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Suppression of late-flowering and semi-dwarf phenotypes in the arabidopsis clock mutant *lhy-12;cca1-101* by *phyB* under continuous light

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Abbreviations: CCA1, CIRCADIAN CLOCK ASSOCIATED 1; CO, CONSTANS; GI, GIGANTEA; ELF3, EARLY FLOWERING 3; FLC, FLOWERING LOCUS C; FT, FLOWERING LOCUS T; LHY, LATE ELONGATED HYPOCOTYL; LD, long-day; LL, continuous light; PHYB, PHYTOCHROME B; SD, short-day; SOC1, SUPPRESSOR OF CO OVEREXPRESSION 1; SVP, SHORT VEGETATIVE PHASE

Photoperiodic flowering in Arabidopsis is controlled not only by floral activators such as GI, CO and FT, but also by repressors such as SVP and FLC. Double mutations in *LHY* and *CCA1* (*lhy;cca1*) accelerated flowering under short days, mainly by the GI-CO dependent pathway. In contrast, *lhy;cca1* showed delayed flowering under continuous light (LL), probably due to the GI-CO independent pathway. This late-flowering phenotype was suppressed by *svp*, *flc* and *elf3*. However, how SVP, FLC and ELF3 mediate LHY/CCA1 and flowering time is not fully understood. We found that *lhy;cca1* exhibited short hypocotyls and petioles under LL, but the molecular mechanism for these effects has not been elucidated.

To address these questions, we performed a screen for mutations that suppress either or both of the *lhy;cca1* phenotypes under LL, using two different approaches. We identified two novel mutations, a dominant (*del1*) and a recessive (*phyB-2511*) allele of *phyB*. The flowering times of single mutants of three *phyB* alleles, *hy3-1*, *del1* and *phyB-2511*, are almost the same and earlier than those of wild-type plants. A similar level of acceleration of flowering time was observed in all three *phyB* mutants tested when combined with the late-flowering mutations *co-2* and *SVPox*. However, the effect of *phyB-2511* on *lhy;cca1* was different from those by *hy3-1* or *del1*. *svp-3* did not strongly enhance the early-flowering phenotypes of *phyB-2511* or *del1*. These results suggest that light signaling via PhyB may affect factors downstream of the clock proteins, controlling flowering time and organ elongation. *phyB* mutations with different levels of effects on *lhy;cca1*-dependent late flowering would be useful to determine a specific role for *PHYB* in the flowering pathway controlled by *lhy;cca1* under LL.

Introduction

In many prokaryotic and eukaryotic organisms, biological clocks mediate the responses of several physiological and molecular processes to diurnal changes in environmental conditions such as light quality and quantity, temperature and humidity. Circadian rhythms persist with a period close to 24 h in the absence of any environmental time cue, and are generated by an endogenous timing mechanism. The basic principles of circadian clocks have been addressed for many organisms, including cyanobacteria, Neurospora, Arabidopsis, mice and human. The clock consists of oscillating molecules that form a negative auto-regulatory feedback loop.[1,2]

In Arabidopsis, the oscillatory molecules thought to compose the clock include the single-MYB transcription factors LATE ELONGATED HYPOCOTYL (LHY) and CIRCADIAN CLOCK ASSOCIATED 1 (CCA1), as well as five PSEUDO RESPONSE REGULATOR (PRR) proteins, PRR1 (also known as TOC1), PRR3, PRR5, PRR7 and PRR9.[3-5] *LHY* and *CCA1* have high sequence similarity, and the transcript and protein levels of both genes cycle with peak expression at dawn.[6-8] Overexpression of either gene abolishes the circadian rhythms of gene expression and leaf movement. In addition, plants overexpressing *LHY* or *CCA1* exhibit long hypocotyls and delayed

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flowering, whereas loss-of-function alleles of either gene cause a shortening of the free-running period (FRP) and early flowering.[4,5,7-10] Furthermore, LHY and CCA1 can suppress their own expression, suggesting that both genes act very close to the oscillator.[7,8] TOC1 contains a domain similar to the receiver domain of two-component response regulators, but lacks the residues essential for the function of typical response regulators.[11,12] In addition, the carboxyl terminus of TOC1 contains a domain implicated in nuclear localization that is also found in the CONSTANS protein, a regulator of flowering time. *TOC1* mRNA abundance peaks in the evening, and mutations in the gene cause a short-period phenotype that is independent of light.[11,12] The *TOC1* transcript level is constant and low in lines overexpressing *LHY* or *CCA1*, which indicates possible transcriptional repression by LHY and CCA1 in a direct or indirect manner.[3]

The genes GIGANTEA (GI), CONSTANS (CO) and FLOWERING LOCUS T (FT) promote flowering in the photoperiodic pathway. Under short days (SD), mutations in the circadian clock genes *LHY* and *CCA1* (*lhy;cca1*) cause misexpression of *GI*, impaired control of circadian and diurnal rhythms, and accelerated flowering through the abundance of *CO* and *FT* mRNAs.[10] In contrast, *lhy;cca1* delays the flowering of Arabidopsis under continuous light (LL).[13,14] Mutations in the *SHORT VEGETATIVE PHASE* (*SVP*), *FLOWERING LOCUS C* (*FLC*) or *EARLY FLOWERING 3* (*ELF3*) genes suppress the late-flowering phenotype of *lhy;cca1* under LL. However, it is still not clear how *lhy;cca1* delays the flowering of Arabidopsis under LL and why even a short dark period can overcome the late-flowering phenotype. Finding the missing link between the light signal and proteins such as LHY, CCA1, ELF3, SVP and FLC is important for understanding how SVP and FLC delay flowering more strongly in *lhy;cca1* plants than in wild-type plants under LL. To this end, we screened for suppressor mutations other than *svp*, *flc* and *elf3* that show the late-flowering and semi-dwarf phenotypes of *lhy;cca1* under LL.

Although there is likely an important step controlled by light that switches flowering time in *lhy;cca1*, we had no direct experimental evidence for it. In Arabidopsis, five phytochromes (phyA-E), two cryptochromes (cry1 and cry2), two phototropins (phot1 and phot2) and three members of the ZEITLUPE (ZTL) family (ZTL, FKF1 and LKP2) have been shown or suggested to function as photoreceptors or light-sensing proteins.[15-17]

Here, we report the identification of the *dominant early flowering and long hypocotyl 1* (*del1*) mutant, which shows early-flowering and long-hypocotyl phenotypes under SD, and is a novel strong allele of *phyB*. Another novel allele of *phyB*, *phyB-2511*, was isolated as a suppressor of the semi-dwarf phenotype of *lhy;cca1* under LL. The expression of clock-component genes such as *LHY*, *CCA1*, *TOC1* and *PRR9* was not significantly affected by the *del1* mutation under either LL or LD. All of the mutations of three *phyB* alleles, *hy3-1*, *del1* and *phyB-2511,* suppressed the short-hypocotyl phenotype of *lhy;cca1* under LL. All three *phyB* mutations partially suppressed the late-flowering phenotype of *co* and *SVPox* plants. However, the suppression of the late-flowering phenotype of *lhy;cca1* by *phyB-2511* was much weaker than that caused by *del1* or *hy3-1*, suggesting that phyB negatively regulates one of the LHY/CCA1-dependent controls of *FT* expression that differ from the GI-CO pathway and the ELF3-SVP pathway. Furthermore, *svp;phyB* double-mutant plants did not flower earlier than *svp* or *phyB* plants. Based on these results, we propose a model in which the phyB-mediated process plays an important role downstream of LHY and CCA1 to control flowering time and organ elongation under LL.

