

Isolation of suppressors of late flowering and abnormal flower shape phenotypes caused by overexpression of the *SHORT VEGETATIVE PHASE* gene in *Arabidopsis thaliana*

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Abstract *SHORT VEGETATIVE PHASE* (*SVP*) encodes a MADS-box transcription factor and function as a floral repressor in *Arabidopsis*. Mutations in two clock genes *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.

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 floral activators, GIGANTEA (GI) and CONSTANS (CO), play key roles in photoperiodic flowering (Fowler et al. 1999; Park et al. 1999), and it has been proposed that GI plays dual roles, namely in regulating period length and circadian phase and in promoting the expression of the circadian clock output pathway that includes CO and FLOWERING LOCUS T (FT) to promote flowering (Mizoguchi et al. 2005). *FLOWERING LOCUS C* (*FLC*) and *SHORT VEGETATIVE PHASE* (*SVP*) encode MADS-box proteins. Both *FLC* and *SVP* negatively regulate the transition, but *FLC* is considered the more central regulator of the flowering enabling pathway (Michaels and Amasino 1999; Hartmann et al. 2000).

In *Arabidopsis*, two closely related MYB proteins with redundant functions, LATE ELONGATED

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.

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HYPOCOTYL (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 *flc*, 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 Sulfonate) 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 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (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 \pm SE ($n \geq 11$). 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 Na_2SO_3 (twice) and distilled water for 30 min (five times). M_2 seeds were collected into pools, with each pool containing ca. 25 M_1 plants. Approximately 12,000 M_2 seeds representing ca. 500 M_1 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'-TCTAGAGGA-TCCATGGCGAG-3' and primer R 5'-TCTTTACTCATTC-GGGCGTG-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'-GGAGAGGAAGTCAAGGACT-3', and 5'-CCATAGG-CAGAACTTACAC-3', Tm: 58°C, cycles: 25) and *TUBULIN* (*TUB*) (5'-CACCATGGAAGAAGTGAAGACG-3' and 5'-GACTGTCTCCAAGGGTTCCAG-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),

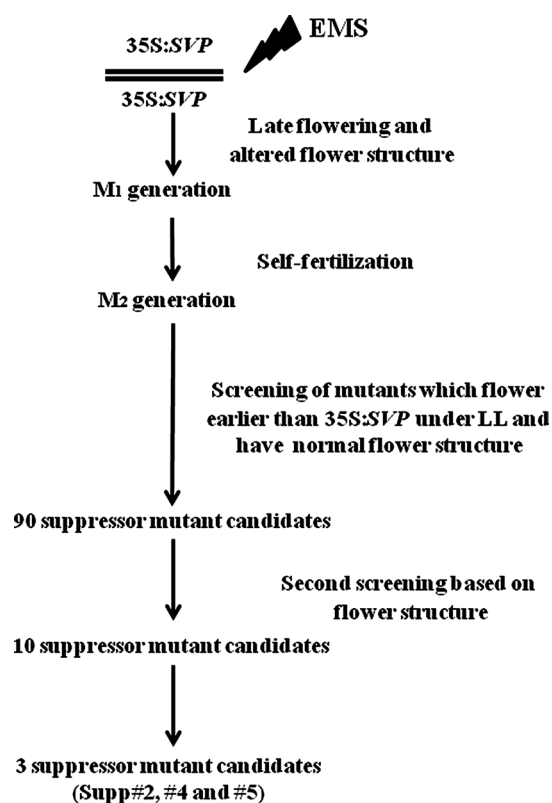


Figure 1. Mutagenesis procedure used to isolate suppressors of late flowering and abnormal flower shape phenotypes of 35S:*SVP* plants. The progenitor line carried the 35S:*SVP* transgene. EMS was used for mutagenesis.

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).

