* expression (Figure 7). All these results indicate that direct regulation of *SOC1* by *AGL24* is an important step during floral induction in *Arabidopsis*. The observation that *AGL24* has little effect on *SOC1* at the early vegetative stage (Figure 4) could be due to the absence of co-factor(s) that are required for the upregulation of *SOC1* by *AGL24*, because overexpression of *AGL24* can only significantly upregulate *SOC1* during floral transition (Figure 4). Before floral transition occurs, the endogenous amount of AGL24 protein is low, which is not sufficient to promote *SOC1* transcription. After it is significantly induced during floral transition, AGL24 may interact with other cofactors to directly promote *SOC1* transcription, which in turn promotes flowering. This strict temporal regulation of *SOC1* needs sufficient amount of AGL24 protein and other cofactor(s) during floral induction.

On the other hand, our experiments also suggest that *AGL24* expression is

directly controlled by *SOC1* (Figure 8 and 9). Thus, the mutual interaction between *SOC1* and *AGL24* may provide a positive feedback regulation of their own expression, which is consistent with previous research on these two genes (Michaels et al., 2003; Yu et al., 2002).

The direct interaction between *SOC1* and *AGL24* could help us to futher understand how flowering signals are integrated together. For example, the vernalization pathway is generally divided into two parts: *FLC*-dependent pathway and *FLC*-independent pathway. In *FLC*-dependent pathway, vernalization treatment promotes flowering through the repression of *FLC*, which in turn controls several target genes including *SOC1*. It has been shown that FLC can bind to a CArG motif in *SOC1* promoter region (Hepworth et al., 2002; Helliwell et al., 2006; Searle et al., 2006) and directly control *SOC1* expression in the shoot apical meristem (Searle et al., 2006). However, *flc* mutants can not completely abolish the upregulation of *SOC1* after vernalization (Moon et al., 2003), suggesting that *SOC1* should receive signals from an *FLC*-independent pathway. *AGL24* (Yu et al., 2002; Michaels et al., 2003) and *AGL19* (Schonrock et al., 2006) are upregulated by vernalization independently of *FLC*. Therefore, direct regulation of *SOC1* by *AGL24* at least partially explains the regulation of *SOC1* in an *FLC*-independent pathway. In addition, we also found that *agl24 soc1* double mutants are still responsive to vernalization under SDs (Figure 10), demonstrating that *AGL19* or other *FLC*-related factors, such as *FT*, can be independently activated by the vernalization pathway.

The effects of *AGL24* and *FLC* on *SOC1* also propose the possibility that *FLC* is

not the only factor regulating *SOC1* expression in the autonomous pathway. It has been suggested that the autonomous pathway represses *FLC* (Michaels and Amasino, 2001) and thus activates *SOC1* expression. Meanwhile, *AGL24* was found to be affected by key factors located in the autonomous pathway (Yu et al., 2002), which is not affected by *FLC* (Michaels et al., 2003). Thus, *SOC1* could receive *AGL24*-mediated autonomous signals in a *FLC*-independent manner.

Similarly, *AGL24* may act as an additional upstream regulator of *SOC1* in the photoperiod pathway, where *CO* is a major regulator (Lee et al., 2000; Samach et al., 2000; Helliwell et al., 2006; Hepworth et al., 2002; Searle et al., 2006; Yoo et al., 2005). *FT* has been identified as a major target gene of *CO* (Samach et al., 2000; Wigge et al., 2005; Yoo et al., 2005) and a mediator integrating floral signals to *SOC1* (Helliwell et al., 2006; Searle et al., 2006; Yoo et al., 2005). On the other hand, *AGL24* can only be affected by *CO*, but not *FT*, implying that *AGL24* and *FT* could be two independent outputs of *CO*, both of which can promote the *SOC1* expression via the photoperiod pathway. Overall, direct regulation from *AGL24* to *SOC1* provides plants a mechanism to precisely control the whole regulatory network of floral transition.