Results

Screen for an arabidopsis mutant with both early-flowering and long-hypocotyl phenotypes under short days. To identify mutations that affect both photoperiodic flowering and organ elongation responses and functionally interact with the clock proteins LHY and CCA1, M_2 populations of EMS-mutagenized L*er* seeds were screened for plants with early-flowering and long-hypocotyl phenotypes under SD.

Of five candidate mutants recovered in four independent pools of M_2 seedlings, one mutant line with the most severe phenotype was named *del1* and studied further. The M_2 plant was self-fertilized, and both the early-flowering (**Fig. 1A**) and long-hypocotyl (**Fig. 1B**) phenotypes of the M_3 progeny were confirmed under SD. These results indicate that the mutant phenotypes of *del1* are heritable.

Circadian and diurnal rhythms of clock gene expression in *del1* mutant plants. To determine whether the phenotypes of *del1* are caused by a disruption of circadian rhythms, we examined the expression patterns of genes encoding clock components under LL conditions. The expression patterns (e.g., amplitude, phase and period) of *CCA1* and *PRR9* in *del1* were similar to those in wild-type plants under LL (**Fig. 2A and B**).

To investigate whether expression of the clock genes *LHY* and *TOC1* was entrained in light-dark cycles, we assessed their expression patterns under LD. The expression patterns of both *LHY* and *TOC1* in *del1* were similar to those in wild-type plants (**Fig. 2C and D**). These results suggest that the early-flowering and long-hypocotyl phenotypes of *del1* are not due to changes in expression patterns of key clock components. The *del1* mutation might affect flowering time and organ elongation via factors downstream of the circadian clock, if the circadian clock and DEL1 function in a common genetic pathway.

***del1* as a novel allele of *phyB*.** To map and identify the *del1* mutation and to test whether it is recessive or dominant, a mutant plant of the M_3 progeny (L*er*) was crossed with Columbia (Col) wild type. The F_1 plants derived from this cross flowered earlier than the wild type and later than *del1* plants under SD (**Fig. 3A–C**). The hypocotyl length of the F_1 plants was also intermediate between those of wild-type and *del1* plants under SD (**Fig. 3E–G**). These results suggest that *del1* behaves as a semi-dominant mutation.

F_2 progeny of the cross between *del1* and Col wild type were grown under SD, and their flowering times were scored and compared to those of *del1* (M_3) and Col wild-type plants (**Fig. 3D**). The hypocotyl lengths of the F_2 progeny were also measured and compared to those of *del1* (M_3) and Col wild-type plants (**Fig. 3H**). The *del1* mutation was mapped to the upper arm of

chromosome 2 based on early-flowering and long-hypocotyl phenotypes similar to those of *del1* under SD. This result was confirmed by a different mapping strategy based on late-flowering and short-hypocotyl phenotypes similar to those of wild-type plants under SD. *PHYB* and *ELF3* loci are located in this region. Both *phyB* and *elf3* mutant plants show early-flowering and long-hypocotyl phenotypes similar to those of *del1*.[18,19] Although *elf3* mutations alter both circadian and diurnal rhythms of clock gene expression, *phyB* mutations do not strongly affect these expression patterns.[20,21] Therefore, we sequenced the *PHYB* gene of *del1*, and found a point mutation (C1078 to T) that causes a premature stop codon and truncates the PHYB protein (Q360 to stop) (**Fig. 4A**).

Screen for a suppressor mutant of the semi-dwarf phenotype of *lhy;cca1* under LL. We recently demonstrated that *lhy-12;cca1-101* plants show a late-flowering phenotype under LL,[13,14] but flower earlier than the wild type under LD and SD.[5,10] We identified three suppressor mutations, *svp*, *flc* and *elf3*, based on flowering time under LL. Furthermore, *lhy-12;cca1-101* plants have a semi-dwarf phenotype under LL.[13,14] The *svp* and *flc* mutations each suppress not only late flowering but also the semi-dwarf phenotype (Niinuma K and Mizoguchi T, unpublished). However, the effect of suppression on the semi-dwarf phenotype is weaker than that on flowering time (Niinuma K and Mizoguchi T, unpublished). Therefore, genetic screening was performed for suppressors of the short-hypocotyl and short-petiole phenotypes of *lhy-12;cca1-101* using EMS-mutagenized *lhy-12;cca1-101* seeds under LL. A total of 76 suppressor candidates were identified and named *lhy;cca1* EMS-mutagenized (*lce*). One mutant line with the longest hypocotyl length, *lce2511*, was subjected to further analysis (**Fig. 4B and C**).

To separate the suppressor mutation from the *lhy-12;cca1-101* mutations, *lce2511* was back-crossed with wild type (L*er*). We obtained segregants without the *lhy;cca1* mutations but with early-flowering and long-hypocotyl/petiole phenotypes similar to those of *phyB* mutants. Then we sequenced the *PHYB* gene of *lce2511* and found a point mutation (G851 to A) in the first exon. This mutation causes an amino acid substitution from G_{284} to E; the allele was named *phyB-2511* (**Fig. 4A**). To test whether the *phyB-2511* mutation was responsible for the long-hypocotyl and early-flowering phenotypes, a complementation test between *phyB-2511* and the *phyB* mutant *hy3-1* was performed. F_1 plants obtained from crosses between *phyB-2511* and *hy3-1* showed phenotypes similar to those of *phyB-2511* and *hy3-1* under LL. The controls, F_1 plants obtained from crosses between wild type (L*er*) and *phyB-2511* or *hy3-1*, showed phenotypes similar to those of wild type (L*er*) under LL (**Fig. 4D and E**). These results indicate that the long-hypocotyl/-petiole and early-flowering mutations present in the *lce2511* line are indeed *phyB* mutant alleles.

The effect of this amino acid substitution of *phyB-2511* on *PHYB* expression was examined. The expression of *PHYB* in *lhy-12;cca1-101* and *lce2511* (*lhy-12;cca-101;phyB-2511*) was compared by RT-PCR. The results suggest that the mutant phenotypes of *phyB-2511* are caused by the amino acid substitution ($G_{284}E$), not a decrease in the *PHYB* mRNA level in *lce2511* (**Fig. 4F**).

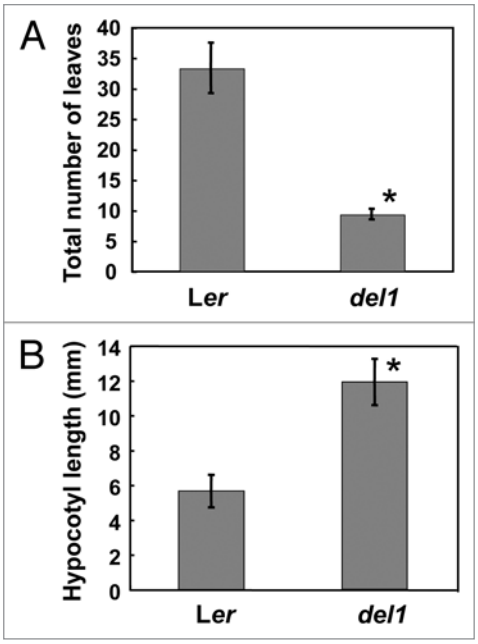

Figure 1. Early-flowering and long-hypocotyl phenotypes of *del1* under SD. Flowering time (A) and hypocotyl length (B) of wild-type (L*er*) and *del1* plants under SD. (A) The number of total leaves at the time of flowering was scored, and the data are presented as mean ± SE (n = 8). (B) Means ± SE (n = 10) of the hypocotyl lengths of wild type (L*er*) and *del1* grown under SD for 14 days. Asterisks (*) represent statistical significance compared to values of the wild type (L*er*) (Student's t test, $p < 0.05$).

