One of the most important events during the life cycle of a plant is the transition from the vegetative to the reproductive state (Bernier et al. 2000). Appropriate timing so that this occurs during the most favorable conditions is crucial in agriculture and horticulture for maximizing reproductive success (Boss et al. 2004). Moreover, to maximize this reproductive success, it is important for flower structure during fertilization to be as complete and intact as possible so that pollination followed by fertilization occurs under the best conditions. In natural conditions in temperate areas, many predictable and unpredictable factors in the environment influence flowering time (Lang 1965; Bernier et al. 1981; Thomas and Vince-Prue 1997). The highly predictable factors include annual changes in day length, photoperiod, and the period of winter cold or vernalization. The ability to detect seasonal changes and to respond to them also confers a selective advantage to plants because it provides a means of anticipating and consequently preventing the adverse effects of a particular seasonal environment. The photoperiodic control of flowering time is tightly linked to the circadian clock and influences the expression of genes regulating the transition from vegetative to reproductive phase.

A genetic approach to investigating the mechanisms required to secure correct timing of the floral transition has mainly been focused on Arabidopsis thaliana. Considering the process of flowering as a default developmental program (Boss et al. 2004; Komeda 2004) that must be suppressed early in the life cycle of the plant, these previous studies divided floral pathways into those that enable the floral transition and those that promote it. Based on their model, the floral enabling pathway would regulate expression of floral repressors.

In Arabidopsis, two closely related MYB proteins with redundant functions, LATE ELONGATED HYPOCOTYL (LHY) and CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) (lhy;cca1) delay flowering time of Arabidopsis under continuous light (LL). Mutation in the SVP gene suppresses the late flowering phenotype of the lhy;cca1 under LL. Here, we describe isolation of three suppressors of late flowering and abnormal flower shape phenotypes of 35S:SVP plants as a first step to understanding molecular mechanisms of late flowering caused by lhy;cca1 and 35S:SVP in Arabidopsis under LL. Genetic analysis suggested that the suppressor phenotypes appeared to be caused by monogenic and recessive mutations.

**Key words:** Circadian rhythm, floral repressor, LHY/CCA1, photoperiodic flowering, SVP.

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**Abbreviations:** CCA1, CIRCADIAN CLOCK ASSOCIATED 1; CO, CONSTANS; FLC, FLOWERING LOCUS C; FT, FLOWERING LOCUS T; GI, GIGANTEA; LD, long day; LHY, LATE ELONGATED HYPOCOTYL; LL, continuous light; SD, short day; SVP, SHORT VEGETATIVE PHASE; TOC1, TIMING OF CAB EXPRESSION 1; TUB, TUBULIN; WT, wild type.

This article can be found at http://www.jspcmb.jp/
Suppressors of 35S:SVP in Arabidopsis

HYOCOTYL (LHY) and CIRCADIAN CLOCK ASSOCIATED 1 (CCA1), are essential components of the circadian clock, and both play important roles in photoperiodic flowering by controlling the rhythmic expression of flowering time genes (Carre and Kim 2002; Mizoguchi et al. 2002, 2005). In particular, LHY and CCA1 regulate a flowering pathway comprising the genes GI, CO, and FT in light/dark cycles such as long days (LD) and short days (SD; Mizoguchi et al. 2002, 2005; Mas 2005). FT gene expression is activated under LD mainly through a conserved pathway consisting of GI and CO (Mizoguchi et al. 2005).

We recently found a novel activity of the circadian clock proteins LHY and CCA1. The lhy;cca1 mutation delayed flowering time of Arabidopsis under continuous light (LL), but accelerated flowering under light/dark cycles such as LD and SD (Mizoguchi et al. 2002, 2005).