Besides direct regulation at the transcriptional level, the common spatial and temporal expression patterns of *SOC1* and *AGL24* (Figure 3; Lee et al., 2000; Samach et al., 2000; Yu et al., 2002; Michaels et al., 2003) raise another interesting question about a possible direct protein interaction between these two regulators. It has been found that SOC1 and AGL24 can form a MADS protein complex in yeast two-hybrid

assay (de Folter et al., 2005). Like another MADS protein complex APETALA3/PISTILLATA (AP3/PI) (Riechmann et al., 1996b), the generation of a possible heterodimer SOC1/AGL24 *in vivo* may produce unique DNA binding capacity and recognize several distinct targets during floral transition.

Finally, it is worthy to point out that alteration of *AGL24* and *SOC1* expression in the background of *soc1* and *agl24* mutants, respectively, could substantially change the flowering time (Yu et al., 2002; Michaels et al., 2003). This observation, together with ChIP assay showing that SOC1-9myc and AGL24-6HA function proteins have different binding capacity to the *LFY* genomic DNA (Figure 26 & unshown data revealing AGL24-6HA does not bind to the *LFY* promoter), suggests that *SOC1* and *AGL24* control different sets of target genes despite their direct interaction.

5.2 *SOC1* and *SVP*

Our finding that *SVP* represses *SOC1* expression is able to explain the early flowering phenotype of *svp* mutants (Figure 11, Hartman et al., 2000), since upregulation of *SOC1* promotes flowering under both LDs and SDs conditions. ChIP assay and *SVP* inducible system have demonstrated that *SVP* directly regulates *SOC1*. Consistent with these findings, mutation of the SVP-6HA binding site in the *SOC1* promoter increases *SOC1* expression (Figure 16). As shown in the expression study (Figure 11 and 12), *SVP* constitutively represses *SOC1* during the whole vegetative stage and floral transitional phase and its expression in the shoot apical meristem is gradually reduced during floral transition, suggesting that this repression is a crucial step in flowering time control. While *SVP* expression is maintained at a significantly high level during the vegetative growth (Figure 12) to prevent early upregulation of *SOC1*, other flowering pathway factors (*AGL24*, *FT*, etc.) gradually strengthen promotive signals to overcome this negative effect. When the overall input signals drive the *SOC1* expression to a threshold level, *SVP* is repressed in the meristem (Figure 12) as a result of feedback-regulation from *SOC1* (Figure 22), which may further in turn cause the derepression of *SOC1* expression and ultimately lead to the activation of floral meristem development.

Like *FLC* (Helliwell et al., 2006; Hepworth et al., 2002; Searle et al., 2006), *SVP* can regulate *SOC1* expression in both the shoot apical meristem and leaf (Figure 13). In the shoot apical meristem, *SOC1* transcription level is greatly elevated with

reduced *SVP* expression, which is consistent with the observation that *SVP* expression decreases in the apical meristem of wild-type plants (Figure 12B; Hartmann et al., 2000). On the other hand, mRNA levels of both *SOC1* and *SVP* steadily increase in the leaf tissue of wild-type plants,even during the floral transition (Figure 12A and 12B), suggesting that *SVP* expression in the leaf may not be as important as that in the apical meristem in terms of flowering time control. Although *SOC1* is also upregulated in the leaf tissues of *svp* mutants (Figure 13), it is likely to be an indirect effect through *FLC*, which is notably downregulated in leaves of *svp-41* mutants (Figure 21). In conclusion, *SVP* primarily suppresses *SOC1* in the shoot apex to regulate flowering.

Expression analysis and genetic data have revealed that *SVP* represses *SOC1* not only constantly but also dominantly. We examined the combined effect of *SVP* and *AGL24*, as well as *SVP* and *FT*, on *SOC1* expression. Mutation in *AGL24* or *FT* can not abolish the upregulation of *SOC1* caused by *svp* mutant, which is consistent with the genetic crossing results (Figure 18&19). Furthermore, our study supports that *SVP* regulates *SOC1* in neither a *FLC*-dependent nor a *FT*-dependent manner (Figure 20 and 21). Taken together, *SOC1* expression is largely dependent on *SVP*, which acts as an internal flowering repressor.