Suppression of the short-hypocotyl phenotype of *lhy;cca1* by *phyB-2511* under LL. Recently, we demonstrated that *lhy-12;cca1-101* plants have a semi-dwarf phenotype under LL.[13] The mutants *svp*, *flc* and *elf3* show a partially suppressed semi-dwarf phenotype of *lhy;cca1* (Niinuma K and Mizoguchi T, unpublished). However, the molecular mechanisms underlying the regulation have not been elucidated. To investigate the relationship between *phyB* mutations and the short hypocotyls of *lhy;cca1* plants, three *phyB* alleles, *hy3-1*, *del1* and *phyB-2511*, were used. Wild type (L*er*), *lhy-12;cca1-101*, and three triple-mutant lines, *lhy-12;cca1-101;hy3-1*, *lhy-12;cca1-101;del1* and *lhy-12;cca1-101;phyB-2511* were grown under LL and the hypocotyl lengths were observed (**Fig. 4G**). The short-hypocotyl phenotype of *lhy;cca1* was suppressed by all three *phyB* mutations. These results indicate that *phyB* is involved at least in part in the control of these developmental processes, along with LHY and CCA1. The suppression of the elongated-organ phenotype of *lhy;cca1* by *phyB-2511* was much weaker than the suppression caused by *hy3-1* or *del1* (**Fig. 4G**).

The suppression of the late-flowering phenotype of *lhy;cca1* by *phyB-2511* is much weaker than that of *del1* and *hy3-1* under LL. To investigate whether the *phyB* mutation affects the late-flowering phenotype of *lhy;cca1* under LL, three *phyB* alleles, *hy3-1*, *del1* and *phyB-2511*, and three triple-mutant lines, *lhy-12;cca1-101;hy3-1*, *lhy-12;cca1-101;del1* and *lhy-12;cca1-101;phyB-2511* were grown under LL and their flowering times were compared (**Fig. 5A**). The three *phyB* mutant plants

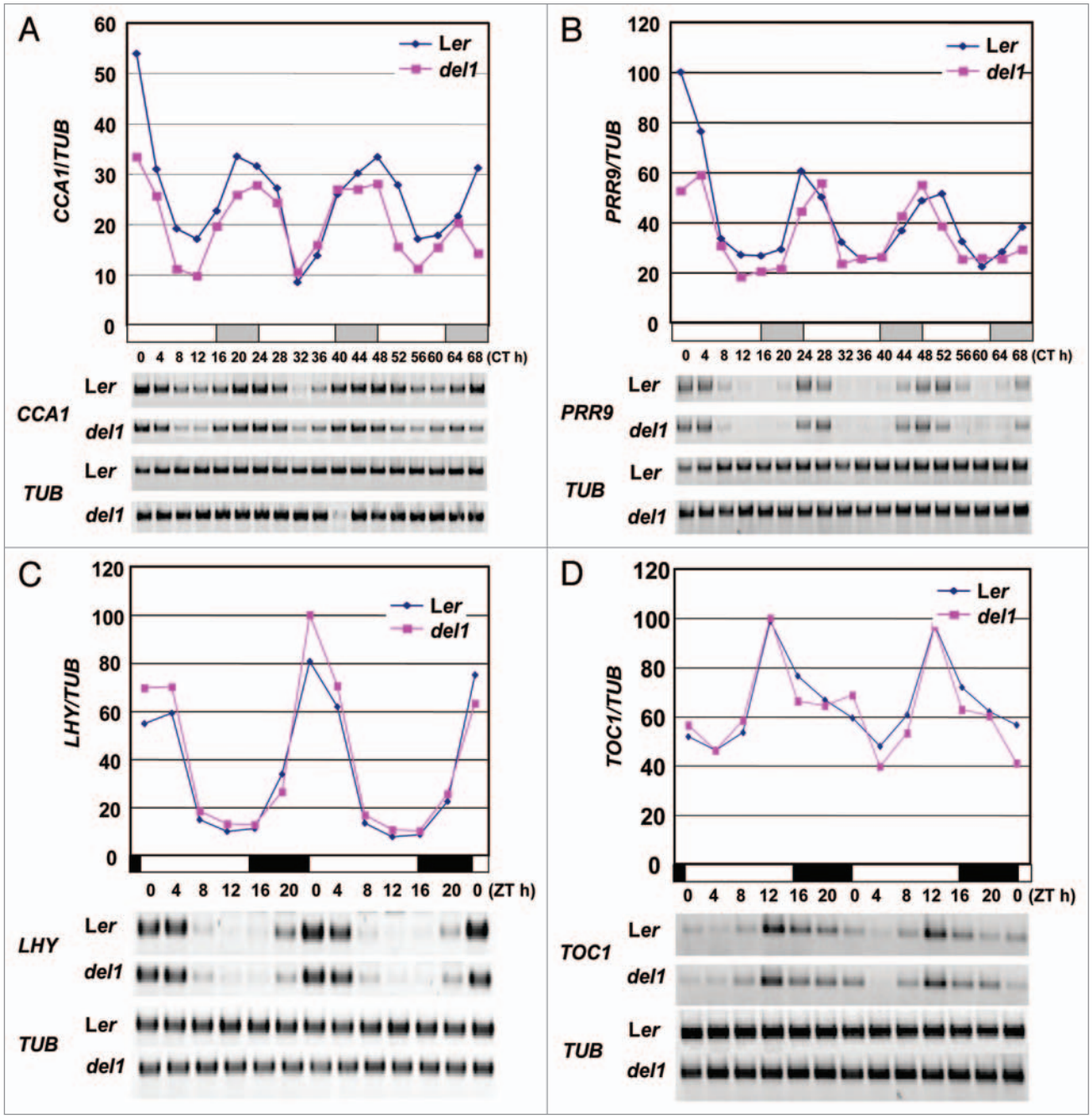

Figure 2. Analysis of *CCA1* and *PRR9* mRNA levels in wild type (L*er*) and *del1* grown under LL, and diurnal expression of the clock genes *LHY* and *TOC1* under light/dark cycles in wild-type (L*er*) and *del*, or *LHY* (C) and *TOC1* (D) under LD (16 h light/8 h dark) cycles. Abundance of *CCA1* (A) and *PRR9* (B) mRNA in wild type (L*er*) and *del1*. The plants were entrained to LD cycles (16 h light/8 h dark) for 8 days and transferred to LL (A and B). The plants were grown under LD cycles for 8 days (C and D). The analysis was performed just before the transfer to LL (circadian time; CT). White and black boxes along the horizontal axis represent light and dark periods, respectively. Zeitgeber Time (ZT) 0 indicates the time point just before lights were turned on. Blue and pink lines represent wild type (L*er*) and *del1*, respectively. Quantification was performed as described in the Materials and Methods section.

exhibited a similar extent of the early-flowering phenotype under LL. *del1* and *hy3-1* suppressed the late-flowering phenotype of *lhy-12;cca1-101* under LL, and both *lhy-12;cca1-101;hy3-1* and *lhy-12;cca1-101;del1* produced fewer leaves than wild-type plants under LL. However, the suppression by *phyB-2511* was much weaker than that by *hy3-1* or *del1* (**Fig. 5A**). These results suggest that the acceleration of flowering time by *phyB-2511*, with the $G_{284}E$ substitution, is different from those of the other two alleles, *hy3-1* and *del1*, in the *lhy;cca1* background under LL.