Our recent genetic studies indicated that two mutations, svp and fle, partially suppress the late-flowering phenotype of lhy;cca1 (Fujiwara et al. 2008). Based on these results, we proposed that both an internal biological clock and external rhythms are required for proper development of Arabidopsis. However, molecular mechanisms underlying the regulation of SVP by the clock and the precise roles of SVP in clock-controlled flowering in Arabidopsis remain unclear. In this work, EMS (Ethane Methyl Sulphonate) mutagenesis of Arabidopsis with SVP overexpression was performed to address the above questions. Suppressors of the late flowering and abnormal flower shape phenotypes were isolated, and three mutant lines were used for further characterization. Genetic analysis of these mutant lines indicates that suppression is caused by a monogenic and recessive mutation in all three lines.

Materials and methods

Plant materials and growth conditions

Arabidopsis thaliana accession Landsberg erecta (Ler) plants were used as the wild type (WT). Transgenic Arabidopsis overexpressing the SVP gene (35S:SVP) has been described previously (Fujiwara et al. 2008). Seeds were imbibed and cold treated at 4°C for 3 days in the dark before germination under light conditions. Plants were grown on soil in controlled environment rooms at 22°C. Light conditions were LD (16 h light/8 h dark), SD (10 h light/14 h dark), or LL (continuous white light) with a photon flux density of ca. 40 μmol m⁻² s⁻¹ (Mizoguchi et al. 2002).

Measurement of flowering time

Flowering time was scored by growing plants on soil under LD, SD, or LL and counting the number of rosette and cauline leaves on the main stem after bolting. Data are presented as means ± SE (n ≥ 11). Measurement of flowering time was performed at least twice, with similar results.

EMS mutagenesis and phenotypic screening for mutations that suppress late flowering and abnormal flower shape phenotypes of 35S:SVP under LL

Approximately 5,000 35S:SVP (Ler) seeds were mutagenized by imbibition in 0.3% EMS (Sigma Aldrich, St. Louis, MO) for 9 h followed by washing with 0.1 M Na₂SO₃ (twice) and distilled water for 30 min (five times). M₂ seeds were collected into pools, with each pool containing ca. 25 M₁ plants. Approximately 12,000 M₂ seeds representing ca. 500 M₁ plants after mutagenesis of 35S:SVP seeds were sown on soil and screened for plants with normal flowering time and flower shape under LL.

Genetic analysis

The suppressor mutations in the 35S:SVP background were backcrossed to the parental line 35S:SVP plants twice before phenotypic analysis.

Detection of SVP transgene by PCR

Standard PCR reactions were performed on genomic DNA to detect the presence of SVP transgene in the suppressor mutants #2, #4 and #5. PCR conditions and sequences of primers were: 92°C for 2 min, 94°C for 1 min, 58°C for 30 s, 72°C for 1 min, 72°C for 7 min, 35 cycles, primer F 5’-TCTAGGAGATCCATGCGAG-3’ and primer R 5’-TCTTTACTCATTGCGGTG-3’.

RT-PCR analysis of gene expression

Plants were sown as described above and grown on soil for 14 days. Aerial leaves were used for RNA preparation. RT-PCR was performed with 1 μg of total RNA using a SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA). cDNA was diluted to 100 μl with TE buffer, and 1 μl of diluted cDNA was used for PCR amplification by GoTaq Green Master Mix (Promega). Sequences of primers and PCR conditions (annealing temperatures and cycles) are as follows: SHORT VEGETATIVE PHASE (SVP) (5’-GAGGAGAACTTTCAAGGACT-3’, and 5’-CCATAGG-CAGAAACTTAC-3’, Tm: 58°C, cycles: 25) and TUBULIN (TUB) (5’-CACCATGGGAAGTAGGACG-3’ and 5’-GACTGTCTCAGGGTCCAG-3’, Tm: 58°C, cycles: 25). The PCR products were separated on 1.5% agarose gels. RT-PCR analyses were performed at least twice with independent RNA samples.

Results

EMS mutagenesis of 35S:SVP and screening of three suppressors of late flowering and abnormal flower shape phenotypes of 35S:SVP plants under LL

The 35S:SVP plants produced more rosette leaves before bolting than WT plants under LD and LL conditions (Fujiwara et al. 2008). Flower shape of the 35S:SVP is also affected by overexpression of the SVP gene (Brill and Watson 2004; Masiero et al. 2004; Liu et al. 2007).