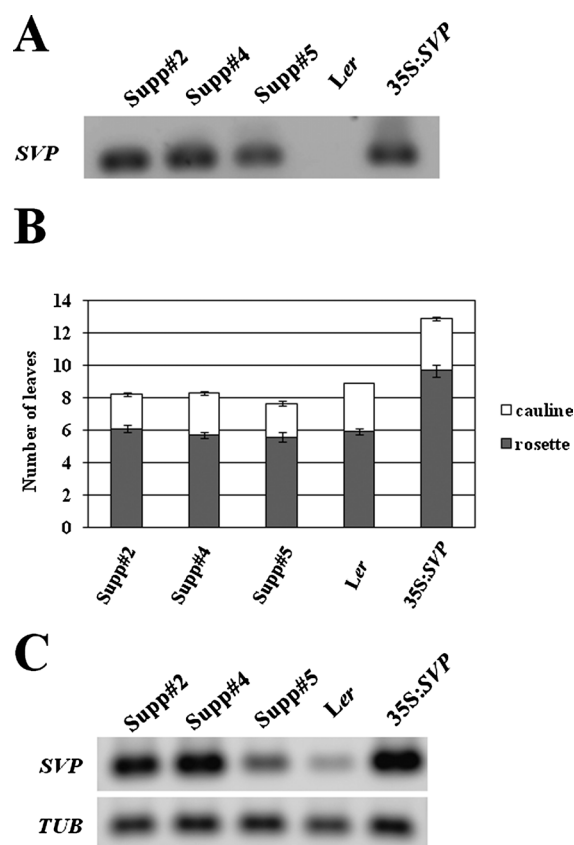


Figure 2. Flowering times and *SVP* expression levels of three suppressor lines (#4, #5, and #2), 35S:*SVP*, and WT (*Ler*) plants under LL. (A) Detection of the *SVP* transgene by PCR. (B) Flowering time was scored by counting the number of rosette (closed boxes) and cauline (open boxes) leaves on the 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.

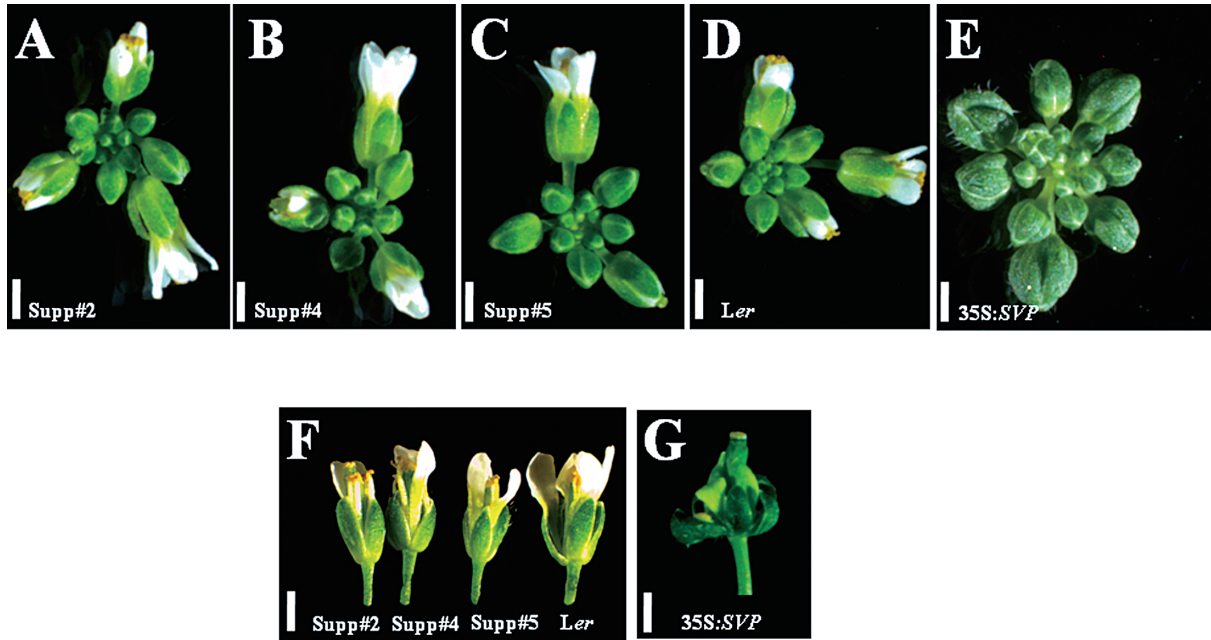


Figure 3. Suppression of the abnormal flower shape of 35S:SVP in the suppressor lines #2, #4 and #5. Close-up of inflorescences and flowers of suppressor lines #2 (A, F), #4 (B, F), #5 (C, F), *Ler* (WT; D, F) and 35S:SVP (E, G) plants grown under LL for 7 weeks. Bars represent 2 mm.

