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The *Fragaria vesca* Homolog of SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1 Represses Flowering and Promotes Vegetative Growth[W]

Katriina Mouhu,[a] Takeshi Kurokura,[a,1] Elli A. Koskela,[a] Victor A. Albert,[b] Paula Elomaa,[a] and Timo Hytönen[a,2]

[a] Department of Agricultural Sciences, University of Helsinki, Helsinki FIN-00014 Finland
[b] Department of Biological Sciences, University at Buffalo, Buffalo, New York 14260

In the annual long-day plant *Arabidopsis thaliana*, *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*) integrates endogenous and environmental signals to promote flowering. We analyzed the function and regulation of the *SOC1* homolog (*Fragaria vesca* [Fv] *SOC1*) in the perennial short-day plant woodland strawberry (*Fragaria vesca*). We found that Fv *SOC1* overexpression represses flower initiation under inductive short days, whereas its silencing causes continuous flowering in both short days and noninductive long days, similar to mutants in the floral repressor Fv *TERMINAL FLOWER1* (Fv *TFL1*). Molecular analysis of these transgenic lines revealed that Fv *SOC1* activates Fv *TFL1* in the shoot apex, leading to the repression of flowering in strawberry. In parallel, Fv *SOC1* regulates the differentiation of axillary buds to runners or axillary leaf rosettes, probably through the activation of gibberellin biosynthetic genes. We also demonstrated that Fv *SOC1* is regulated by photoperiod and Fv *FLOWERING LOCUS T1*, suggesting that it plays a central role in the photoperiodic control of both generative and vegetative growth in strawberry. In conclusion, we propose that Fv SOC1 is a signaling hub that regulates yearly cycles of vegetative and generative development through separate genetic pathways.

INTRODUCTION

The molecular control of flowering has been studied in detail in the annual model plants *Arabidopsis thaliana* and rice (*Oryza sativa*; Kim et al., 2009; Tsuji et al., 2011; Turnbull, 2011), whereas less is known about perennial species. In *Arabidopsis*, four major genetic pathways (i.e., the photoperiodic, vernalization, autonomous, and gibberellin [GA] pathways) regulate flowering time (Simpson, 2004; Turck et al., 2008; Kim et al., 2009; Mutasa-Göttgens and Hedden, 2009). Many genes with sequence similarity to known flowering genes are found in different plant families, but their functions may differ between species (Suárez-López et al., 2001; Hayama et al., 2003; Hecht et al., 2005, 2011; Mouhu et al., 2009). In perennials, which undergo repeated cycles of vegetative and reproductive phases, flowering time is controlled by seasonal regulation of flowering genes (Böhlenius et al., 2006; Wang et al., 2009; Koskela et al., 2012). Moreover, homologous genes, or genes classified in the same gene family, may have roles during the seasonal cycle beyond those that are known in annual species (Böhlenius et al., 2006; Hsu et al., 2011). Therefore, careful analysis of gene functions in the different phases of seasonal cycles is required to better understand perennial growth.

We use diploid woodland strawberry (*Fragaria vesca*) as a model to study the environmental control of perennial growth. Strawberry belongs to the Rosaceae family, which includes many economically important fruit crops, such as apple (*Malus domestica*), pear (*Pyrus communis*), peach (*Prunus persica*), plum (*Prunus domestica*), and cherry (*Prunus avium*), and ornamental genera, such as *Rosa*, *Potentilla*, and *Spiraea* (Potter et al., 2007). Woodland strawberry and other strawberry species are perennial rosette herbs. Most accessions of strawberry and cultivars of the garden strawberry (*Fragaria* × *ananassa*) are seasonal flowering short-day (SD) plants (Heide, 1977; Heide and Sønsteby, 2007). During the vegetative phase under long days (LDs), strawberries spread clonally through aboveground stolons called runners, which are formed from the axillary buds of the rosette stem, called the crown (Figure 1; Konsin et al., 2001; Hytönen et al., 2004; Heide and Sønsteby, 2007), and consist of two long internodes followed by a daughter plant. Under SDs in the autumn, runner formation ceases and the uppermost axillary buds differentiate to axillary leaf rosettes called branch crowns (Figure 1; Konsin et al., 2001; Hytönen et al., 2004), and vegetative growth is reduced as characterized by decreased petiole elongation (Guttridge and Thompson, 1964; Wiseman and Turnbull, 1999; Konsin et al., 2001). At the same time, SDs activate flower initiation in the shoot apex of the main crown as well as in branch crowns that have become competent for floral development before or during the inductive SDs (Figure 1; Hytönen et al., 2004). However, the youngest axillary shoots remain vegetative, enabling the next seasonal growth cycle.

Early genetic studies have shown that different single genes, *SEASONAL FLOWERING LOCUS* (*SFL*) and *RUNNERING LOCUS* (*RL*), regulate seasonal flowering and runner formation

[1] Current address: Faculty of Agriculture, Utsunomiya University, Tochigi 321-8505, Japan.
[2] Address correspondence to timo.hytonen@helsinki.fi.
The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: Timo Hytönen (timo.hytonen@helsinki.fi).
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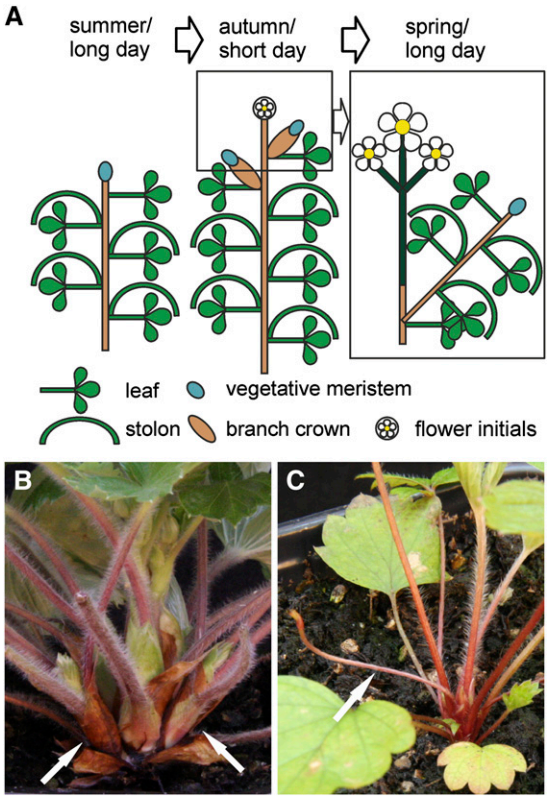

Figure 1. Development of Strawberry Shoot.

(A) Schematic representation of the shoot structure and development in seasonal flowering strawberry (SD *F. vesca*). Under LDs in summer, the plant grows vegetatively and axillary buds typically differentiate into runners (stolons). Autumn SDs cause flower initiation in the apical meristem and the development of axillary branch crowns. The terminal inflorescence emerges in the next season, and newly formed axillary branch crowns continue vegetative development. Note that branch crowns formed in the autumn often produce terminal inflorescences in the next spring.

(B) Close-up of a strawberry crown with the main crown in the middle and axillary branch crowns (arrows) in both sides. An Fv *SOC1*-RNAi line, which produces only a few runners, was photographed.

(C) Close-up of a young strawberry seedling with a newly emerged runner (arrow). Simple leaves in the figure are juvenile leaves.

in strawberry, and recessive alleles of these loci cause perpetual (continuous) flowering and runner-less phenotypes, respectively (Brown and Wareing, 1965; Albani et al., 2004). *RL* has not been identified. However, it is known that the photoperiodic control of GA biosynthesis is involved in the axillary bud differentiation to runners and branch crowns (Hytönen et al., 2009). Iwata et al. (2012) showed that *SFL* and the *RECURRENT BLOOMING* locus in roses encode homologs of TERMINAL FLOWER1 (TFL1), a member of the phosphatidyl ethanolamide binding protein family. We confirmed the function of Fv TFL1 as a major floral repressor and showed that its photoperiodic control in the shoot apex explains seasonal flowering in strawberry (Koskela et al., 2012). LDs in summer activate Fv *TFL1* mRNA expression, and flower initiation only occurs in autumn, when Fv *TFL1* is downregulated by SDs. In spring, high Fv *TFL1* expression is restored in the apices of new branch crowns to allow the production of new vegetative shoots (Koskela et al., 2012). Perpetual-flowering strawberry accessions, by contrast, do not require SDs for flower induction because of a frame shift mutation, which prevents the production of the functional Fv TFL1 (Iwata et al., 2012; Koskela et al., 2012). In these accessions, LD strongly advances flowering by activating another phosphatidyl ethanolamide binding protein homolog, Fv *FLOWERING LOCUS T1* (Fv *FT1*) (Koskela et al., 2012). Also in seasonal flowering strawberry, Fv *FT1* is expressed specifically under LDs, correlating negatively with the photoperiodic flower induction.

FT is a major activator of photoperiodic flowering in many LD and SD plants, including perennials (Turck et al., 2008; Pin and Nilsson, 2012). It is activated by CONSTANS (CO) in the leaf vascular tissues under flower-inductive conditions (Suárez-López et al., 2001; An et al., 2004). Consequently, FT protein relocates through phloem to the shoot apex, where it forms a complex with a bZIP protein FD and 14-3-3 proteins to induce *APETALA1* (*AP1*) and flowering (Abe et al., 2005; Wigge et al., 2005; Corbesier et al., 2007; Tamaki et al., 2007; Taoka et al., 2011). In *Arabidopsis*, TFL1 has not been shown to control photoperiodic flowering, although it can also bind FD (Hanano and Goto, 2011). TFL1 functions to maintain an indeterminate inflorescence meristem by repressing floral meristem identity genes *AP1* and *LEAFY* (*LFY*), whereas AP1 and LFY downregulate *TFL1* in the flanks of the inflorescence meristem to specify floral meristems (Liljegren et al., 1999; Ratcliffe et al., 1999).