To isolate novel genes that functionally interact with
SVP to control flowering time and flower shape, EMS mutagenesis was performed on 35S:SVP (Fujiwara et al. 2008) seeds, and M2 populations were screened for suppressors of late flowering and abnormal flower shape phenotypes of 35S:SVP (Figure 1). In total 12,000 M2 seedlings were screened for individuals that flowered earlier than 35S:SVP plants and had normal flower shapes similar to WT (Ler) plants under LL. Of 90 candidate mutants recovered in ten independent pools of M2 seedlings, suppression of the late-flowering phenotype of 35S:SVP was most significant in lines #2, 4, and 5. These three lines were isolated from three independent M2 pools and therefore considered as independent suppressor mutants. These three lines were used for further analysis (Figure 1).

**Suppression of late-flowering phenotypes of 35S:SVP in lines #2, 4, and 5**

The three lines #2, 4, and 5 were self-fertilized, and M3 progeny were confirmed to carry the 35S:SVP transgene by PCR (Figure 2A) and sequencing (data not shown); therefore, lines #2, 4, and 5 obtained from the M2 plants that produced significantly fewer leaves than the 35S:SVP were derived from the 35S:SVP. The M3 progeny exhibited an early-flowering phenotype similar to lines #2, 4, and 5 under LL (Figure 2B), indicating that the suppressor phenotypes were heritable. Overexpression of SVP in the suppressor #2 and #4 lines was not affected by the suppressor mutations (Figure 2C), suggesting that the mutations might be extragenic and suppress the late flowering of 35S:SVP without affecting the gene expression level of SVP. Level of SVP expression in the suppressor #5 was higher than wild type but lower than 35S:SVP plants.

**Suppression of abnormal flower shape phenotype of 35S:SVP in lines #2, 4, and 5**

35S:SVP plants showed not only the late-flowering phenotype but also abnormal flower shape (Figure 3). Petals of WT plants are white and longer than the sepals (Figure 3), while sepals and petals of the 35S:SVP plants were larger and much greener, respectively, than those of WT plants (Figure 3). The abnormal flower shape and late flowering of the 35S:SVP plants were suppressed in lines #2, 4, and 5 (Figures 2 and 3).

![Figure 1. Mutagenesis procedure used to isolate suppressors of late flowering and abnormal flower shape phenotypes of 35S:SVP plants. The progenitor line carried the 35S:SVP transgene. EMS was used for mutagenesis.](image-url)

![Figure 2. Flowering times and SVP expression levels of three suppressor lines (#4, #5, and #2), 35S:SVP, and WT (Ler) plants under LL. (A) Detection of the SVP transgene by PCR. (B) Flowering time was scored by counting the number of rosette (closed boxes) and cauline (open boxes) leaves on the main stem. Error bars represent SE (n=10). Each experiment was performed at least twice, with similar results. (C) Expression levels of SVP and TUBULIN (TUB) were analyzed by RT-PCR.](image-url)
To test whether suppressor mutations were recessive or dominant, lines #2, #4, and #5 were crossed with the 35S:SVP progenitor line. The F1 plants derived from these crosses flowered almost at the same time as 35S:SVP and flowered later than WT plants under LL (Figure 4). F2 progeny of each crossing between the three lines and the 35S:SVP were grown under LL, and their flowering times were scored and compared to those of three suppressor lines (M3) and 35S:SVP plants (Figure 5). The ratio between the late-flowering plants with abnormal flowers and other plants with normal flowers was close to 3 : 1 (p<0.05; Table 1). These results indicate that the suppressors behaved as monogenic recessive mutations in 35S:SVP to suppress the late-flowering phenotype under LL.