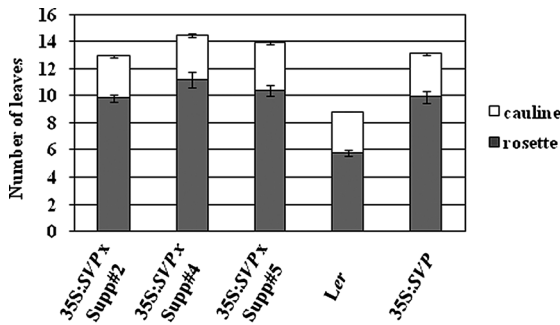


Figure 4. Flowering times of the F₁ plants derived from crosses between three suppressor lines and 35S:SVP. Plants were grown under LL, and flowering time was scored by counting the number of rosette (closed boxes) and cauline (open boxes) leaves on the main stem. Error bars represent SE ($n \geq 10$). Each experiment was performed at least twice, with similar results.

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

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

F ₂ progeny	Flowering time		χ^2	<i>P</i>
	Late flowering	Early flowering		
Supp#2 × 35S:SVP	57	12	2.2	>0.1
Supp#4 × 35S:SVP	58	12	2.3	>0.1
Supp#5 × 35S:SVP	49	19	0.3	>0.5

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 (χ^2) and corresponding probabilities (*p*) are indicated ($p > 0.5$).

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*).