CO and FT have been shown to promote flowering also through the MADS box transcription factor *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*; Samach et al., 2000; Moon et al., 2005; Yoo et al., 2005). *SOC1* may be the primary target of FT, since it is the first gene to be activated after a single inductive LD in the shoot apex (Torti et al., 2012). In addition to the photoperiodic pathway, *SOC1*, as well as *FT*, integrates signals from the vernalization, autonomous, and GA flowering pathways (Lee et al., 2000; Moon et al., 2003, 2005; Li et al., 2008; Jung et al., 2012). SOC1 interacts with multiple MADS box proteins, including AGAMOUS LIKE24 (AGL24), FRUITFUL (FUL), and AP1, and regulates the expression of several flowering genes, such as *SHORT VEGETATIVE PHASE*, *AGL15*, and *AGL18*, by directly binding to their regulatory sequences (de Folter et al., 2005; Lee et al., 2008; Seo et al., 2009; Immink et al., 2012; Tao et al., 2012).

Here, we report the functional analysis of the *SOC1* homolog in the perennial SD plant strawberry. We show evidence that Fv FT1 may mediate the photoperiodic regulation of Fv *SOC1*. This regulation of Fv *SOC1* plays an important role in the photoperiodic development of strawberry, since both overexpression and silencing of Fv *SOC1* strongly compromise the regulation of both vegetative and generative development by daylength. We also demonstrate that Fv SOC1 activates the expression of the floral repressor Fv *TFL1*, which prevents flower induction under LD conditions. In addition, Fv SOC1 promotes vegetative development by activating the expression of several GA biosynthetic genes. Our results suggest that Fv SOC1 is a general regulator of photoperiodic development that mediates photoperiodic signaling

to regulate flowering and vegetative growth through separate genetic pathways.

RESULTS

Fv *SOC1* Is an Ortholog of *SOC1*

We previously cloned Fv *SOC1*, which is the closest strawberry homolog of *Arabidopsis SOC1*, sharing 66% identity at the amino acid sequence level (Mouhu et al., 2009; Shulaev et al., 2011). We performed a maximum likelihood phylogenetic analysis using randomized axelerated maximum likelihood (Stamatakis et al., 2008), which showed that Fv SOC1 groups with other known SOC1 homologs from Rosaceae (e.g., Trainin et al., 2013), within the same clade as SOC1 homologs from other rosids (e.g., Hecht et al., 2005; see Supplemental Figure 1 and Supplemental Data Set 1 online). Moreover, a syntenic view generated by CoGe (Lyons and Freeling, 2008) showed that the microsynteny around *SOC1* and Fv *SOC1* is conserved (see Supplemental Figure 2 online). The protein sequence alignment of Fv SOC1 and several SOC1-like proteins showed that Fv SOC1 contains the highly conserved MADS box domain, the K domain, and the SOC1 domain characteristic of these proteins (see Supplemental Figure 3 online; Riechmann and Meyerowitz, 1997; Vandenbussche et al., 2003; Nakamura et al., 2005). We also overexpressed Fv *SOC1* in the *Arabidopsis* Columbia ecotype under the cauliflower mosaic virus *35S* promoter and analyzed flowering time. Similarly to the constitutive expression of *Arabidopsis SOC1* (Lee et al., 2000), heterologous overexpression of Fv *SOC1* advanced flowering in both LD and SD conditions in Columbia-0 (see Supplemental Figure 4 online). Therefore, we conclude that Fv SOC1 is the ortholog of SOC1, acting as a floral activator in *Arabidopsis*.

Fv *SOC1* Is under Photoperiodic Regulation in SD Strawberry

We first analyzed the daily rhythm of Fv *SOC1* in seasonal flowering SD strawberry plants (PI551792; National Clonal Germplasm Repository, Corvallis, OR; abbreviated to VES in the figures). Results indicated that Fv *SOC1* is slightly downregulated at ZT8 and ZT12 (zeitgeber time) compared with other time points under LDs, whereas no clear rhythm was found under SDs (see Supplemental Figure 5 online). Based on these data, all samples for gene expression analyses were collected at ZT4. To examine the spatial expression pattern of Fv *SOC1*, we collected tissue samples from plants exposed to a 6-week SD flower induction treatment followed by LD conditions. We found that Fv *SOC1* was highly expressed in all tissues except flower buds and flowers (Figures 2A to 2C). In the shoot apex, Fv *SOC1* mRNA was abundant in both apical and axillary meristems as well as in the vascular tissue and leaf primordia (see Supplemental Figure 6 online). To analyze if daylength has an effect on Fv *SOC1* expression, we subjected young SD strawberry seedlings to photoperiodic treatments and collected leaf and shoot apex samples. We found that under LDs, the expression of Fv *SOC1* was high in both shoot apices and leaves. However, strong downregulation of Fv *SOC1* was found in both tissues of plants grown under flower-inductive SDs (Figures 2B and 2C). Time-course analysis showed that Fv *SOC1* mRNA levels stayed at high levels in the shoot apex under LDs (Figure 2D). By contrast, during a 6-week SD treatment, Fv *SOC1* expression gradually decreased and reached low levels after 4 weeks of SDs, similarly to the major floral repressor Fv *TFL1* in the same sample set (as shown in Koskela et al., 2012). To see if exposure to LDs after SD treatment restores Fv *SOC1* expression, we collected shoot apex samples from vegetative axillary shoots that developed after the SD treatment. In these samples, Fv *SOC1* expression was increased to a level of expression similar to that detected before SD treatment (Figure 2D). Therefore, our results suggest that Fv *SOC1* is seasonally regulated similarly to Fv *TFL1*, and its expression correlates negatively with flower initiation in SD strawberry.

Regulation of Fv *SOC1* in the Perpetual Flowering Mutant

We also analyzed the expression of Fv *SOC1* in the Hawaii-4 accession (PI551572; National Clonal Germplasm Repository, Corvallis, OR; called H4 hereafter). H4 lacks functional Fv *TFL1*; therefore, it flowers perpetually after flower induction and its photoperiodic requirement for flower induction is reversed (i.e., it flowers earlier under LDs than SDs) (Koskela et al., 2012). Also in H4, Fv *SOC1* was regulated by photoperiod. However, our data indicated that under LDs, the mRNA level of Fv *SOC1* was slightly lower in H4 leaf samples compared with SD strawberry, and the downregulation of Fv *SOC1* under SDs was less dramatic in H4 (Figures 2B and 2C). Therefore, we tested if Fv TFL1 regulates the expression of Fv *SOC1* by analyzing previously reported Fv *TFL1* transgenic lines in the SD strawberry background (Koskela et al., 2012). Neither RNA interference (RNAi) silencing nor overexpression of Fv *TFL1* affected the expression of Fv *SOC1*, suggesting that Fv *SOC1* is not regulated by Fv TFL1 (see Supplemental Figure 7 online). Therefore, Fv SOC1 may act upstream of Fv TFL1 or independently of it.

To examine if Fv *FT1* regulates Fv *SOC1*, we studied previously reported Fv *FT1*-RNAi lines in the H4 background (Koskela et al., 2012). We found about a threefold downregulation of Fv *SOC1* mRNA levels in the shoot apices of three independent RNAi lines (Figure 2E). Given that both Fv *FT1* and Fv *SOC1* are activated by LDs (Koskela et al., 2012), our results indicate that Fv *FT1* is likely involved in the photoperiodic regulation of Fv *SOC1* mRNA expression, which correlates positively with flowering in H4. In SD strawberry, these genes are similarly regulated, but their expression correlates negatively with flowering.

RNAi Silencing of Fv *SOC1* Downregulates Fv *TFL1* and Causes Day-Neutral Flowering in Strawberry

Since our real-time PCR and in situ hybridization analyses showed that Fv *SOC1* has spatial and temporal expression patterns similar to the floral repressor Fv *TFL1* in the shoot apex (Figures 2C and 2D; see Supplemental Figure 6 online; Koskela et al., 2012), we hypothesized that Fv SOC1 may regulate Fv *TFL1* expression to repress flowering in strawberry. Therefore, we silenced Fv *SOC1* in the SD strawberry background using an RNAi construct expressed under the *35S* promoter. Analysis

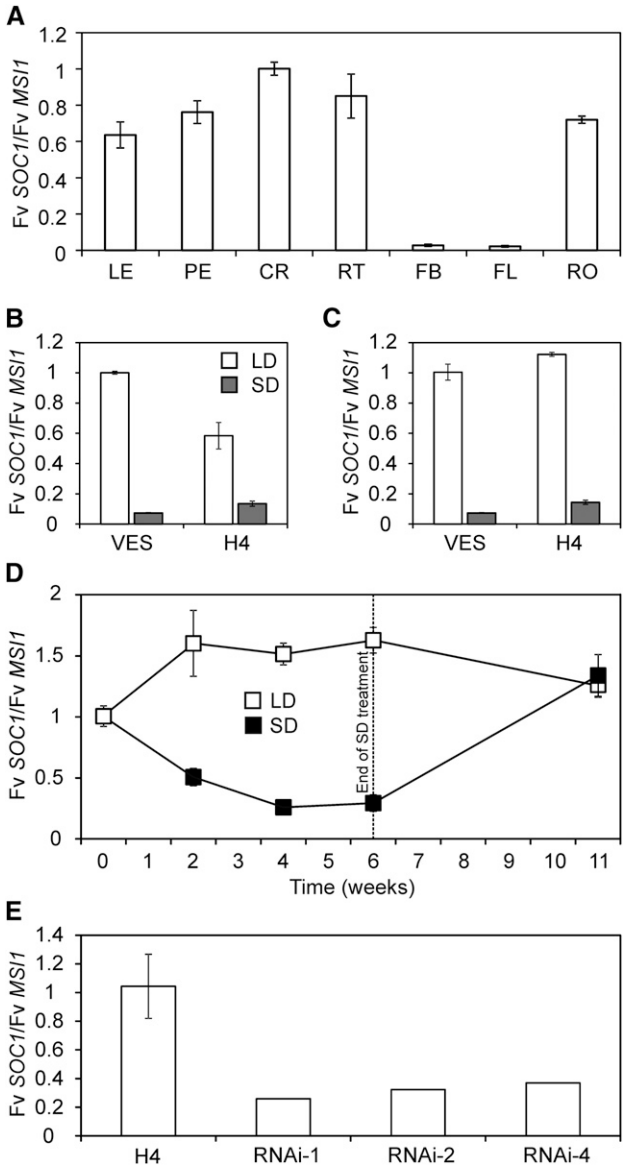

Figure 2. Spatial Expression Pattern and Regulation of Fv *SOC1*.