To test whether these suppressor mutations were intragenic or extragenic, lines #2, 4, and 5 were backcrossed with Ler WT plants. The F1 plants derived from these crosses flowered almost at the same time as 35S:SVP and flowered later than WT plants under LL (Figure 6). F2 progeny of each crossing between the three lines and the 35S:SVP were grown under SD, and their flowering times were scored and compared to those of three suppressor lines (M3), WT, and 35S:SVP plants (Figure 7). The presence of segregants with the late-flowering phenotype similar to 35S:SVP indicates that these three suppressor mutations were extragenic (Table 2). Plants that flowered earlier than the WT and did not carry the 35S:SVP transgene were candidates of single suppressor mutants and tentatively named suppressor of late flowering of SVP-overexpression 1-3 (sls1-3).

Table 1. Segregation of the flowering time phenotype in F2 progeny of suppressor #2, 4 and 5 back-crossed with 35S:SVP background.

<table>
<thead>
<tr>
<th>F2 progeny</th>
<th>Flowering time</th>
<th>χ²</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Late flowering</td>
<td>Early flowering</td>
<td></td>
</tr>
<tr>
<td>Supp#2×35S:SVP</td>
<td>57</td>
<td>12</td>
<td>2.2</td>
</tr>
<tr>
<td>Supp#4×35S:SVP</td>
<td>58</td>
<td>12</td>
<td>2.3</td>
</tr>
<tr>
<td>Supp#5×35S:SVP</td>
<td>49</td>
<td>19</td>
<td>0.3</td>
</tr>
</tbody>
</table>

Segregation data were evaluated with the Chi-square goodness-of-fit test by using 3:1 segregation of the late and early flowering phenotype as the null hypothesis. Chi-square values (χ²) and corresponding probabilities (p) are indicated (p>0.5).

Genetic analysis

To test whether suppressor mutations were recessive or dominant, lines #2, #4, and #5 were crossed with the 35S:SVP progenitor line. The F1 plants derived from these crosses flowered almost at the same time as 35S:SVP and flowered later than WT plants under LL (Figure 4). F2 progeny of each crossing between the three lines and the 35S:SVP were grown under LL, and their flowering times were scored and compared to those of three suppressor lines (M3) and 35S:SVP plants (Figure 5). The ratio between the late-flowering plants with abnormal flowers and other plants with normal flowers was close to 3 : 1 (p<0.05; Table 1). These results indicate that the suppressors behaved as monogenic recessive mutations in 35S:SVP to suppress the late-flowering phenotype under LL.

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Figure 5. Frequency of distribution of rosette leaf number at flowering. Data from Ler WT (A), 35S:SVP (B), line #2 (C), line #4 (D), line #5 (E), and plants from segregating populations (F₂) derived from crosses between the suppressor lines (M₃) and 35S:SVP (F–H) are shown. (F–H) F₂ populations showed a 3:1 ratio of plants that flowered similarly to the 35S:SVP and those that flowered significantly earlier than the 35S:SVP (p>0.05, Table 1). F₂ progeny with normal flowers surrounded by dotted lines in (F–H) were judged as early-flowering plants. Other plants with abnormal flowers similar to those of 35S:SVP plants were judged as late-flowering plants.
Suppressors of late flowering of 35S:SVP plants have not been fully elucidated. Identification of the genes for the suppressor mutations of the 35S:SVP and their characterization will be important to understand how the over-accumulation of SVP protein affects the conversion of floral organs or floral reversion as seen in 35S:SVP plants have not been fully elucidated.