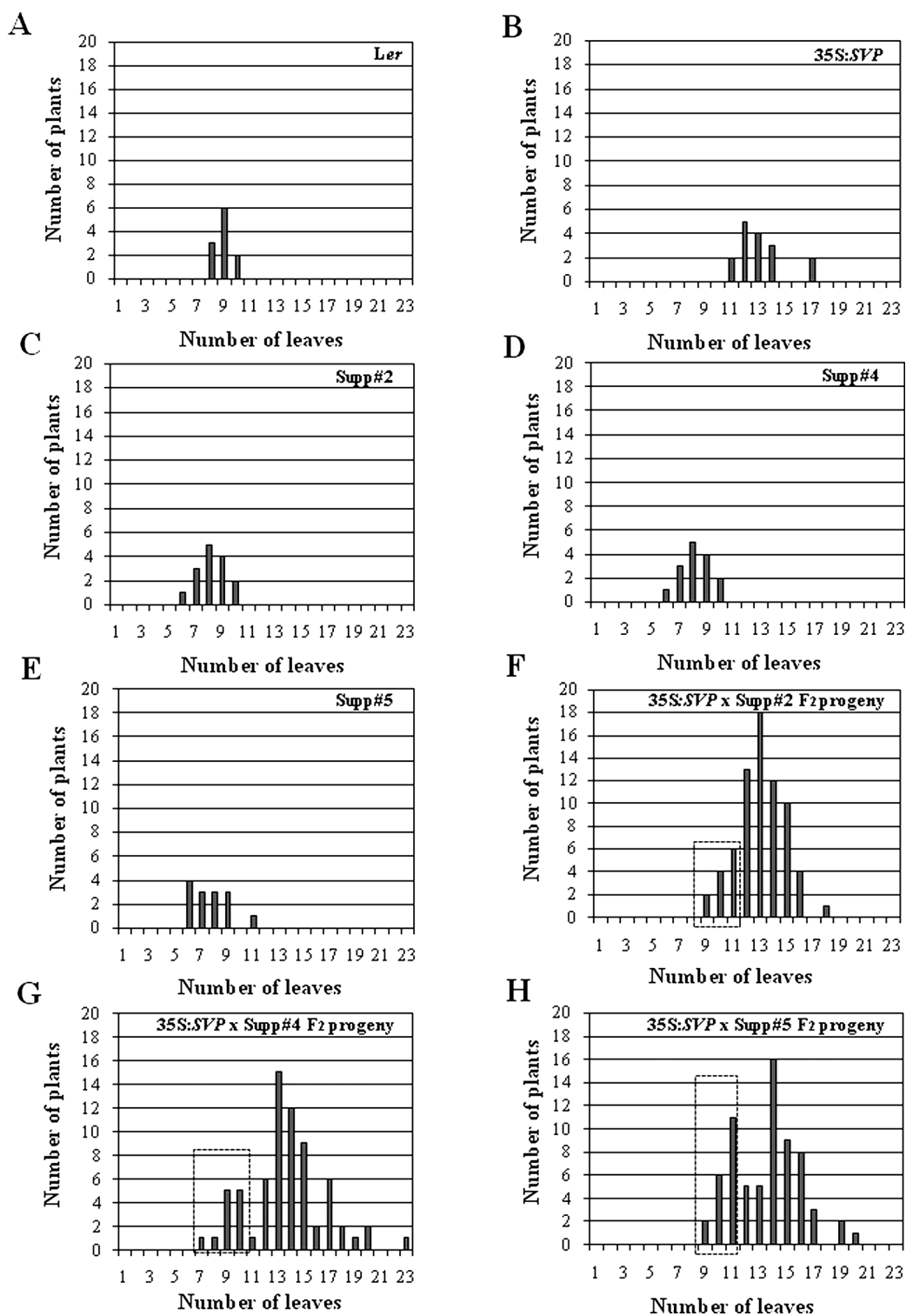


Figure 5. Frequency of distribution of rosette leaf number at flowering. Data from *Ler* WT (A), *35S:SVP* (B), line #2 (C), line #4 (D), line #5 (E), and plants from segregating populations (F₂) derived from crosses between the suppressor lines (M₃) and *35S:SVP* (F–H) are shown. (F–H) F₂ populations 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.

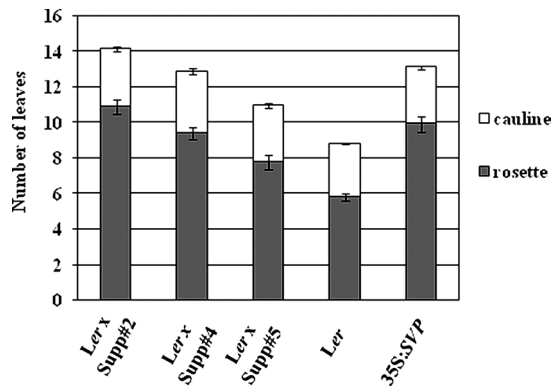


Figure 6. Flowering times of the F_1 plants derived from crosses between three suppressor lines and *Ler* (WT). Plants were grown under LL, and flowering time was scored by counting the number of rosette (closed boxes) and cauline (open boxes) leaves on the main stem after bolting. Error bars represent SE ($n \geq 10$). Each experiment was performed at least twice, with similar results.

Table 2. Segregation of the flowering time phenotype in F_2 progeny of Suppressor #2, 4 and 5 back-crossed with *Ler* background under SD conditions.

F_2 progeny	Flowering time		χ^2	p
	Late flowering	Early flowering		
Supp#2 \times <i>Ler</i>	100	81	0.074	>0.7
Supp#4 \times <i>Ler</i>	91	78	0.397	>0.5
Supp#5 \times <i>Ler</i>	121	92	0.027	>0.8

Segregation data were evaluated with the Chi-square goodness-of-fit test by using 9 : 7 segregation of the late and early flowering phenotype as the null hypothesis. Chi-square values (χ^2) and corresponding probabilities (p) are indicated ($p > 0.5$).