(A) Relative expression of Fv *SOC1* in different plant organs. Clonally propagated 2-month-old SD strawberry plants were grown under SDs for 6 weeks followed by standard LDs until anthesis. LE, leaf; PE, petiole; CR, axillary branch crown; RT, runner tip; FB, flower bud; FL, opened flower; RO, root. Mean fold change was calculated relative to axillary branch crown. Error bars represent SE; $n = 2$.

(B) and **(C)** Relative expression of Fv *SOC1* in leaves **(B)** and shoot apices **(C)** of SD strawberry (VES) and Hawaii-4 (H4) seedlings. Samples were collected from plants grown under LDs or SDs until the three-leaf stage. Mean fold change was calculated relative to SD strawberry grown under LDs. Error bars represent SE; $n = 3$ to 4.

(D) Time-course analysis of Fv *SOC1* relative expression under SDs and LDs. Clonally propagated 5-week-old plants of SD strawberry were subjected to photoperiodic treatments for 6 weeks (weeks 0 to 6) followed by LDs for 5 weeks. Primary shoot apices were analyzed during weeks 0 to 6, and newly formed branch crowns were analyzed at week 11. Mean fold change was calculated relative to week 0. Error bars represent SE; $n = 3$.

showed that Fv *SOC1* was strongly downregulated in three independent transgenic lines (see Supplemental Table 1 online). We propagated these lines clonally from runner cuttings and subjected the plants and nontransgenic controls to LD and SD treatments for 6 weeks followed by standard LDs (see Methods). All plants flowered after inductive SD treatment, but only Fv *SOC1*-RNAi plants flowered under noninductive LD conditions (Figures 3A and 3B). Compared with LDs, SDs advanced flowering by 2 to 3 weeks in two Fv *SOC1*-RNAi lines, whereas the line Fv *SOC1*-RNAi#1 flowered at the same time in both photoperiods. SD-treated nontransgenic plants produced on average six terminal inflorescences on the top of the primary and axillary leaf rosettes during weeks 11 to 16 after the beginning of the photoperiodic treatment, before returning to the vegetative growth phase (Figure 3C). By contrast, RNAi#1 and RNA#3 lines continuously produced new inflorescences in both photoperiods (Figures 3C and 3D; see Supplemental Figure 8 online), similarly to perpetual-flowering H4, which lacks functional Fv TFL1 (Koskela et al., 2012).

To test if Fv SOC1 regulates the expression of Fv *TFL1* in the shoot apex, we analyzed its expression levels in young Fv *SOC1*-RNAi plants in the SD strawberry background. Fv *TFL1* was downregulated in the shoot apices of LD-grown RNAi plants compared with nontransgenic SD strawberry. The level of downregulation correlated with the strength of Fv *SOC1* RNAi silencing (Figure 3E). To confirm that these differences were not caused by changes in the meristem identity in these plants, we analyzed the expression of the floral meristem identity gene Fv *AP1*, which is strongly upregulated at the time of flower induction (Mouhu et al., 2009; Koskela et al., 2012). Fv *AP1* was expressed at low levels in all RNAi plants compared with flower-induced SD-grown nontransgenic plants (see Supplemental Figure 9 online). This result confirmed that the apical meristems of LD-grown plants were vegetative at this stage and that the low Fv *TFL1* expression levels in RNAi plants were not caused by the activation of Fv *AP1*.

Next, we studied gene expression levels in SD strawberry and in Fv *SOC1*-RNAi line #1 in the SD strawberry background after an additional 4 weeks of photoperiodic treatments. In SD strawberry, both Fv *SOC1* and Fv *TFL1* were downregulated under SDs compared with LDs, correlating negatively with the expression of Fv *AP1*, which was activated under SDs (Figures 3F to 3H). In RNAi plants, by contrast, downregulation of both Fv *SOC1* and Fv *TFL1* was detected in both photoperiods. In addition, compared with LD-grown nontransgenic plants, Fv *AP1* was ~20 and 160 times upregulated in LD- and SD-grown RNAi plants, respectively, and plants flowered in both photoperiods. These results strongly suggest that, under LDs, Fv SOC1 promotes

(E) Relative expression of Fv *SOC1* in shoot apices of Fv *FT1*-RNAi lines in the H4 background. H4 and three independent Fv *FT1*-RNAi lines were grown under standard LD conditions and samples were collected at the three-leaf stage. Fold change was calculated relative to H4. Error bar represents SE; $n = 3$ for H4, and $n = 1$ for transgenic lines, which are shown as biological replicates. See Koskela et al. (2012) for the Fv *FT1* expression levels.

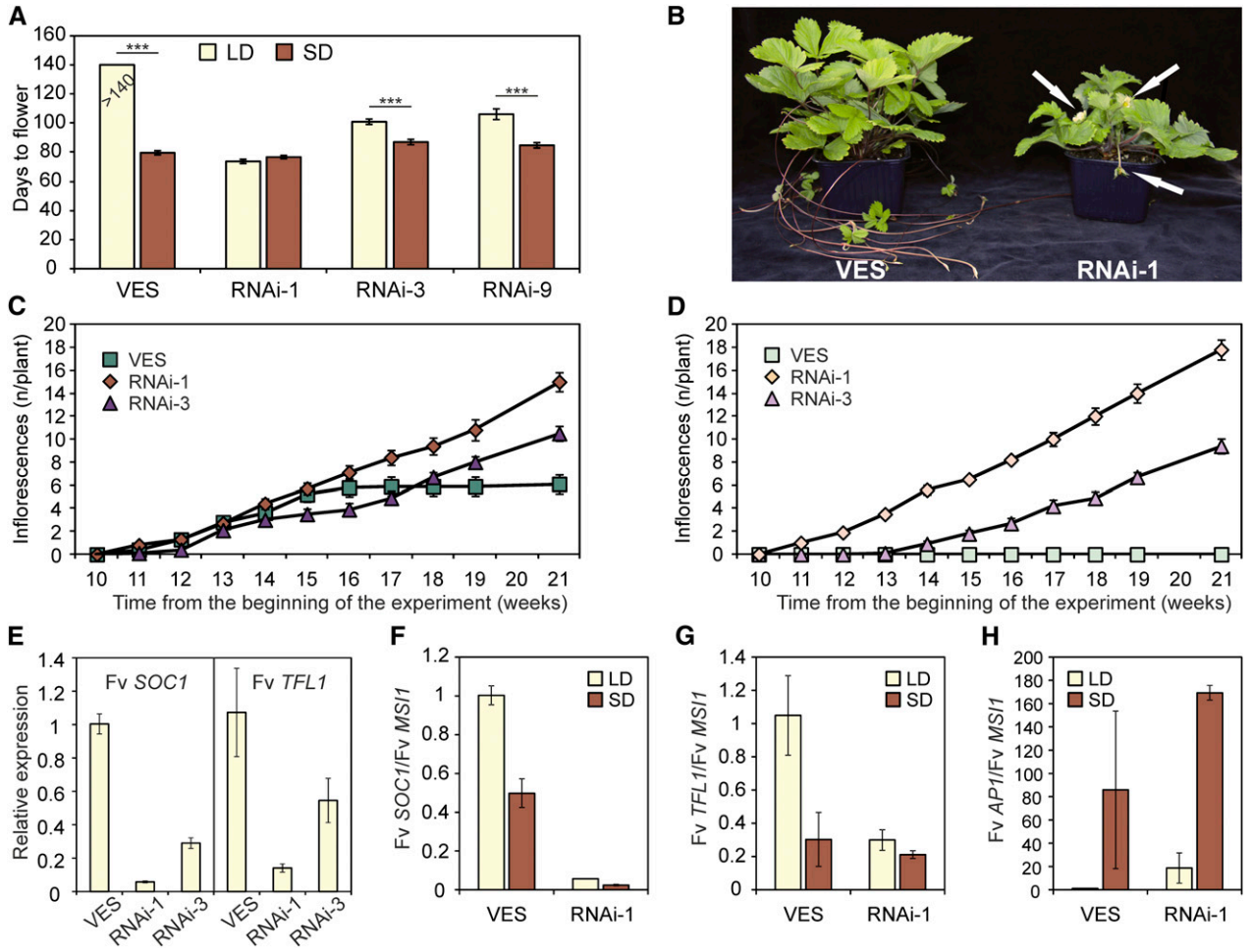

Figure 3. Silencing of Fv *SOC1* Causes Day-Neutral Flowering in Strawberry.

(A) Flowering time of SD strawberry (VES) and Fv *SOC1*-RNAi lines #1, #3, and #9. Clonally propagated 5-week-old plants were subjected to photoperiodic treatments for 6 weeks followed by standard LD conditions. Time from the beginning of the SD treatment to the first open flower was observed. Horizontal bars represent statistically significant differences between photoperiodic treatments (separately for SD strawberry and transgenic lines); ***$P < 0.001$. Error bars represent SE; $n = 10$.

(B) The phenotype of LD-grown SD strawberry and Fv *SOC1*-RNAi plants. SD strawberry is vegetative and produces runners, whereas the RNAi plant is flowering and has no runners. Arrows point to flowers.

(C) and **(D)** Time-course analysis of inflorescence formation in SD strawberry and Fv *SOC1*-RNAi lines #1 and #3. Cumulative number of inflorescences is shown. Clonally propagated 5-week-old plants were subjected to 6 weeks of SDs followed by LDs **(C)** or grown under continuous LDs **(D)**. Error bars represent SE; $n = 10$.

(E) The expression of Fv *SOC1* and Fv *TFL1* in 5-week-old clonally propagated SD strawberry and Fv *SOC1*-RNAi plants grown under LDs. Mean fold change was calculated relative to SD strawberry. Error bars represent SE; $n = 3$.