**Discussion**

**SVP as a floral repressor**

The *SVP* gene encodes a transcription factor with a MADS-box domain and acts as a floral repressor. The *SVP* gene is highly expressed in young leaves and the shoot apical meristem, but its expression level is quite low in the inflorescence apical meristem (Hartmann et al. 2000). During flower development, *SVP* gene expression decreases to an undetectable level prior to the emergence of the sepals (Hartmann et al. 2000). The first report on isolation of an *svp* mutant demonstrated that it had an early-flowering phenotype without displaying other distinguishing features (Hartmann et al. 2000), and thus the *svp* mutation seemed to pass more rapidly through the vegetative development stage. Recently we found that double loss-of-function of two clock genes, *LHY* and *CCA1*, caused late flowering under LL (Fujiwara et al. 2008). We have also shown that *svp* and *flc* mutations suppress the late-flowering phenotype under LL (Fujiwara et al. 2008). We proposed that SVP and FLC may mediate between the circadian clock and flowering time regulation (Fujiwara et al. 2008). A tight connection between photoperiodic flowering and circadian clock function has been shown previously (Suarez-Lopez et al. 2001; Mas 2005). A possible function of SVP in photoperiodic flowering has been proposed based on genetic analysis (Scortecci et al. 2003). Isolation of the *svp* mutation as one suppressor of the late-flowering phenotype of the clock mutant, *lhy;cca1*, under LL, and suppression of downregulation of the floral activator genes, *FT* and *SOC1*, support the suggestion that SVP might be a key regulator in the photoperiodic flowering pathway (Fujiwara et al. 2008).

The precise mechanisms underlying the negative regulation of flowering in *lhy;cca1* under LL are still not clear, because *lhy;cca1* mutations did not greatly affect the mRNA levels of *SVP* or *FLC*, and we did not detect protein–protein interactions between LHY/CCA1 and SVP or FLC (Fujiwara et al. 2008). Therefore, how SVP and FLC delay flowering more strongly in *lhy;cca1* mutants than WT plants under LL is unknown. To find a missing link between LHY/CCA1 and SVP/FLC, screening for more mutations that cause *lhy;cca1* to flower earlier than WT plants under LL are currently underway.

Suppressors of late flowering of 35S:SVP plants have been isolated in this work. SVP protein suppressed the *FT* expression via direct binding to the *FT* gene (Lee et al. 2007). Therefore, suppressors identified here may include regulators of SVP protein.

**SVP as a modulator of flower meristem identity**

Besides its role in controlling flowering time, the *SVP* gene also functions as a modulator of meristem identity. Ectopic expression of the *SVP* gene inhibits floral meristem identity in *Arabidopsis*, causing floral abnormalities such as the conversion of sepals and petals to leaf-like structures (Brill and Watson 2004; Masiero et al. 2004) and floral reversion (Tooke et al. 2005) through the production of inflorescence-like structures within the flowers (Brill and Watson 2004).

Protein–protein interactions between SVP and other MADS-box proteins have been reported (De Folter et al. 2005). Genetic studies have shown that *svp* mutation suppressed the late flowering caused by overexpression of the *FLM/MAF1* gene, and that *svp;flm* double mutants behaved like single mutants (Scortecci et al. 2003). These results indicate that FLM/MAF1 and SVP appear to function in a single genetic pathway, which interacts with the photoperiodic pathway. Therefore, the *flm/maf1* mutation is a candidate for the suppressor mutation of 35S:SVP.

However, precise mechanisms underlying the conversion of floral organs or floral reversion as seen in 35S:SVP plants have not been fully elucidated. Identification of the genes for the suppressor mutations of the 35S:SVP and their characterization will be important to understand how the over-accumulation of SVP protein affects the conversion of floral organs or
Figure 7. Frequency of distribution of total leaf number at flowering. Data from Ler WT (A), 35S:SVP (B), line #2 (C), line #4 (D), line #5 (E), and plants from segregating populations (F$_2$) derived from crosses between the suppressor lines (M$_3$) and Ler (F–H) are shown. (F–H) F$_2$ populations showed a 9 : 7 ratio of plants that flowered similarly to the 35S:SVP and those that flowered significantly earlier than the 35S:SVP ($p$ < 0.05, Table 2). F$_2$ progeny with normal flowers surrounded by dotted lines in (F–H) were judged as early-flowering plants. Other plants with abnormal flowers similar to those of 35S:SVP plants were surrounded by solid lines and judged as late-flowering plants.
suppressors of 35S: SVP in Arabidopsis

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