The late-flowering phenotype of *co-2* is partially suppressed by *phyB* mutations under LL. *CO* was the first gene identified as a key regulator of the photoperiodic control of flowering time. Since then, many studies have demonstrated how CO acts as a central molecule in controlling flowering under different photoperiodic conditions.

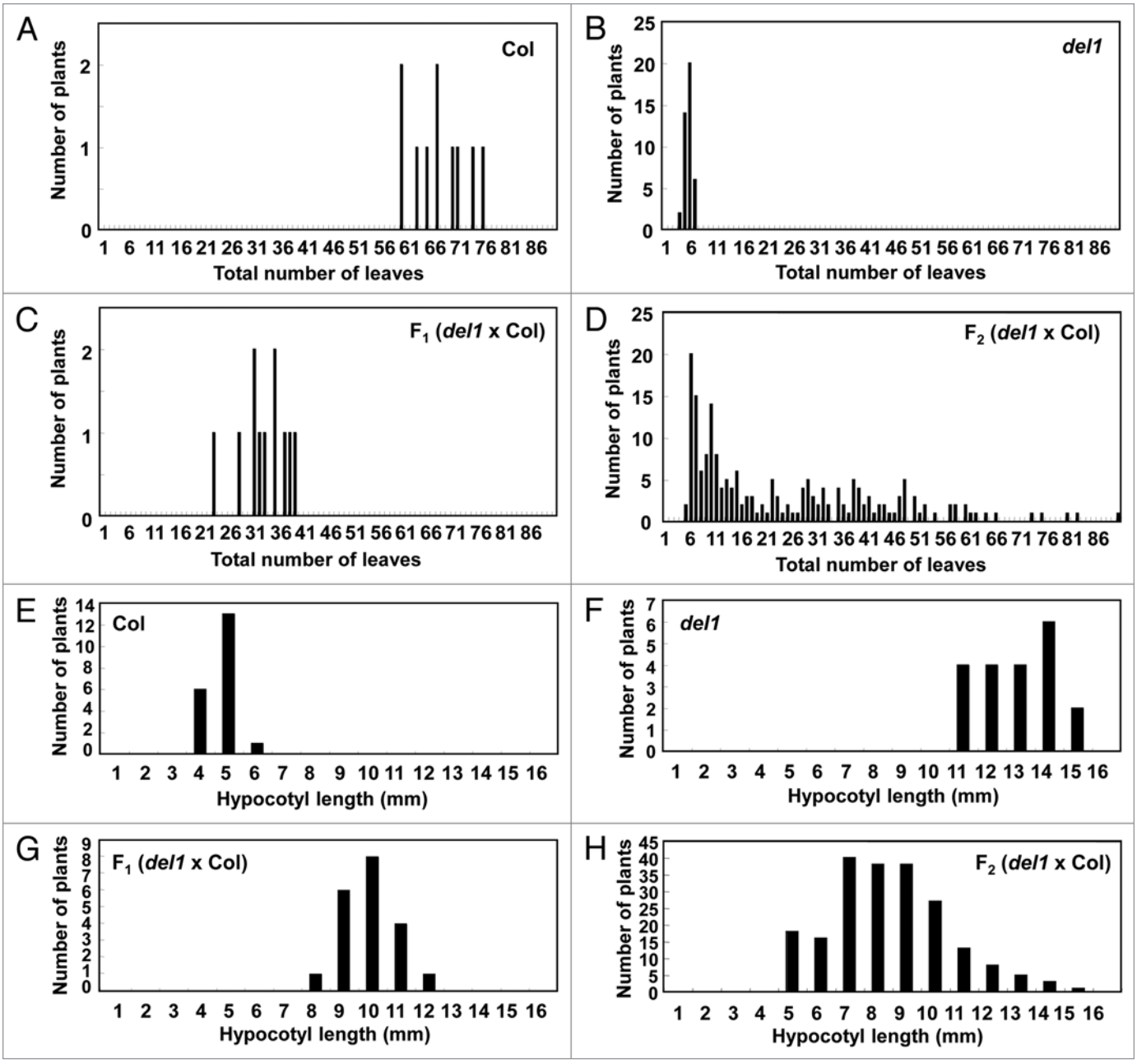

Figure 3. Frequency of distribution of flowering time and hypocotyl length. Data from wild-type (Col; A), *del1* (B), F_1 (C) and F_2 (D) plants are derived from crosses between *del1* (L*er*) and wild-type (Col) grown under SD. Data from wild type (Col; E), *del1* (F), F_1 (G) and F_2 (H) plants are derived from crosses between the *del1* (L*er*) and wild-type (Col) grown under SD for 14 days.

Mutations in the *CO* gene delay flowering under LD but not SD. However, the *phyB*-dependent acceleration of flowering is partially but not completely suppressed by the *co* mutation even under SD.[22] The accumulation of CO protein in *phyB* mutant plants even under SD explains how the *co* mutation affects the early-flowering phenotype of *phyB*.[23] One possible explanation for the weak suppression by *phyB-2511* was that the activation of the CO protein is more impaired in *phyB-2511* than in *hy3-1* or *del1*. To test this possibility, we constructed *co-2;phyB* double mutants using three *phyB* alleles. *co-2* partially suppressed the early-flowering phenotypes of *hy3-1*, *del1* and *phyB-2511* under LL. There were no significant differences in the flowering times of the three *co-2;phyB* mutants under LL (**Fig. 5B**). These results are consistent with previous findings that *phyB* can accelerate flowering in both CO-dependent and -independent pathways.[22]

The late-flowering phenotype of *SVPox* is partially suppressed by *phyB* mutations under LL. Overexpression of *SVP* (*SVPox*) delays flowering under LL, LD and SD.[14,24] *SVPox;co-2* produces more leaves than either *SVPox* or *co-2*, indicating that *SVPox* and *co-2* show delayed flowering, at least in part, in distinct pathways.[14] We expected that *SVPox;phyB-2511* would produce significantly more leaves than *SVPox;hy3-1* and