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* mutant 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

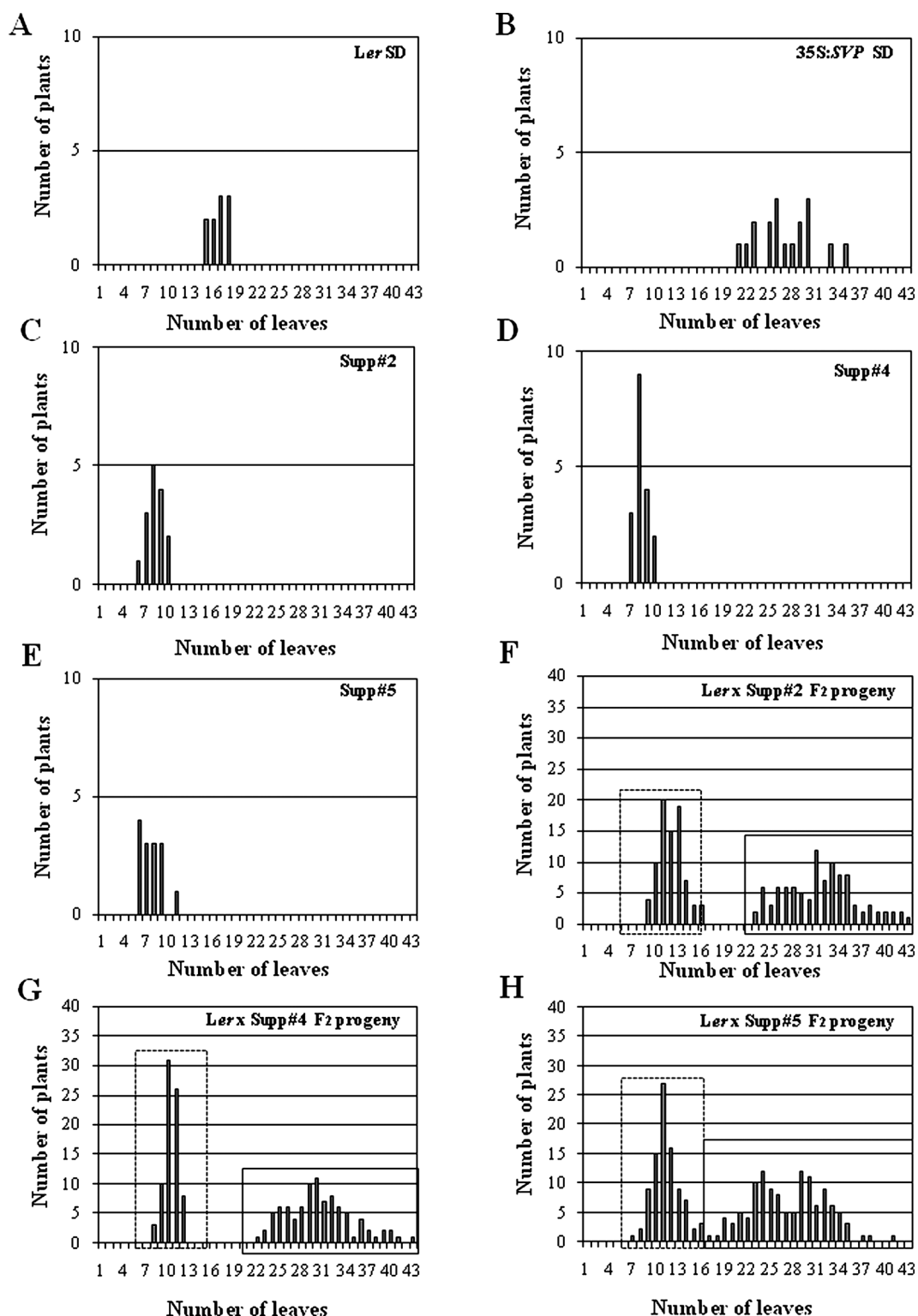


Figure 7. Frequency of distribution of total leaf number at flowering. Data from *Ler* WT (A), *35S:SVP* (B), line #2 (C), line #4 (D), line #5 (E), and plants from segregating populations (F₂) derived from crosses between the suppressor lines (M₃) and *Ler* (F–H) are shown. (F–H) F₂ populations showed 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). 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 surrounded by solid lines and judged as late-flowering plants.

floral reversion.

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