(F) to **(H)** Relative expression of Fv *SOC1* **(F)**, Fv *TFL1* **(G)**, and Fv *AP1* **(H)** in the shoot apices of SD strawberry and Fv *SOC1*-RNAi line #1. Clonally propagated 5-week-old plants were subjected to LDs or SDs for 4 weeks before sampling. Mean fold change was calculated relative to LD-grown SD strawberry. Error bars represent SE; $n = 3$; for SD-grown strawberry, $n = 2$.

the expression of Fv *TFL1*, which prevents the activation of Fv *AP1* and flower initiation in SD strawberry.

Fv *SOC1* Overexpression Represses Flowering in Strawberry

We also overexpressed Fv *SOC1* under the *35S* promoter in SD strawberry (Fv *SOC1*-OX). We selected transgenic lines based on Fv *SOC1* expression levels (see Supplemental Table 1 online) and subjected clonally propagated plants to SD treatment for 45 d followed by standard LDs. Only a few Fv *SOC1*-OX plants flowered after SD treatment, whereas all SD-grown nontransgenic control plants flowered and produced several inflorescences (Table 1). Moreover, opposite to Fv *SOC1*-RNAi plants, all LD-grown Fv *SOC1*-OX plants and nontransgenic control plants remained vegetative. SDs strongly repressed Fv

Table 1. Overexpression of Fv *SOC1* Represses Flowering in Strawberry

	Flowering Plants (Total Plants)		Inflorescences (*n*/Plant)	
Transgenic Line	LD	SD	LD	SD
VES	0 (9)	7 (7)	0	3.7 ± 0.5
OX-7	0 (10)	1 (10)	0	0.1 ± 0.1
OX-10	0 (10)	0 (10)	0	0
OX-11	0 (10)	2 (10)	0	0.3 ± 0.2

Five-week-old plants of SD strawberry (VES) and three independent Fv *SOC1*-OX lines (OX-7, OX-10, and OX-11) were subjected to SD or LD treatments for 6 weeks followed by standard LD growing conditions, and their flowering phenotypes were observed. The number of inflorescences (*n*/plant) is indicated as mean ± SE.

SOC1 and Fv *TFL1* expression in the shoot apices of nontransgenic control plants, whereas the expression of floral marker Fv *AP1* was ninefold upregulated in SDs compared with LDs, correlating with flowering (Figures 4A to 4C). In Fv *SOC1*-OX lines, consistent with their flowering phenotypes, upregulation of Fv *TFL1* was found in both photoperiods compared with control plants grown under the same photoperiods. However, in contrast with nontransgenic SD strawberry, Fv *SOC1* and Fv *TFL1* expression levels did not fully correlate in transgenic lines (Figures 4A and 4B). Although Fv *SOC1* expression remained at high levels in Fv *SOC1*-OX lines in both photoperiods, clear downregulation of Fv *TFL1* mRNA levels by SDs were still seen in these plants, suggesting the presence of both Fv SOC1-dependent and -independent regulatory mechanisms of Fv *TFL1*. In *Arabidopsis*, AP1 is known to downregulate *TFL1* (Liljegren et al., 1999; Ratcliffe et al., 1999), and we observed mild increases of Fv *AP1* mRNA levels in parallel with reduced Fv *TFL1* expression levels in SD-grown Fv *SOC1*-OX lines compared with LD-grown plants. The analysis of Fv *SOC1*-OX plants further supported the hypothesis that Fv SOC1 activates Fv *TFL1* to repress flowering in SD strawberry. However, based on these data, the role of Fv AP1 as a negative regulator of Fv *TFL1* cannot be excluded.

We also analyzed the mRNA levels of Fv *LFY* and Fv *AGL24*, since SOC1 has been reported to bind directly to the promoters of *LFY* and *AGL24* to activate their expression and to advance flowering in *Arabidopsis* (Lee et al., 2008; Liu et al., 2008). Constitutive overexpression of Fv *SOC1* did not affect Fv *LFY* mRNA expression in the SD strawberry background, whereas Fv *AGL24* tended to be slightly upregulated in Fv *SOC1*-OX lines compared with nontransgenic SD strawberry (Figures 4D and 4E). However, gene expression analysis in Fv *SOC1*-RNAi plants suggested that Fv SOC1 does not regulate Fv *AGL24* or Fv *LFY* (see Supplemental Figure 10 online).

Fv SOC1 Does Not Repress Flowering in Hawaii-4

Since our results indicated that Fv SOC1 regulates flowering through Fv *TFL1*, we explored whether Fv SOC1 regulates flowering time in H4, which lacks functional Fv TFL1 (Koskela et al., 2012). We transformed H4 plants with the Fv *SOC1*-RNAi or Fv *SOC1*-OX construct, selected transgenic lines according to Fv *SOC1* expression levels (see Supplemental Table 1 online), and produced T1 seedlings by self-pollination. To observe the flowering time of T1 seedlings, the plants were subjected to SD and LD treatments for six weeks. As observed earlier by Koskela et al. (2012), H4 plants flowered earlier under LDs than under SDs. Fv *SOC1*-OX lines and H4 control plants produced equal number of leaves before developing a terminal inflorescence under LDs, whereas Fv *SOC1*-RNAi plants were late flowering compared with nontransgenic H4 (Figure 5A). Under SDs, however, no differences were found in the flowering time of Fv *SOC1*-RNAi lines and H4 control plants, whereas one overexpression line produced slightly more leaves than H4. Our results, which show that Fv SOC1 does not repress flowering under LDs in the absence of functional Fv TFL1 in H4, in contrast with SD strawberry, which contains functional Fv TFL1, support the hypothesis that Fv SOC1 regulates flowering upstream of Fv TFL1. Moreover, the late flowering of Fv *SOC1*-RNAi lines under

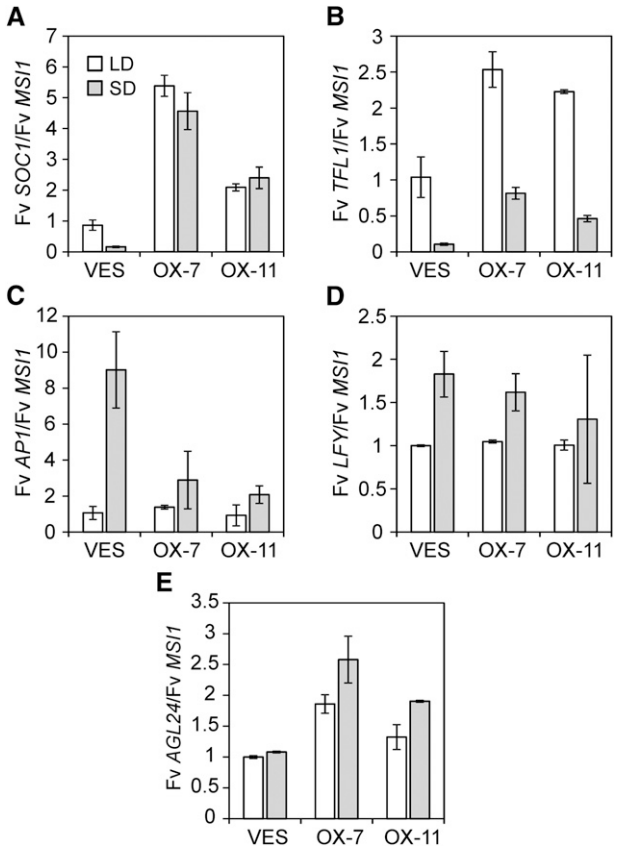

Figure 4. Overexpression of Fv *SOC1* Activates Fv *TFL1* and Represses Fv *AP1*.

Relative expression of Fv *SOC1* **(A)**, Fv *TFL1* **(B)**, Fv *AP1* **(C)**, Fv *LFY* **(D)**, and Fv *AGL24* **(E)** in shoot apices of SD strawberry (VES) and Fv *SOC1*-OX lines #7 and #11. Clonally propagated 5-week-old plants were grown for 4 weeks under LDs or SDs before sampling. Mean fold change was calculated relative to LD-grown SD strawberry. Error bars represent SE; $n = 2$.

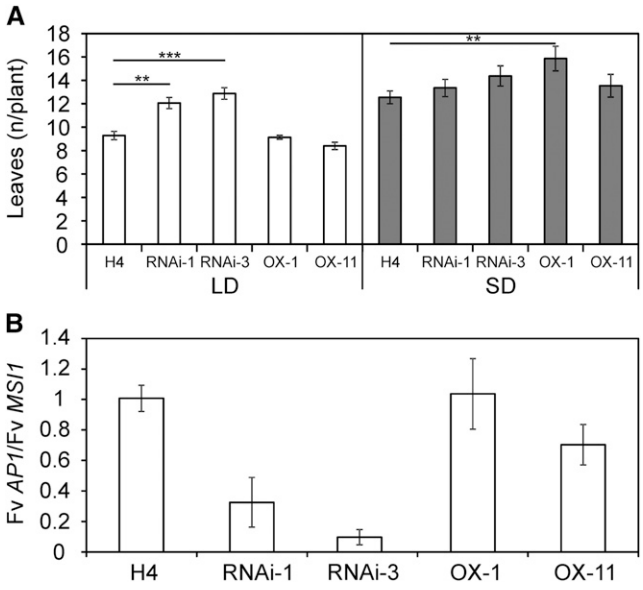

Figure 5. Silencing of Fv *SOC1* Delays Flowering in Hawaii-4 under LDs.

(A) Flowering time of Fv *SOC1*-RNAi lines #1 and #3 and Fv *SOC1*-OX lines #1 and #11 in the Hawaii-4 (H4) background. T1 seedlings were subjected to LDs or SDs for 6 weeks followed by standard LD conditions. Flowering time was calculated as the total number of leaves formed in the main crown below the terminal inflorescence. Error bars represent SE; $n = 7$ to 16. Horizontal bars represent statistically significant differences to H4 (separately for LD and SD); $^{**}P < 0.01$ and $^{***}P < 0.001$.