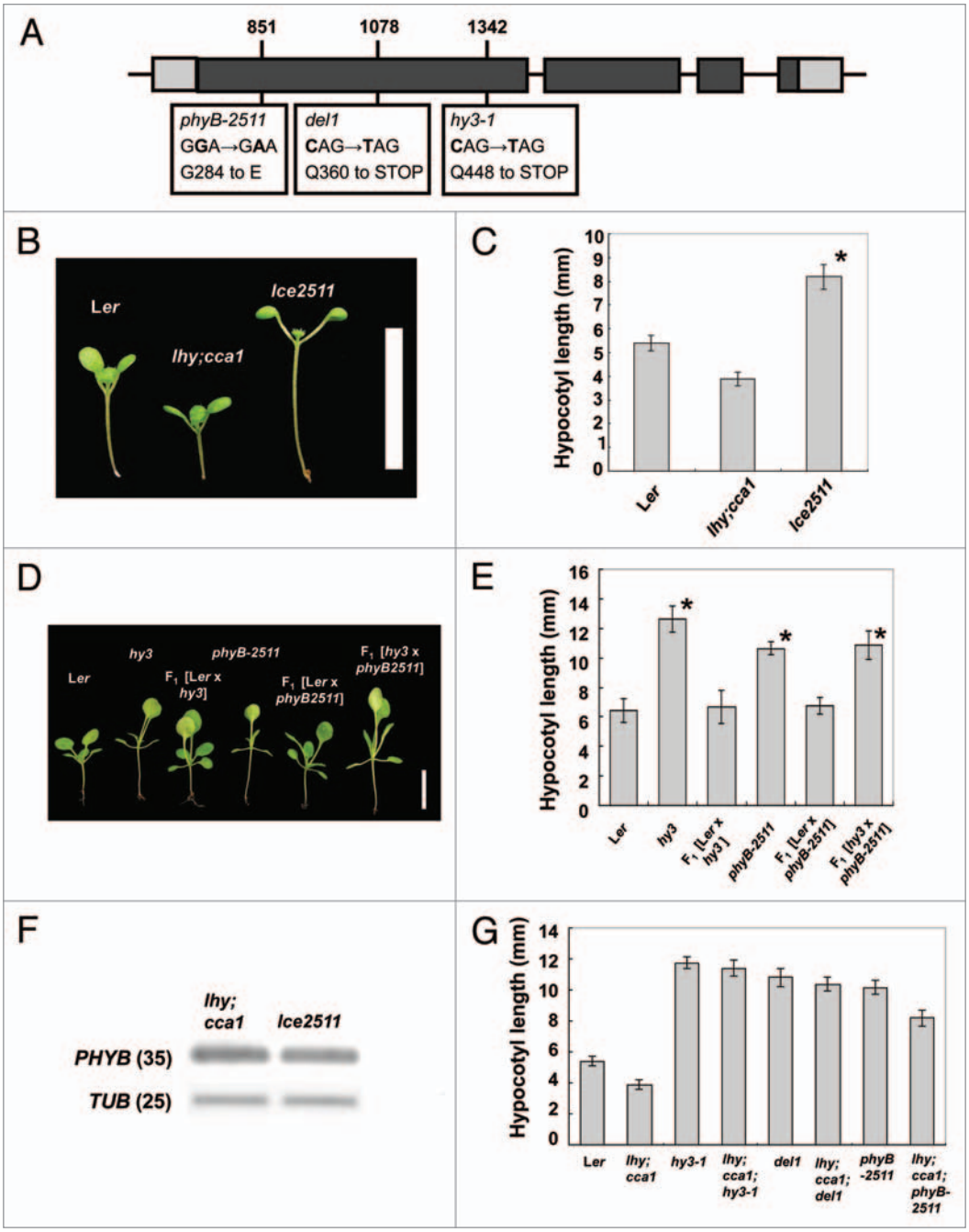

Figure 4. Isolation of a suppressor of the semi-dwarf phenotype of *lhy-12;cca1-101* under LL, and identification of the *del1* and *phyB-2511* mutations as new *phyB* alleles. (A) Positions of the *del1*, *hy3-1* and *phyB-2511* mutations. (B) A photo of wild -type (L*er*), *lhy-12;cca1-101* and *lce-25111* grown under LL for 10 days. The short-hypocotyl phenotype of *lhy-12;cca1-101* was suppressed by *phyB-2511* under LL. Bar indicates 1 cm. (C) Means ± SE (n = 15) of the hypocotyl lengths of the plants shown in (B) grown under LL for 10 days. Asterisks (*) represent statistical significance compared to the values of *lhy-12;cca1-101* (Student's t test, $p < 0.01$). (D) A photo of wild-type (L*er*), *hy3-1*, F_1 (L*er*x*hy3-1*), *phyB-2511*, F_1 (L*er*x *phyB-2511*) and F_1 (*phy B-2511* x *hy3-1*). Bar indicates 1 cm. (E) Means ± SE (n = 10) of the hypocotyl lengths of the plants shown in (D) grown under LL for 14 days. Asterisks (*) represent statistical significance compared to values of the wild type (L*er*) (Student's t test, $p < 0.01$). (F) Analysis of *PHYB* mRNA levels in *lhy-12;cca1-101* and *lce-2511* grown under LL. (G) Means ± SE (n = 15) of the hypocotyl lengths of wild type (L*er*), *lhy-12;cca1-101*, *hy3-1*, *lhy-12;cca1-101;hy3-1*, *phyB-2511*, *lhy-12;cca1-101;phyB-2511*, *del1* and *lhy-12;cca1-101;del1* grown under LL for 10 days.

SVPox;del1, if the lower activation of the CO protein in *phyB-2511* than in *del1* or *hy3-1* were responsible for the phenotype of *lhy;cca1;phyB-2511*. However, similar numbers of leaves were produced by *SVPox;phyB-2511* and *SVPox;hy3-1* under LL (**Fig. 5C**). This result suggests that the weak suppression of the late-flowering phenotype of *lhy;cca1* by *phyB-2511* cannot simply be explained by possible differences in the CO activation mechanism.

To test whether phyB and SVP regulate flowering time in a common genetic pathway, we constructed three *svp;phyB* double mutants, *svp-3;hy3-1*, *svp-3;del1*, and *svp-3;phyB-2511*, and compared their flowering times to those of control plants. The *svp-3* mutation did not enhance the early-flowering phenotype of the *del1* (**Fig. 6A**). These results suggest that SVP regulates flowering time in one of the phyB-dependent pathways (**Fig. 6B**).

Discussion

The third flowering pathway controlled by LHY and CCA1 may be active in *phyB-2511* but not in *hy3-1* or *del1*. We recently demonstrated that the clock proteins LHY and CCA1 control flowering time through at least two independent pathways. One is the GI-CO pathway, which functions as an activator of *FT* expression and is highly conserved in plants.[10] LHY and CCA1 set the phase of *GI* expression under light/dark cycles such that the downstream events, including expression of the *CO* mRNA and activation of the CO protein by light, can occur at precise times of the day. The other is the SVP-FLC pathway, which negatively regulates *FT* expression, thus delaying flowering time.[13,14] To understand the molecular mechanisms underlying the developmental control by LHY and CCA1 in Arabidopsis, we performed many types of genetic screens using clock mutants and the wild type. From this process, we obtained two novel *phyB* mutant alleles, *del1* and *phyB-2511* (**Fig. 4A**).

Each of the *phyB* mutations *hy3-1*, *del1* and *phyB-2511* accelerated flowering time under LL as single mutations (**Fig. 5**). However, interestingly, the suppression of the late-flowering phenotype of *lhy;cca1* by *phyB-2511* under LL was much weaker than that by *hy3-1* and *del1* (**Fig. 5A**). To elucidate why *phyB-2511* did not strongly suppress the late flowering of *lhy;cca1*, we compared the effects of these *phyB* alleles on the CO-dependent and SVP-FLC pathways. All three *phyB* mutations suppressed the late-flowering phenotype of *co-2* under LL. However, there were no significant differences among the flowering times of the *co-2;del1* and *co-2;phyB-2511* mutants under LL (**Fig. 5B**). The late-flowering phenotype of *SVPox* was also suppressed by the three *phyB* mutations. Although the suppression by *del1* was stronger than that by *hy3-1* or *phyB-2511*, there were no significant differences between the flowering times of the *SVPox;hy3-1* and *SVPox;phyB-2511* mutants under LL (**Fig. 5C**). These results suggest that different activities to control SVP or CO in the three *phyB* mutants are unlikely to be responsible for the weaker suppression of the late-flowering phenotype of *lhy;cca1* by *phyB-2511* (**Fig. 5A**). They also suggest that the clock proteins LHY and CCA1 control