SVP protein shows high sequence homology to AGL24, but exerts an opposite effect on *SOC1* expression according to our study. The alignment of amino acid sequence reveals a major difference in the C-terminal regions of SVP and AGL24, which may determine the capacity of protein-protein interaction of MADS domain

transcription factors (Figure 17). AGL24 and SVP could recruit distinct cofactor(s) to perform their opposite functions. It will also be interesting to clarify the whole transcription protein complex to examine potential competitive binding of AGL24 and SVP to *SOC1* since the distance between the SVP and AGL24 bind sites of *SOC1* promoter sequence is only around 200bp (Figure 6 and 15).

A recent publication claimed that *SVP* responds to ambient temperature changes by negatively regulating *FT* (Lee et al., 2007), which mediates thermal induction by elevated growth temperature (Balasubramanian et al., 2006). Although we have not found this regulatory relationship between *SVP* and *FT* in our expression study (Figure 20), this discrepancy might be the result of using different *svp* mutant lines. Furthermore, the reported effect of *SVP* on *FT* partly explains the early flowering phenotype of *soc1-2 svp-41* double mutants as loss of *SVP* could derepress *FT* expression, which in turn directly regulates *AP1* expression to promote flowering independent of *SOC1* (Abe et al., 2005; Takada and Goto, 2003). Another target candidate of *SVP* is *AG* since the early onset of *AG* has been observed in *svp-41* mutants (Figure 24) and overexpression of *AG* results in early flowering. Nevertheless, this idea needs further investigation because ectopic expression of *AG* also generates floral organ defects, which is, however, absent in *svp-41* mutants (Mizukami and Ma, 1992). In addition, early flowering of *svp-41* mutants may cause early formation of floral meristems and thus indirectly induce upregulation of *AG*.

5.3 Identified novel floral pathways

As discussed above, *SOC1*-mediated flowering regulatory network is partially clarified through our studies. Importantly, we identified *SVP* as a novel repressor of *SOC1* and proved that *LFY* is one of the direct targets of *SOC1*. Meanwhile, the mutual interaction between *AGL24* and *SOC1* provides a new aspect of floral signals integration. However, further investigation is necessary for this research work in order to validate other possible regulatory pathways (Figure 27).
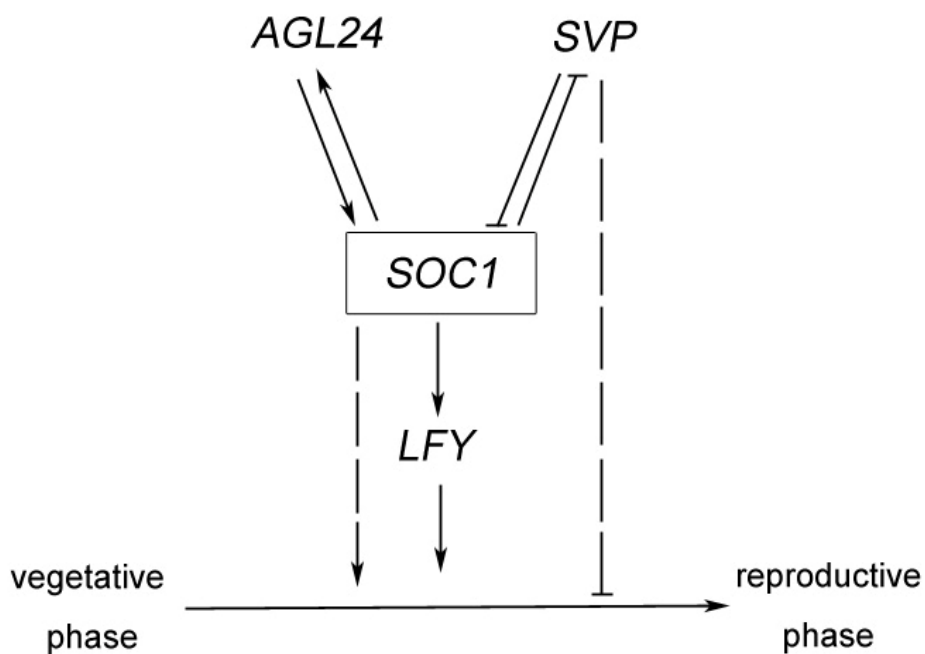
Figure 27. The schematic flowering pathways identified from our studies. Arrows and T-lines indicate positive and negative regulations, respectively. Dotted lines mean that target genes need to be validated.

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