(B) Relative expression of Fv *AP1* in shoot apices of H4 and transgenic lines grown under LDs. Samples were collected at the three-leaf stage. Mean fold change was calculated relative to H4. Error bars represent SE; $n = 3$.

LDs suggests that Fv *SOC1* is required for the LD flowering response in H4.

To understand the molecular control of flowering time in H4 transgenic lines, we analyzed the expression of Fv *AP1*, Fv *AGL24*, and Fv *LFY* under LDs. Consistent with flowering time, we found similar Fv *AP1* expression levels in the shoot apices of H4 and Fv *SOC1*-OX lines. However, Fv *AP1* was downregulated in Fv *SOC1*-RNAi lines, indicating that Fv SOC1 may be required for the activation of Fv *AP1* under LDs in H4 (Figure 5B). In contrast with Fv *AP1*, neither the expression of Fv *AGL24* nor Fv *LFY* was affected in H4 transgenic lines (see Supplemental Figure 11 online). To verify that the mutation in H4 Fv *TFL1* does not convert the repressor into an activator that is still upregulated by Fv SOC1, we analyzed transgenic lines overexpressing the mutated Fv *TFL1* in SD strawberry and H4 backgrounds. Flowering was not advanced in these transgenic lines in either SD strawberry (see Supplemental Figure 12 online) or H4 (Koskela et al., 2012), ruling out the proposed new function of mutated Fv *TFL1*.

Fv *SOC1* Regulates Vegetative Growth in Strawberry

We also monitored the vegetative development of Fv *SOC1* transgenic lines in the SD strawberry background. Overexpression and RNAi silencing of Fv *SOC1* affected vegetative vigor, as indicated by the length of the petioles (see Supplemental Figure 13 online). Moreover, Fv *SOC1*-OX plants grown in the greenhouse for more than a year became elongated, whereas Fv *SOC1*-RNAi plants had a bushy growth habit (see Supplemental Figure 8 online).

To examine the effect of Fv *SOC1* on the photoperiodic regulation of vegetative development, we performed 6 weeks of photoperiodic treatment on clonally propagated plants of Fv *SOC1*-RNAi and Fv *SOC1*-OX lines in the SD strawberry background and observed runner formation. Nontransgenic control plants halted runner production after a few weeks under SDs, whereas Fv *SOC1*-OX plants formed runners continuously during the observation period, regardless of the photoperiod (Figures 6A and 6B). By contrast, plants of the strongest Fv *SOC1*-RNAi line stopped runner formation after 2 to 3 weeks in both SDs and LDs (Figures 6C and 6D). Also in the weaker Fv *SOC1*-RNAi line, runner formation ceased in both photoperiods, but this occurred a few weeks later in LDs compared with SDs. Consistent with reduced runner formation, most axillary buds developed into branch crowns in Fv *SOC1*-RNAi lines, and the plants became highly branched even under LDs (see Supplemental Figure 8 online). Moreover, Fv *SOC1*-OX lines produced only a few branch crowns even under SDs, where nontransgenic control plants formed several branch crowns (see Supplemental Figure 14 online). Taken together, in addition to the regulation of flowering, we conclude that Fv *SOC1* is involved in the photoperiodic regulation of axillary bud differentiation into runners and branch crowns in SD strawberry.

Fv *SOC1* Activates GA Biosynthesis

In strawberry, GA affects petiole elongation (Guttridge and Thompson, 1964; Wiseman and Turnbull, 1999) and promotes axillary bud differentiation into runners (Hytönen et al., 2009). To analyze if the enhanced vegetative growth of Fv *SOC1*-OX lines is GA dependent, we treated LD-grown plants with the inhibitor of GA biosynthesis prohexadione-calcium (Evans et al., 1999). We found that prohexadione-calcium completely inhibited runner formation in both SD strawberry and Fv *SOC1*-OX plants in the SD strawberry background (Figure 7A), confirming that a normal level of GA biosynthesis is also required for runner formation in Fv *SOC1*-OX lines. Next, we treated LD-grown Fv *SOC1*-RNAi and SD strawberry plants with GA_3. Nontransgenic control plants produced runners continuously, and GA_3 had no effect on runner formation (Figure 7B). However, runner formation ceased in nontreated RNAi plants within a few weeks, whereas GA_3 strongly enhanced the production of new runners.

Growth regulator treatments of transgenic lines suggested that Fv *SOC1* may activate GA biosynthesis to control vegetative development in SD strawberry. Therefore, we studied the mRNA levels of GA biosynthetic gene homologs (Hedden and Thomas, 2012; Kang et al., 2013). We found opposite changes in the expression of several genes in Fv *SOC1*-OX and Fv *SOC1*-RNAi lines in the SD strawberry background. Two putative *GA20-oxidase* (*GA20ox*) and four *GA3ox* homologs were activated in Fv *SOC1*-OX plants (Figures 7C and 7D). By contrast, an opposite trend in the expression of these genes was

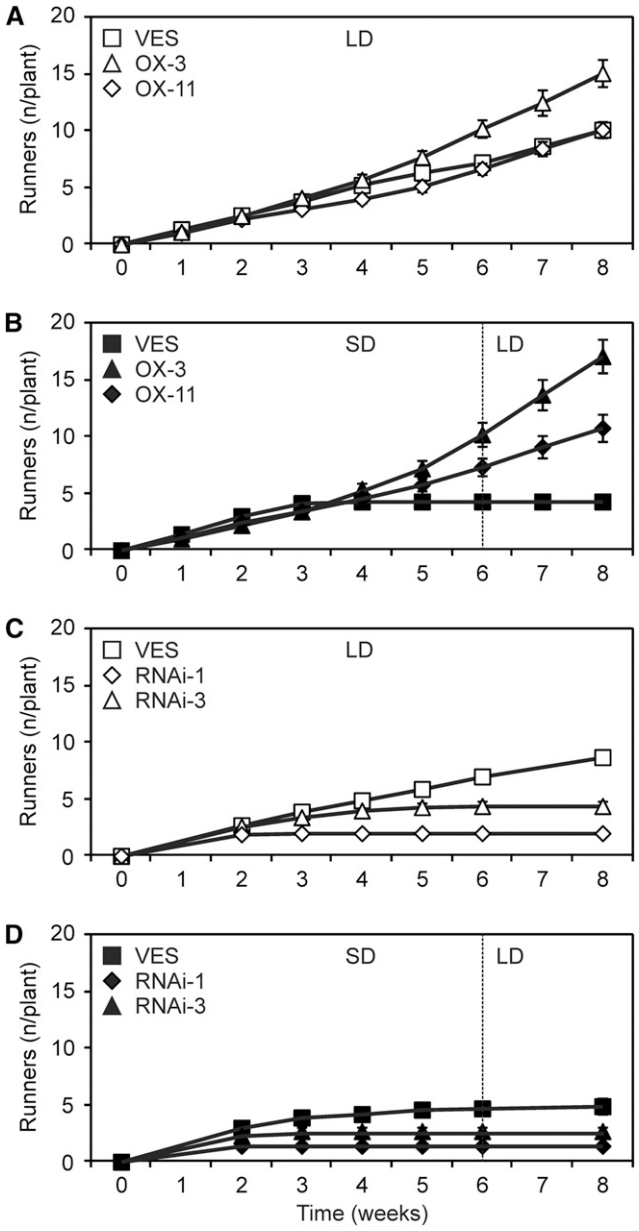

Figure 6. Fv *SOC1* Enhances Runner Formation in Strawberry.

(A) and **(B)** Cumulative number of runners (n/plant) in clonally propagated plants of SD strawberry (VES) and Fv *SOC1*-OX lines #3 and #11. Five-week-old plants were grown under LDs **(A)** and under SDs for 6 weeks followed by LDs **(B)**. Error bars represent SE; $n = 7$ to 10.

(C) and **(D)** Cumulative number of runners in SD strawberry and Fv *SOC1*-RNAi lines #1 and #3. Five-week-old plants were grown under LDs **(C)** and under SDs for 6 weeks followed by LDs **(D)**. Error bars represent SE; $n = 10$.

observed in the strong Fv *SOC1*-RNAi line #1 (Figures 7E and 7F). In addition, the mRNA level of the strawberry homolog of *GA2ox*, which encodes a GA degradation enzyme (Hedden and Thomas, 2012), was slightly increased in Fv *SOC1*-OX lines and decreased in the Fv *SOC1*-RNAi line (see Supplemental Figure 15 online).

Next, we analyzed the expression of the strawberry homolog of *GIBBERELLIC ACID INSENSITIVE* (*GAI*), which encodes a DELLA growth repressor in the GA pathway (Peng et al., 1997). Consistent with earlier results that genes encoding DELLA proteins are feed-forward regulated by GA (Hytönen et al., 2009; Hedden and Thomas, 2012), we found that Fv *GAI* was upregulated in Fv *SOC1* overexpression plants compared with SD strawberry and, again, that the response was opposite in the RNAi line (see Supplemental Figure 15 online). Strawberry homologs of positive regulators of GA signaling, *GIBBERELLIN INSENSITIVE DWARF1* and *SLEEPY* (Harberd et al., 2009), were not clearly affected in transgenic lines (see Supplemental Figure 15 online). In conclusion, our results indicate that Fv SOC1 may activate the expression of many genes of the GA biosynthetic pathway to regulate vegetative development in strawberry.

The Activation of Fv *TFL1* by Fv SOC1 Is GA Independent

Our results indicated that Fv SOC1 promotes the expression of both Fv *TFL1* and several genes of the GA biosynthetic pathway. This raised the question of whether Fv SOC1 upregulates Fv *TFL1* through the GA pathway. In such a scenario, GA_3 treatment should restore the normal Fv *TFL1* expression level in Fv *SOC1*-RNAi plants in the SD strawberry background. Therefore, we treated SD strawberry and Fv *SOC1*-RNAi plants with GA_3 and analyzed the expression of Fv *TFL1* in the shoot apex samples. GA_3 had no clear effect on the expression of Fv *TFL1* in either RNAi or nontransgenic control plants (Figure 8; see Supplemental Figure 16 online), although it affected runner formation in RNAi plants (Figure 7B). Therefore, Fv SOC1 likely regulates vegetative and floral development in strawberry through GA-dependent and -independent pathways, respectively.