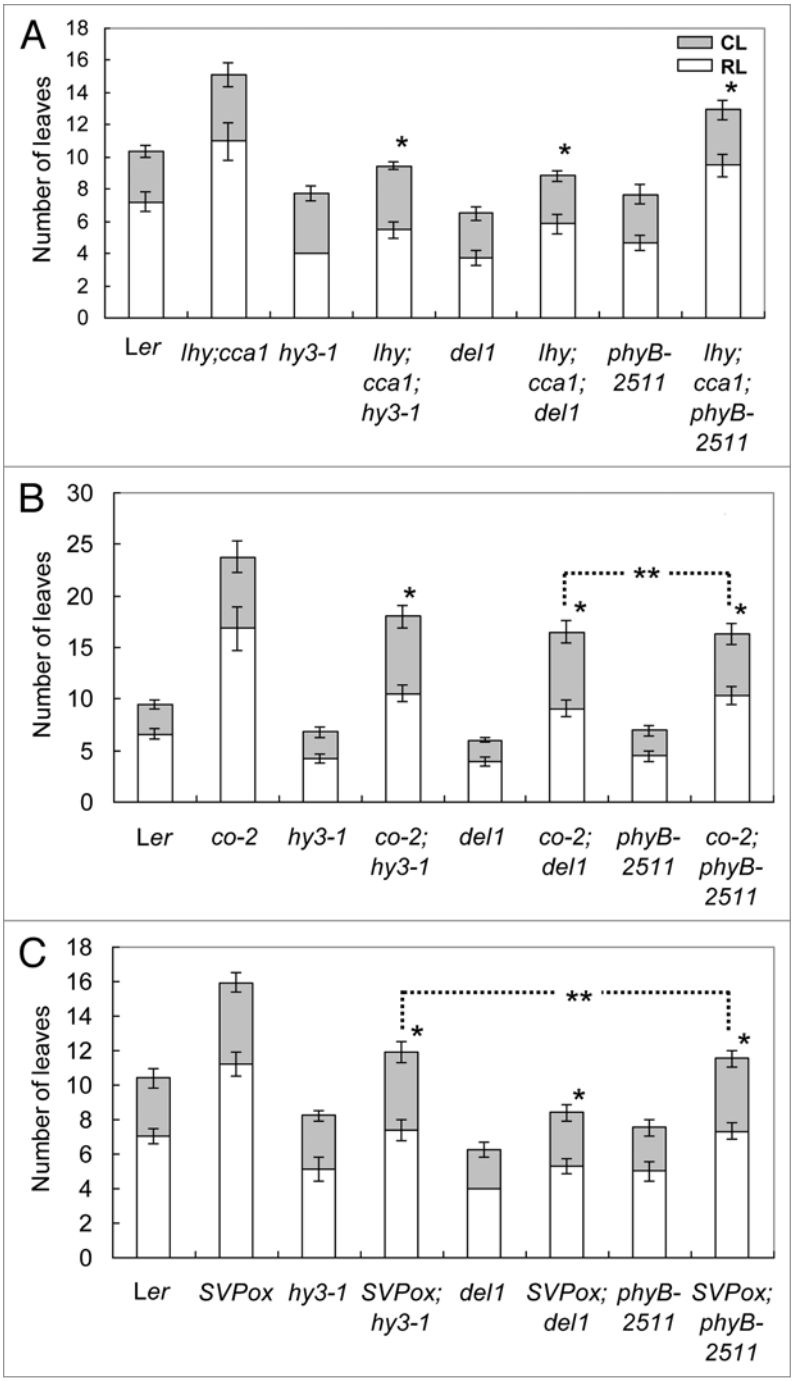

Figure 5. Suppression of the late flowering of *lhy-12;cca1-101*, *co-2* and *SVPox* by *hy3-1*, *phyB-2511* and *del1* under LL. (A–C) The numbers of cauline (CL) and rosette (RL) leaves at the time of flowering were scored, and the data are presented as mean ± SE (n = 20). (A) Wild type (L*er*), *lhy-12;cca1-101*, *hy3-1*, *lhy-12;cca1-101;hy3-1 phyB-2511*, *lhy-12;cca1-101;phyB-2511*, *del1* and *lhy-12;cca1-101;del1*. Asterisks (*) represent statistical significance compared to the values of *lhy-12;cca1-101* (Student's t test, $p < 0.01$). (B) Wild type (L*er*), *co-2*, *hy3-1*, *co-2;hy3-1 phyB-2511*, *co-2;phyB-2511*, *del1* and *co-21;del1*. Asterisks (*) represent statistical significance compared to the values of *co-2* (Student's t-test, $p < 0.01$) and double asterisks (**) represent no significance between two plants (Student's t test, $p > 0.01$). (C) Wild type (L*er*), *SVPox*, *hy3-1*, *SVPox;hy3-1 phyB-2511*, *SVPox;phyB-2511*, *del1* and *SVPox;del1* plants grown under LL. Asterisks (*) represent statistical significance compared to the values of *SVPox* (Student's t test, $p < 0.01$) and double asterisks (**) represent no significance between two plants (Student's t test, $p > 0.01$).

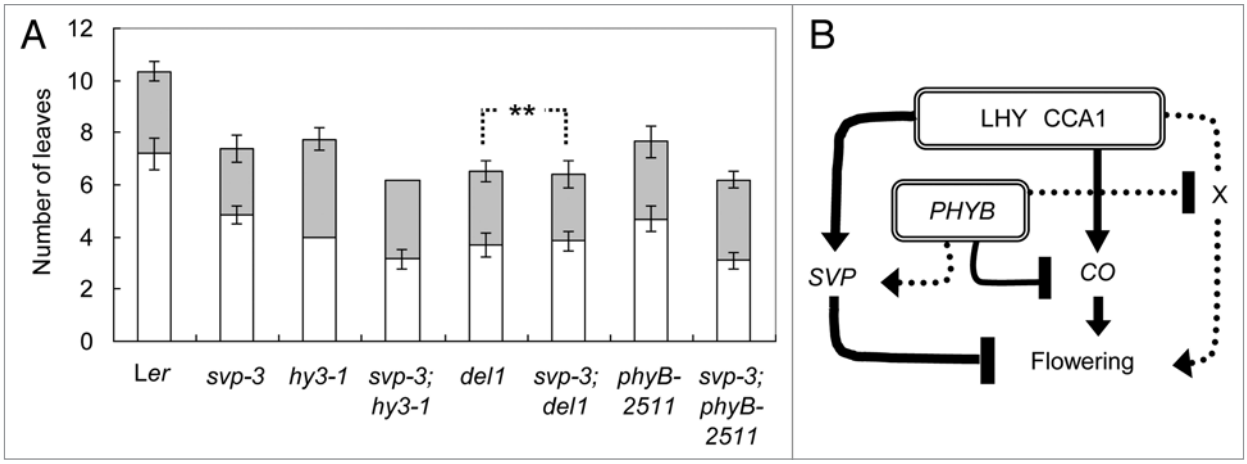

Figure 6. Flowering time of *svp;phyB* and a hypothetical model of the regulation of flowering by LHY, CCA1 and PHYB. (A) The numbers of cauline (CL) and rosette (RL) leaves at the time of flowering were scored, and the data are presented as mean ± SE (n = 20). (A) Wild-type (*Ler*), *svp-3*, *hy3-1*, *svp-3;hy3-1 phyB-2511*, *svp-3;phyB-2511*, *del1* and *svp-3;del1* plants grown under LL. Double asterisks (**) represent no statistical significance between two plants (Student's t test, p > 0.01). (B) A hypothetical model. For details, see text.

flowering time via at least three distinct pathways (**Fig. 6B**). The early-flowering phenotype of the *phyB* mutants was not enhanced by *svp-3*, suggesting that SVP functions downstream of PhyB in one of the PhyB-dependent processes (**Fig. 6B**). All three pathways appear to be impaired in *hy3-1* and *del1*. In *phyB-2511*, the regulation of the SVP pathway and the CO pathway by PhyB seemed to be impaired, but that of the pathway including the unknown factor X did not.

PhyB proteins consist of the N-terminal chromophoric and the C-terminal dimerization moieties. These are further divided into a few sub-domains. The open tetrapyrrole chromophore is covalently attached to the GAF (cGMP phosphodiesterase, adenylate cyclase, FhlA) domain residing in the center of the N-terminal moiety. The GAF domain is flanked by the N-terminal PAS (PER, ARNT and SIM) and C-terminal PHY (phytochrome-specific GAF-related) domains. In the C-terminal half of the PhyB, two successive PAS domains and the histidine-kinase-related domain (HKRD) are found.

The point mutations caused premature stop codons both in *del1* and *hy3-1*, and most part of the C-terminal moiety of the PhyB was lost in these mutants. By contrast, the amino acid substitution occurred in the GAF domain in *phyB-2511*. The domains outside of the GAF of phyB-2511 protein may regulate flowering time via the pathway X.

Functional interactions between the clock proteins LHY and CCA1 and the photoreceptor PhyB in the control of organ elongation in arabidopsis. The overexpression of PhyB delays flowering, shortens the length of the hypocotyl, and causes increased sensitivity to red light. *lhy;cca1* mutant plants have similar phenotypes, such as late flowering and short hypocotyls, under LL, and greater sensitivity to continuous red light.[5,10,13] The late-flowering and short-hypocotyl phenotypes of *lhy;cca1* were suppressed by the new strong allele *del1* under LL (**Figs. 4G and 5A**). These results suggest that increased levels of *phyB* mRNA or PhyB protein, or enhanced PhyB signaling activity, occur in *lhy;cca1* under LL.

The effect of *phyB-2511* on the short-hypocotyl phenotype of *lhy;cca1* was also weaker than the effects of *hy3-1* and *del1* (**Fig. 4G**). The X shown in **Figure 6B** might be involved in the control of both flowering time and hypocotyl elongation.

Although *del1* suppressed both the late-flowering and short-hypocotyl phenotypes of *lhy;cca1* under LL, *del1* single mutant plants flowered earlier and had longer hypocotyls than the wild type (**Fig. 1**). The phenotypes of *lhy-12;cca1-101;del1* were intermediate between those of wild-type and *del1* plants under LL (**Figs. 4G and 5A**). Therefore, an alternative explanation is also possible: the two clock proteins LHY and CCA1 and the photoreceptor PhyB are not in a single linear genetic pathway, but function in parallel pathways that affect a common downstream target directly or indirectly. For example, some plant hormones have been shown to be involved in hypocotyl elongation. Both the circadian clock and PhyB have been reported to affect the expression levels of key genes involved in the biosynthesis or signaling of GA and auxin. These plant hormone signaling events are candidates for the common downstream targets of LHY/CCA1 and PhyB.

The partial suppression of the *lhy;cca1* phenotypes by *phyB* can also be explained by multiple functions for these proteins. We prefer the idea that LHY/CCA1 and PhyB have both common and distinct targets for the control of organ elongation in Arabidopsis. If this is true, mutations other than *svp*, *flc* and *phyB* that suppress the *lhy;cca1* phenotypes under LL should be recovered in further genetic screenings. If the proteins identified in screening interact with LHY/CCA1, SVP/FLC and PhyB, and function as hubs, these proteins might be candidates for the common targets. The X shown in **Figure 6B** would be one candidate for the common targets.

This is the first demonstration to identify suppressors of the semi-dwarf phenotype of the *lhy;cca1* under LL. Although one may think that LL condition is artificial, we think that this condition is quite important to investigate effects of loss-of-function of clock on developmental controls of plants. This is simply because light/dark cycles have strong impact on the entrainment of the

clock and the effects of the clock-mutation can be masked by LD or SD conditions. If LHY, CCA1 and phyB function in a single linear genetic pathway, complete suppression should be observed in phenotypes in the *lhy;cca1;phyB* triple mutants. However, as already known, LHY/CCA1 and phyB are multi-functional proteins and therefore have shown to control flowering time and organ elongation via more than two independent pathways. Phenotypes of *phyB-2511* could be explained by (1) a potential novel function of it on the LHY/CCA1-dependent pathway or (2) weaker allele of it than *del1* and *hy3-1*. In **Figure 6B**, we have shown that LHY/CCA1 seems to control flowering time via more than three independent pathways: the SVP-pathway, CO-pathway and X-pathway. Presence of the X-pathway has been suggested based on findings in this work, for the first time. Recently, we have found that SVP controls both flowering time and organ elongation in the downstream of LHY/CCA1 (Niinuma K and Mizoguchi T, unpublished). The mechanism includes one of the plant hormone pathways and this is based on our findings on protein-protein interaction between SVP and a positive factor of the plant hormone pathway. Also, we have identified a candidate of the X shown in **Figure 6B** as an enhancer of the phenotypes of *lhy;cca1* under LL (Miyata K and Mizoguchi T, unpublished). This enhancer is a novel protein without any homology to proteins reported in public databases. A comparison between *phyB-2511* and other *phyB* mutants such as *hy3-1* and *del1* would be useful for identifying such common targets.

Materials and Methods

Plant material, growth conditions and analysis of the organ-elongation phenotypes of the *phyB* mutants. *Arabidopsis thaliana* accessions Landsberg *erecta* (L*er*) and Columbia (Col) were used as the wild-type (WT) plants. The mutant lines *lhy-12;cca1-101*, *SVPox* (L*er*),[13] *hy3-1* (L*er*),[18] and *co-2* (L*er*),[22] were described previously. Seeds were imbibed and cold-treated at 4°C for 3 days in darkness before germination under light. Plants were grown in controlled-environment rooms at 22°C. The 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 about 40 $\mu molm^{-2}s^{-1}$. The hypocotyl lengths of 14-day-old plants were measured. The leaf blades and petioles of the third and fifth leaves were measured 3 weeks and 30 days after sowing, respectively.[25]

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

Preparation of RNA and semiquantitative RT-PCR. Plants were sown as described above and grown on soil for 10 days. Aerial parts were used for RNA preparation. RT-PCR was performed with 1 μg total RNA using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA). cDNA was diluted to 100 μl with TE buffer, and 1 μl diluted cDNA was used for PCR amplification by TaKaRa Extaq (TaKaRa, Shiga, Japan). The primer sequences used were as follows: *CCA1* forward, 5'-CG TGA AAG GTG GAC TGA GGA AGA AC-3', *CCA1* reverse, 5'-GCG GAA AGT GCT TGC GTT TGA TGT C-3'; *FT* forward, 5'-ACA ACT GGA ACA AAC CTT TGG CAA TG-3', *FT* reverse, 5'-ACT ATA TAG GCA TCA TCA CCG TTC GTT ACT CG-3'; *LHY* forward, 5'-GCC TGG GAA CAA CGG TAC A-3', *LHY* reverse, 5'-GGT CTT ACT TGT TTC AAT GTC G-3'; *PRR9* forward, 5'-TTT AGG CTT TGT TGG TTT TAC-3', *PRR9* reverse, 5'-CTT TGA GCA TGA GCA GTA GGA-3'; *TOC1* forward, 5'-GAA TCC CTG TGA TAA TGA TCT T-3', *TOC1* reverse, 5'-CAA GAC CAC CAT CAC GAG CAT GAA C-3'; *PHYB* forward, 5'-TGA GCT GCA GCA AGC TTT AC-3', *PHYB* reverse, 5'-CAA GGA AAA ACT CTT CCC TC-3'; *TUB* forward, 5'-CTC AAG AGG TTC TCA GCA GTA-3', *TUB* reverse: 5'-TCA CCT TCT TCA TCC GCA GTT-3'. The cycles used for amplification were as follows: 27 cycles for *CCA1*, *LHY*, *PRR9* and *TOC1*; 33 cycles for *FT*; 35 cycles for *PHYB*; and 25 cycles for *TUB*. The PCR products were separated on 1.5% agarose gels and expression was quantified using a Bio-Rad Molecular Imager (Molecular Imager Fx, 1998, Bio-Rad Laboratories Inc.). The data are represented relative to the maximum value among all data sets after normalization to the *TUB* control. RT-PCR analyses were performed at least twice and usually with independent RNA samples. Similar results were obtained from two experiments.