DISCUSSION

Fv SOC1 Activates Fv *TFL1* to Repress Flowering under LDs

SOC1 and *SOC1*-like genes encode MADS box transcription factors that are reported to function as floral activators in annual LD and SD plants (Menzel et al., 1996; Lee et al., 2000; Ferrario et al., 2004; Lee et al., 2004) and in a perennial species *Cardamine flexuosa* (Zhou et al., 2013). Here, we show that in strawberry, which is a seasonal flowering perennial SD plant (SD strawberry), the overexpression of the strawberry ortholog of *SOC1* suppresses photoperiodic flowering, whereas Fv *SOC1*-RNAi plants flower continuously without inductive SD treatment, similarly to perpetual flowering mutants, which lack the functional floral repressor Fv TFL1 (Figures 3A to 3D; Iwata et al., 2012; Koskela et al., 2012). Our result that Fv SOC1 does not repress flowering under LDs in the perpetual flowering H4 genotype (Figure 5A) suggests that Fv SOC1 may repress flowering through Fv TFL1. Consistent with this idea, Fv *TFL1* was upregulated in Fv *SOC1*-OX lines and downregulated in RNAi lines in the SD strawberry background, whereas the floral meristem identity gene Fv *AP1* was oppositely regulated, correlating with flowering time. These results suggest that Fv SOC1 may activate Fv *TFL1* to repress Fv *AP1* and flowering under LDs in SD strawberry. Under SDs, however, both Fv *SOC1* and Fv *TFL1*

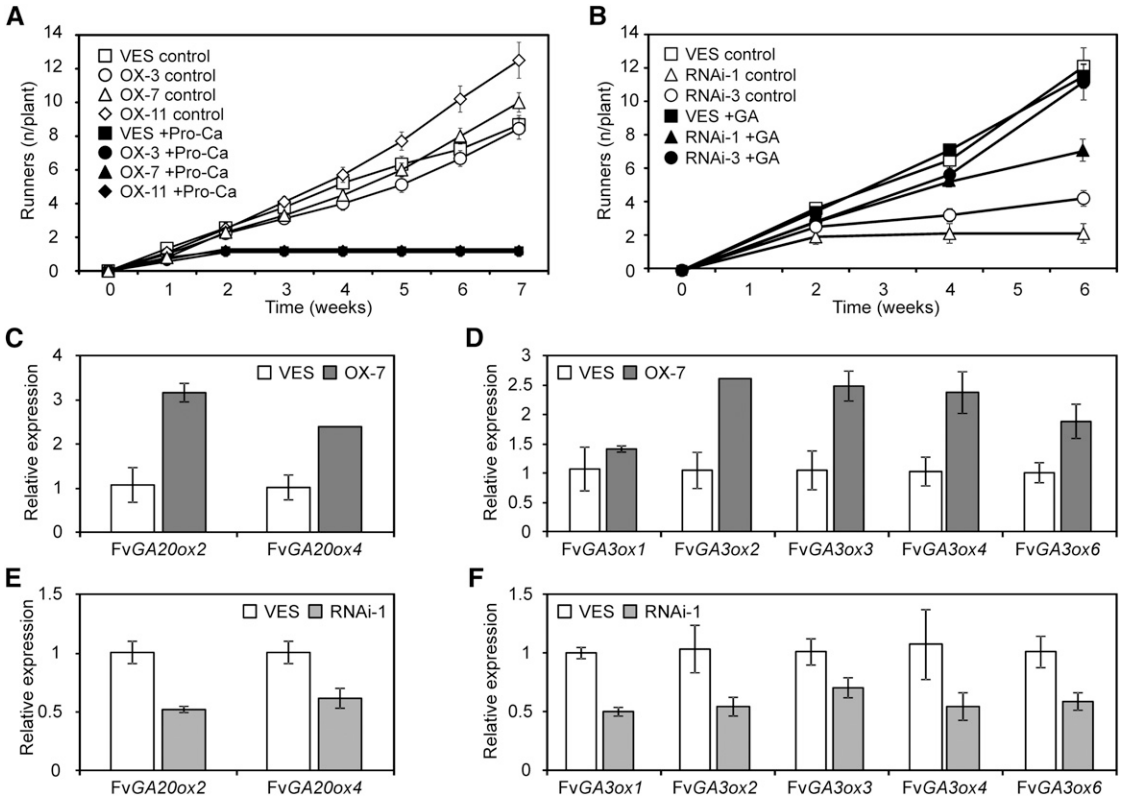

Figure 7. Fv *SOC1* Activates GA Biosynthesis to Promote Runner Growth in Strawberry.

(A) The effect of the inhibitor of GA biosynthesis, prohexadione-calcium (Pro-Ca), on runner formation in SD strawberry (VES) and Fv *SOC1*-OX lines. The cumulative number of runners (n/plant) is shown. Five-week-old plants were grown under LDs and treated with 100 ppm Pro-Ca or water (control) at week 0. Error bars represent SE; $n = 9$ to 10.

(B) The effect of GA_3 on runner formation in SD strawberry and Fv *SOC1*-RNAi lines. Cumulative number of runners is shown. Five-week-old plants were grown under LDs and treated with 50 ppm GA_3 solution or mock treated (-GA) at weeks 0 and 2. Error bars represent SE; $n = 10$ to 11; for RNAi #1, $n = 5$.

(C) and **(D)** Relative expression of Fv *GA20ox* **(C)** and Fv *GA3ox* **(D)** genes in the leaves of clonally propagated SD strawberry and Fv *SOC1*-OX line #7 grown under LDs. Mean fold change was calculated relative to the expression in SD strawberry separately for each gene. Error bars represent SE; $n = 2$.

(E) and **(F)** Relative expression of Fv *GA20ox* **(E)** and Fv *GA3ox* **(F)** genes in the leaves of clonally propagated SD strawberry and Fv *SOC1*-RNAi line #1 grown under LDs. Mean fold change was calculated relative to the expression in SD strawberry separately for each gene. Error bars represent SE; $n = 3$.

are downregulated and flower induction occurs (Figures 2B to 2D; Koskela et al., 2012).

Guttridge and Thompson (1964) showed that exogenous GA application delays flowering in strawberry. Since we observed changes in the expression of several GA biosynthetic genes and GA-dependent vegetative phenotypes in Fv *SOC1* transgenic lines in the SD strawberry background (see below), we tested if Fv SOC1 controls Fv *TFL1* through GA. Although a recent study suggested that GA may activate the rose *TFL1* homolog *KOUSHIN* (*KSN*) through GA responsive *cis*-elements, which are also present in the promoter of Fv *TFL1* (Randoux et al., 2012), GA_3 did not activate the expression of Fv *TFL1* in our Fv *SOC1*-RNAi lines or in SD strawberry. These results suggest that GA is not involved in the regulation of Fv *TFL1* mRNA expression. However, further studies are needed to explore whether changes in endogenous GAs have an effect on flowering time in Fv *SOC1* RNAi and overexpression lines or in nontransgenic strawberry.

In *Arabidopsis*, TFL1 regulates both flowering time and inflorescence architecture (Bradley et al., 1997). *TFL1* is highly expressed in the center of the inflorescence meristem in order to maintain indeterminacy of the meristem. However, AP1 and LFY downregulate *TFL1* in the flanks of the inflorescence meristem to specify floral meristems (Liljegren et al., 1999; Ratcliffe et al., 1999). AP1 binds to the MADS box element downstream of the *TFL1* coding sequence (Kaufmann et al., 2010), and a recent report showed that SOC1 is involved in the AP1-dependent regulation of *TFL1* homologs in both *Arabidopsis* and rice (Liu et al., 2013). Our time-course gene expression analysis suggests that at least initial downregulation of Fv *TFL1* in the shoot apex of SD strawberry does not depend on the activation of floral meristem identity genes. In SD strawberry, Fv *TFL1* is downregulated after only 2 weeks of SD in parallel with Fv *SOC1*, but both Fv *AP1* and Fv *FUL1* are unaffected by SDs at this time point (Figure 2D; Koskela et al., 2012). However, Fv *TFL1* mRNA levels still decrease during the following two weeks

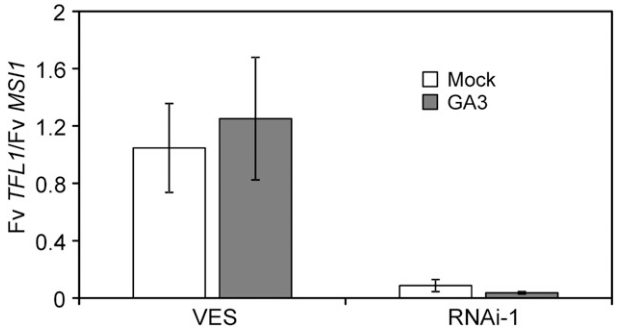

Figure 8. GA Does Not Activate Fv *TFL1*.

Effect of GA_3 on the relative expression of Fv *TFL1* in SD strawberry (VES) and Fv *SOC1*-RNAi line #1. Shoot apex samples of plants grown under LDs for 5 weeks were collected 7 d after 50 ppm GA_3 or mock treatment. Mean fold change was calculated relative to SD strawberry. Error bars represent SE; $n = 2$.

of SDs, when Fv *AP1* and flowering, but not Fv *FUL1*, are induced (Heide and Sønsteby, 2007; Koskela et al., 2012). Therefore, the downregulation of Fv *SOC1* by SDs may cause an initial decrease of Fv *TFL1* mRNA levels independently of Fv AP1, but the role of Fv AP1 in the later stages cannot be excluded. Strawberry produces a cymose inflorescence, which does not have an indeterminate inflorescence meristem (Jahn and Dana, 1970), suggesting that the possible role of Fv AP1 in the regulation of Fv *TFL1* is at least spatially different from that of the homologs in *Arabidopsis*. In addition, Fv LFY may play a role in later floral development, since its mRNA expression follows Fv *AP1* with a delay in the shoot apex (Mouhu et al., 2009).