EMS mutagenesis. *del1* was initially isolated from an M_2 population of EMS-mutagenized *lhy-12* seeds, as a mutant with early-flowering and long-hypocotyl phenotypes under SD. The procedure used for the mutagenesis has been reported in reference 13. *del1;lhy-12* was backcrossed with L*er* wild type, and the *del1* single mutant was obtained. *lhy-12* did not affect the early-flowering or long-hypocotyl phenotypes of *del1* under SD, LD or LL.

For the isolation of *phyB-2511* as a suppressor of the semi-dwarf phenotype of *lhy-12;cca1-101* under LL, an M_2 population of EMS-mutagenized *lhy-12;cca1-101* seeds was used. The procedure used for this mutagenesis has been reported in reference 13.

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References

1. Bell-Pedersen D, Cassone VM, Earnest DJ, Golden SS, Hardin PE, Thomas TL, et al. Circadian rhythms from multiple oscillators: lessons from diverse organisms. Nat Rev Genet 2005; 6:544-56.
2. Young MW, Kay SA. Time zones: a comparative genetics of circadian clocks. Nat Rev Genet 2001; 2:702-15.
3. Alabadí D, Oyama T, Yanovsky MJ, Harmon FG, Más P, Kay SA. Reciprocal regulation between *TOC1* and *LHY/CCA1* within the Arabidopsis circadian clock. Science 2001; 293:880-3.
4. Alabadí D, Yanovsky MJ, Más P, Harmer SL, Kay SA. Critical role for CCA1 and LHY in maintaining circadian rhythmicity in Arabidopsis. Curr Biol 2002; 12:757-61.
5. Mizoguchi T, Wheatley K, Hanzawa Y, Wright L, Mizoguchi M, Song HR, et al. *LHY* and *CCA1* are partially redundant genes required to maintain circadian rhythms in Arabidopsis. Dev Cell 2002; 2:629-41.
6. Kim JY, Song HR, Taylor BL, Carré IA. Light-regulated translation mediates gated induction of the Arabidopsis clock protein LHY. EMBO J 2003; 22:935-44.
7. Schaffer R, Ramsay M, Samach A, Corden S, Putterill J, Carré IA, et al. The *late elongated hypocotyl* mutation of Arabidopsis disrupts circadian rhythms and the photoperiodic control of flowering. Cell 1998; 93:1219-29.
8. Wang ZY, Tobin EM. Constitutive expression of the *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*) gene disrupts circadian rhythms and suppresses its own expression. Cell 1998; 93:1207-17.
9. Green RM, Tobin EM. Loss of the circadian clock-associated protein 1 in Arabidopsis results in altered clock-regulated gene expression. Proc Natl Acad Sci USA 1999; 96:4176-9.
10. Mizoguchi T, Wright L, Fujiwara S, Cremer F, Lee K, Onouchi H, et al. Distinct roles of *GIGANTEA* in promoting flowering and regulating circadian rhythms in Arabidopsis. Plant Cell 2005; 17:2255-70.
11. Makino S, Kiba T, Imamura A, Hanaki N, Nakamura A, Suzuki T, et al. Genes encoding pseudo-response regulators: insight into His-to-Asp phosphorelay and circadian rhythm in *Arabidopsis thaliana*. Plant Cell Physiol 2000; 41:791-803.
12. Strayer C, Oyama T, Schultz TF, Raman R, Somer DE, Más P, et al. Cloning of the Arabidopsis clock gene *TOC1*, an autoregulatory response regulator homolog. Science 2000; 289:768-71.
13. Fujiwara S, Oda A, Yoshida R, Niinuma K, Miyata K, Tomozoe Y, et al. Circadian clock proteins LHY and CCA1 regulate SVP protein accumulation to control flowering in Arabidopsis. Plant Cell 2008; 20:2960-71.
14. Yoshida R, Fekih R, Fujiwara S, Oda A, Miyata K, Tomozoe Y, et al. Possible role of EARLY FLOWERING 3 (ELF3) in clock-dependent floral regulation by SHORT VEGETATIVE PHASE (SVP) in *Arabidopsis thaliana*. New Phytologist 2009; 182:838-50.
15. Demarsy E, Fankhauser C. Higher plants use LOV to perceive blue light. Curr Opin Plant Biol 2009; 12:69-74.
16. Franklin KA, Quail PH. Phytochrome functions in Arabidopsis development. J Exp Bot 2010; 61:11-24.
17. Li QH, Yang HQ. Cryptochrome signaling in plants. Photochem Photobiol 2007; 83:94-101.
18. Reed JW, Nagpal P, Poole DS, Furuya M, Chory J. Mutations in the gene for the red/far-red light receptor phytochrome B alter cell elongation and physiological responses throughout Arabidopsis development. Plant Cell 1993; 5:147-57.
19. Zagotta MT, Hicks KA, Jacobs CI, Young JC, Hangarter RP, Meeks-Wagner DR. The Arabidopsis *ELF3* gene regulates vegetative photomorphogenesis and the photoperiodic induction of flowering. Plant J 1996; 10:691-702.
20. Hicks KA, Millar AJ, Carré IA, Somers DE, Straume M, Meeks-Wagner DR, et al. Conditional circadian dysfunction of the Arabidopsis *early-flowering 3* mutant. Science 1996; 274:790-2.
21. Somers DE, Devlin PF, Kay SA. Phytochromes and cryptochromes in the entrainment of the Arabidopsis circadian clock. Science 1998; 282:1488-90.
22. Putterill J, Robson F, Lee K, Simon R, Coupland G. The *CONSTANS* gene of arabidopsis promotes flowering and encodes a protein showing similarities to zinc finger transcription factors. Cell 1995; 80:847-57.
23. Valverde F, Mouradov A, Soppe W, Ravenscroft D, Samach A, Coupland G. Photoreceptor regulation of CONSTANS protein in photoperiodic flowering. Science 2004; 303:1003-6.
24. Fekih R, Miyata K, Yoshida R, Ezura H, Mizoguchi T. Isolation of suppressors of late flowering and abnormal flower shape phenotypes caused by overexpression of the *SHORT VEGETATIVE PHASE* gene in *Arabidopsis thaliana*. Plant Biotechnol 2009; 26:217-24.
25. Niinuma K, Nakamichi N, Miyata K, Mizuno T, Kamada H, Mizoguchi T. Roles of Arabidopsis *PSEUDO-RESPONSE REGULATOR* (*PRR*) genes in the opposite controls of flowering time and organ elongation under long-day and continuous light conditions. Plant Biotechnol 2008; 25:65-72.