The promoter region of Fv *TFL1* contains two predicted binding sites of MADS domain transcription factors called CArG boxes 2178 bp (TT[ACTTTTTAGT]C) and 1159 bp (TT[TCTTTTATGG]CAAA) upstream of Fv *TFL1* start codon, supporting the hypothesis that Fv SOC1 may directly bind to the Fv *TFL1* promoter. Furthermore, the latter putative CArG box is almost identical with the SOC1 binding site in the *AGL24* promoter (Liu et al., 2008) and has an adjacent triple AAA, which is probably required for MADS box protein binding (Deng et al., 2011; Tao et al., 2012). A detailed study of these promoter elements should be performed to reveal their potential role in the regulation of Fv *TFL1* mRNA expression.

The Function of Fv SOC1 Depends on Fv TFL1

In contrast with SD strawberry, perpetual flowering accessions containing nonfunctional Fv TFL1 flower earlier under LDs than under SDs (Sønsteby and Heide, 2008; Mouhu et al., 2009). We previously reported that Fv FT1 promotes flowering in H4 under LDs and proposed that in SD strawberry, this LD promotion pathway is masked by the repressor Fv TFL1 (Koskela et al., 2012). Our results for Fv *SOC1*-RNAi lines in the H4 background further support the presence of a LD-promoting pathway. In H4, opposite to SD strawberry, RNAi silencing of Fv *SOC1* caused reduced Fv *AP1* mRNA levels and delayed flowering specifically under LD conditions. However, the overexpression of Fv *SOC1* had no effect on flowering time under LDs, suggesting that Fv *SOC1* mRNA levels higher than a specific threshold have no additional effect on flowering time. We also found that the RNAi silencing of Fv *FT1* in the H4 background downregulated Fv *SOC1* and Fv *AP1* (Figure 2E; Koskela et al., 2012), indicating that Fv FT1 may function upstream of Fv *SOC1* to promote flowering in H4, similarly to *FT* and *SOC1* in *Arabidopsis* (Samach et al., 2000; Moon et al., 2005; Yoo et al., 2005). Given that Fv FT1 activates Fv *SOC1* in H4, it may also upregulate Fv *TFL1* through Fv SOC1 in SD strawberry. Therefore, the activation of Fv *TFL1* by Fv SOC1 may account for the divergence in the photoperiodic pathways between the LD plant *Arabidopsis* and the SD plant strawberry. In this scenario, H4, which lacks functional Fv TFL1, uses the default pathway. This default pathway may be present also in SD strawberry, but it is probably masked by the activation of Fv *TFL1* by Fv SOC1. Also in *Arabidopsis*, the upregulation of *TFL1* by FT was recently predicted by modeling, and a clear positive correlation was found in the expression of these genes (Jaeger et al., 2013), but the functional significance of this interaction remains to be shown.

How Fv *SOC1* regulates flowering in H4 is an open question. In *Arabidopsis*, SOC1 directly activates the expression of *LFY* and *AGL24* by binding to their promoters (Moon et al., 2005; Lee et al., 2008; Liu et al. 2008), but we did not find clear evidence that Fv SOC1 would regulate strawberry *LFY* and *AGL24* homologs. Similarly, in mustard (*Sinapis alba*) and evergreen azalea (*Rhododendron* × *pulchrum*), *SOC1* and *LFY* homologs are expressed independently of each other (D'Aloia et al., 2009; Cheon et al., 2012). One possibility is that Fv SOC1 directly activates Fv *AP1*. However, Fv SOC1 is likely to have several roles in the floral regulatory pathways in strawberry, since SOC1 interacts with several other MADS box proteins and binds to the promoters of multiple floral regulators in *Arabidopsis* (de Folter et al., 2005; van Dijk et al., 2010; Immink et al., 2012; Tao et al., 2012).

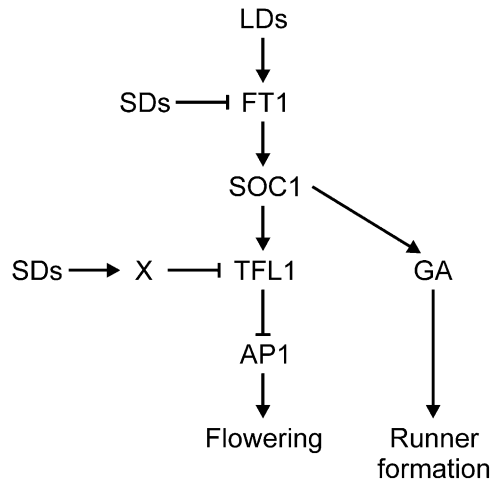

Figure 9. Model Showing the Photoperiodic Regulation of Flowering and Runner Formation in Strawberry.

Arrows indicate activation, and bars indicate repression.

Fv SOC1 Regulates Vegetative Development by Activating GA Biosynthesis

In strawberries, flower initiation is tightly connected to changes in vegetative development within yearly growth cycles (Hytönen and Elomaa, 2011). Under LDs, strawberries continuously produce runners from axillary buds, whereas under flower inductive SDs, axillary buds differentiate into branch crowns (Konsin et al., 2001; Hytönen et al., 2004, 2009). We show here that in addition to its role in the regulation of flowering, Fv *SOC1* plays a role in the photoperiodic regulation of vegetative development. While runner formation in SD strawberry ceased after a few weeks of SDs, Fv *SOC1*-OX plants in the SD strawberry background continued to produce runners under these conditions. By contrast, Fv *SOC1*-RNAi lines were dwarfed, with several branch crowns. Young RNAi plants formed a few runners before they completely stopped runner development in both LDs and SDs. Since both Fv *SOC1*-OX and RNAi lines lost the photoperiodic response of vegetative development, we reason that normal photoperiodic control of Fv *SOC1* may be crucial for vegetative responses. However, we showed earlier that Fv TFL1 has no effect on the photoperiodic regulation of axillary bud differentiation to runners or branch crowns (Koskela et al., 2012); therefore, Fv SOC1 may regulate this process through other genes. *RUNNERING LOCUS*, which is an unknown but dominant regulator of runner formation (Brown and Wareing, 1965), is one candidate for such a gene.

Vegetative phenotypes of Fv *SOC1* transgenic lines suggest that Fv *SOC1* may affect the activity of the GA biosynthetic pathway (Guttridge and Thompson, 1964; Wiseman and Turnbull, 1999; Hytönen et al., 2009). This hypothesis is supported by the findings that prohexadione-calcium, the inhibitor of GA biosynthesis, stopped runner formation in Fv *SOC1*-OX plants, and GA_3 induced runner development in Fv *SOC1*-RNAi lines in the SD strawberry background. We also revealed the role of Fv SOC1 as the putative regulator of GA biosynthetic genes. In Fv *SOC1*-OX plants in the SD strawberry background, several strawberry homologs of *GA20ox* and *GA3ox* genes were activated and an opposite trend was found in Fv *SOC1*-RNAi plants. Since GA-20 oxidation is a rate-limiting step in GA biosynthesis (Appleford et al., 2006; Middleton et al., 2012), twofold to severalfold changes in the expression levels of Fv *GA20ox* genes in our transgenic lines is likely to affect GA levels and, consequently, growth. In *Arabidopsis*, SOC1 is known to mediate the effect of GA on flowering (Moon et al., 2003; Searle et al., 2006), but its role in the regulation of GA biosynthesis is not clear. In concordance with our findings, the expression of *GA20ox1* is slightly downregulated in the *Arabidopsis soc1* mutant (Dorca-Fornell et al., 2011), but the effect of this change on growth is unknown. SOC1 may also regulate the GA biosynthetic pathway by repressing *TEMPRANILLO1* (Tao et al., 2012), which downregulates *GA3ox1* and *GA3ox2* by binding to their promoters (Osnato et al., 2012). Although our results strongly suggest that Fv SOC1 controls the GA biosynthetic pathway in strawberry, genetic experiments combining silencing of GA biosynthetic genes with Fv *SOC1* overexpression or vice versa would be necessary to confirm our findings.

Seasonal Regulation of Vegetative and Generative Development in Strawberry

We recently presented a model suggesting that the seasonal regulation of Fv *TFL1* mRNA expression regulates yearly flowering cycles in SD strawberry (Koskela et al., 2012). Here, we extend this genetic model and show evidence that branching of the flowering pathway may explain the seasonal regulation of vegetative development (Figure 9). We found that both Fv *SOC1* and Fv *TFL1* are highly expressed in the shoot apex under LDs, whereas under SDs, their gradual downregulation is followed by the induction of Fv *AP1* and flowering. Under subsequent LDs, the expression of both Fv *SOC1* and Fv *TFL1* is reactivated in the newly emerged vegetative axillary shoots (Figure 2D; Koskela et al., 2012). Given that Fv SOC1 likely activates Fv *TFL1* mRNA expression (Figures 3E, 3G, and 4B) and that Fv *SOC1* is downregulated in Fv *FT1* RNAi lines (Figure 2E), we propose that Fv FT1 may mediate the photoperiodic regulation of Fv SOC1, which regulates Fv *TFL1* expression according to seasonal changes in photoperiod; therefore, flower initiation only occurs during short photoperiods in autumn, whereby plants flower the next spring. However, additional regulators are likely involved in the photoperiodic regulation of Fv *TFL1* (indicated by x in Figure 9), or the activity of Fv SOC1 is modulated by light, since the overexpression of Fv *SOC1* did not fully nullify the photoperiodic regulation of Fv *TFL1*. Our data indicate that the photoperiod sensing and transmission pathway is shared between *Arabidopsis* and strawberry. However, the divergence of the response pathway downstream of SOC1 orthologs may cause opposite photoperiodic flowering responses in *Arabidopsis* and SD strawberry.

Independently of its effect on flowering, Fv SOC1 may also promote GA biosynthesis (Figures 7 and 9), which is involved in the photoperiodic regulation of axillary bud differentiation to runners or branch crowns (Hytönen et al., 2009). The LD activation of Fv *SOC1* could increase GA levels in summer to promote runner development, whereas the downregulation of Fv *SOC1* and the GA biosynthetic pathway in autumn could cause the differentiation of axillary buds into branch crowns. In conclusion, we revealed the role of Fv SOC1 as a central signaling hub that regulates photoperiodic development in strawberry. A detailed analysis of Fv SOC1/GA and Fv SOC1/Fv TFL1 pathways may open new possibilities to control vegetative and generative development in strawberries and other rosaceous crops.

METHODS

Plant Material

Strawberry (*Fragaria vesca*) experiments were performed with seasonal flowering SD strawberry and perpetual flowering accession Hawaii-4 (H4). SD strawberry (PI551792) was obtained from the National Clonal Germplasm Repository, Corvallis, OR, and H4 (National Clonal Germplasm Repository accession number PI551572) was provided by Kevin Folta. Either seedlings or plants clonally propagated from runner cuttings were used for the experiments as indicated in the text and figure legends. For *Arabidopsis thaliana* experiments, Columbia-0 ecotype was used.

Growth Conditions and Phenotyping

Plants were grown in a greenhouse at the University of Helsinki, Finland. The greenhouse temperature was 18 ± 2°C, and plants were illuminated by high-pressure sodium lamps (HPS; Airam 400 W) for 18 h daily with a light intensity of ~150 μmol m^{-2} s^{-1} (standard LD growing conditions). Photoperiodic experiments were conducted in greenhouse rooms during

the winter season (October to March) when the natural light irradiance is low in Finland. In the photoperiodic treatments, strawberry plants were subjected to 12-h SD and 18-h LD photoperiods. In both photoperiods, plants were illuminated with 12 h of HPS (~150 μmol m^{-2} s^{-1}) light. In the LD treatment, 12 h of HPS illumination was extended with low-intensity incandescent light (~8 μmol m^{-2} s^{-1}) for 6 h in the evening. Darkening curtains were used to exclude any additional light during daylength extension treatment and to ensure darkness during the night. Temperature in photoperiodic experiments was constant 18 ± 1°C. Flowering time was measured either as the number of leaves initiated in the main crown below the terminal inflorescence or as the number of days to the first open flower. For *Arabidopsis* flowering observations, plants were grown under 8-h SD or 18-h LD at 20 ± 1°C.

Growth Regulator Treatments.

For prohexadione-calcium (BAS125; BASF) treatment, 100 mg L^{-1} solution was prepared in milli-Q water. GA_3 (Duchefa) was first dissolved in ethanol at a concentration of 5 μg μL^{-1}, and a 50 mg L^{-1} dilution was made in milli-Q water (Millipore). Growth regulators were sprayed on clonally propagated plants until drip-off. Prohexadione-calcium treatment was performed once and GA_3 treatment twice with a 14-d interval. Mock treatments without growth regulators were performed for control plants.

Expression Analysis

Leaf and shoot apex samples were frozen in liquid nitrogen and stored at −70°C before total RNA was extracted using a modification of the pine tree method (Monte and Somerville, 2002). cDNAs were synthesized from 1 μg of total RNA using Superscript III reverse transcriptase (Invitrogen). SYBR Green Master (Roche) was used for real-time PCR reactions, which were performed in the Light Cycler 480 (Roche) instrument as described previously (Mouhu et al., 2009). Real-time PCR reactions were performed with three technical replicates and two to four biological replicates as mentioned in the figure legends. Relative expression levels were calculated by the ΔΔCt (cycle threshold) method with Fv *MSI1* as the normalization gene as described previously (Mouhu et al., 2009). Primers used in the real-time PCR are listed in Supplemental Table 2 online. Primer efficiencies were almost equal for all primer pairs.

In Situ Hybridization

In situ hybridization was performed on longitudinal sections of the apex of the main shoot as described previously (Kurokura et al., 2006). To avoid cross-hybridization, the probe was designed in the 3′-region of the gene, which is less conserved among MADS box transcription factor encoding genes. Fv *SOC1* probe template fragment was amplified by RT-PCR using forward and reverse primers 5′-GAAGGCACAGGTTTTCAAGG-3′ and 5′-CAGCCTTGGCTTGGATAGAG-3′, respectively, and cloned into the pDrive cloning vector (Qiagen). Antisense probe was synthesized using the SP6 promoter (Qiagen). Fv *TFL1* sense probe described earlier was used as a control (Koskela et al., 2012).

Plasmid Constructs

Plasmid constructs for overexpression and RNAi silencing lines were created according to Gateway technology with Clonase II (Invitrogen). For Fv *SOC1* overexpression and RNAi constructs, cDNA from *F. vesca* var *semperflorens* 'Baron Solemacher' was amplified with primer pairs 5′-AAAAAGCAGGCTGGTTGCGCTCATAATCTTCTCT-3′ (attB1) and 5′-AGAAAGCTGGGTTGTTCACACTCCTCTCCAACTG-3′ (attB2), and 5′-AAAAAGCAGGCTTCTCAAGAACTTGCTGGGTTCA-3′ (attB1) and 5′-AGAAAGCTGGGTGCTAGTGCTTCGATCTCCTTTCTG-3′ (attB2), respectively. The construct reported by Koskela et al. (2012) was used to overexpress mutated Fv *TFL1* in SD strawberry. The destination vectors were p7WG2D for overexpression and PK7GWIWG2(II) for RNAi silencing (Karimi et al., 2002). Both vectors contain green fluorescent protein as a positive selection marker.

Transformation

Vectors carrying overexpression and RNAi constructs were electroporated into *Agrobacterium tumefaciens* strain GV3101 and transformed into SD strawberry and H4 as described previously (Oosumi et al., 2006). Several transgenic lines were generated for both constructs in each genetic background. Transgenic lines were selected for the experiments based on their phenotypes and Fv *SOC1* expression levels. *Arabidopsis* Columbia-0 ecotype was transformed using the floral dip method, and transgenic T1 seedlings were selected based on green fluorescent protein fluorescence (Zhang et al., 2006).

Statistical Analyses

When appropriate, averages were subjected to analysis of variance using the general linear model procedure and differences between means were tested using contrasts in the SAS/STAT software (version 9.2 of the SAS System for Windows; SAS Institute).

Phylogenetic Analysis

Maximum likelihood phylogenetic analysis was performed to gain insight into the relationships among *SOC1*-like genes. Amino acid sequences of Fv SOC1 and other selected eudicots were aligned using Muscle (Edgar, 2004). A single most-optimal tree was computed using the randomized axelerated maximum likelihood BlackBox Web server (Stamatakis et al., 2008). Default settings were used with a gamma distribution and the Whelan and Goldman model of molecular evolution. One hundred bootstrap samples were generated to assess support for the inferred relationships.

Accession Numbers

Sequence data from this article can be found in the GenBank/National Center for Biotechnology Information data library under the following accession numbers: Fv *SOC1* (FJ531999), Fv *TFL1* (JN172097), Fv *FT1* (JN172098), and Fv *LFY* (FJ532000). Predicted gene models (Hybrid V2) can be found in the Genome Database for Rosaceae (http://www.rosaceae.org): Fv *AP1* (gene04564), Fv *AGL24* (gene30741), Fv *MSI1* (gene03001), Fv *GA20ox2* (gene19438), *Fv GA20ox4* (gene09034), Fv *GA3ox1* (gene06004), Fv *GA3ox2* (gene01056), Fv *GA3ox3* (gene01058), Fv *GA3ox4* (gene01059), and Fv *GA3ox6* (gene11192).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Maximum Likelihood Tree of Amino Acid Sequences Showing Phylogenetic Relationships of SOC1-Like Proteins in Eudicots.

Supplemental Figure 2. Syntenic Analysis of Fv *SOC1* and *SOC1* from *Arabidopsis*.

Supplemental Figure 3. The Alignment of SOC1-Like Proteins.

Supplemental Figure 4. Overexpression of Fv *SOC1* Advances Flowering in *Arabidopsis*.

Supplemental Figure 5. Diurnal Rhythm of Fv *SOC1* mRNA Expression.

Supplemental Figure 6. Localization of Fv *SOC1* mRNA in the Shoot Apex.

Supplemental Figure 7. The Expression of Fv *SOC1* in Fv *TFL1* Transgenic Lines.

Supplemental Figure 8. Phenotypes of Strong Fv *SOC1*-OX and RNAi Lines.

Supplemental Figure 9. The Expression of Fv *AP1* in Young Fv *SOC1*-RNAi Plants.

Supplemental Figure 10. The Expression of Fv *AGL24* and Fv *LFY* in Fv *SOC1*-RNAi Plants.

Supplemental Figure 11. The Expression of Fv *AGL24* and Fv *LFY* in Fv *SOC1* Transgenic Lines in the Hawaii-4 Background.

Supplemental Figure 12. Overexpression of Mutated Fv *TFL1* Does Not Affect Flowering Time in *Fragaria vesca*.

Supplemental Figure 13. Fv *SOC1* Regulates Vegetative Growth in *Fragaria vesca*.

Supplemental Figure 14. Overexpression of Fv *SOC1* Suppresses the Formation of Branch Crowns in *Fragaria vesca*.

Supplemental Figure 15. The Effect of Fv *SOC1* on the Expression of Gibberellin Pathway Genes in *Fragaria vesca*.

Supplemental Figure 16. The Effect of GA_3 Treatment on the Expression of Fv *TFL1*.

Supplemental Table 1. Relative Expression of Fv *SOC1* in Leaves of Fv *SOC1*-RNAi and OX Lines.

Supplemental Table 2. List of Primers Used in Real-Time PCR Reactions.

Supplemental Data Set 1. Alignments Used to Generate the Phylogeny in Supplemental Figure 1 Online.

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AUTHOR CONTRIBUTIONS

K.M., T.K., P.E., and T.H. designed the experiments. K.M., T.K., E.A.K., V.A.A., and T.H. performed the research. K.M. and T.H. wrote the article with input from all the authors.

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