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Note

A loss-of-function mutation in the *DWARF4*/*PETANKO5* gene enhances the late-flowering and semi-dwarf phenotypes of the *Arabidopsis* clock mutant *lhy-12;cca1-101* under continuous light without affecting *FLC* expression

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Abstract The circadian clock plays important roles in the control of photoperiodic flowering in *Arabidopsis*. Mutations in the *LATE ELONGATED HYPOCOTYL* (*LHY*) and *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*) genes (*lhy;cca1*) accelerate flowering under short days, whereas *lhy;cca1* delays flowering under continuous light (LL). The *lhy;cca1* mutant also exhibits short hypocotyls and petioles under LL. However, the molecular mechanisms underlying the regulation of both flowering time and organ lengths in the LHY/CCA1-dependent pathway are not fully understood. To address these questions, we performed EMS mutagenesis of the *lhy-12;cca1-101* line and screened for mutations that enhance the *lhy;cca1* phenotypes under LL. In this screen, we identified a novel allele of *dwarf4* (*dwf4*) and named it *petanko 5* (*pta5*). A similar level of enhancement of the delay in flowering was observed in these two *dwf4* mutants when combined with the *lhy;cca1* mutations. The *lhy;cca1* and *dwf4* mutations did not significantly affect the expression level of the floral repressor gene *FLC* under LL. Our results suggest that a defect in brassinosteroid (BR) signaling delayed flowering independent of the *FLC* expression level, at least in plants with the *lhy;cca1* mutation grown under LL. The *dwf4*/*pta5* mutation did not enhance the late-flowering phenotype of plants overexpressing *SVP* under LL, suggesting that SVP and BR function in a common pathway that controls flowering time. Our results suggest that the *lhy;cca1* mutant exhibits delayed flowering due to both the BR signaling-dependent and -independent pathways under LL.

Key words: brassinosteroid, CCA1, DWF4, flowering time, LHY.

Photoperiodic flowering responses are classified into three main groups: long-day (LD), short-day (SD), and day-neutral. *Arabidopsis thaliana* (*Arabidopsis*) is a facultative LD plant and flowers much earlier in a daily regime with a long light period and a short dark period (e.g., 16 h light/8 h dark) than in one with a short light period and a long dark period (e.g., 8 h light/ 16 h dark or 10 h light/14 h dark). In *Arabidopsis*, two related MYB proteins, LATE ELONGATED HYPOCOTYL (LHY) and CIRCADIAN CLOCK ASSOCIATED 1 (CCA1), are essential clock components with redundant functions; both play important roles in photoperiodic flowering by controlling the rhythmic expression of flowering-time genes (Mizoguchi et al. 2002). In particular, the clock proteins LHY and CCA1 regulate a flowering pathway comprising the genes *GIGANTEA* (*GI*), *CONSTANS* (*CO*), *FLOWERING LOCUS T* (*FT*), and *SUPPRESSOR OF OVEREXPRESSION OF CO 1* (*SOC1*) in light/dark cycles such as LD and SD (Mizoguchi et al. 2002; Mizoguchi et al. 2005). The expression of *FT*, which encodes a florigen, is increased under LDs mainly through a conserved pathway consisting of *GI* and *CO*.

Abbreviations: Arabidopsis, *Arabidopsis thaliana*; BR, brassinosteroid; CCA1, CIRCADIAN CLOCK ASSOCIATED 1; Col, Columbia; DWF4, DWARF4; FLC, FLOWERING LOCUS C; FT, FLOWERING LOCUS T; L*er*, Landsberg *erecta*; LHY, LATE ELONGATED HYPOCOTYL; LL, continuous light; PTA5, PETANKO5; SVP, SHORT VEGETATIVE PHASE; SOC1, SUPPRESSOR OF OVEREXPRESSION OF CO 1; TUB, TUBULIN; Ws, Wassilewskija.

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Lines containing the *lhy;cca1* mutations cause altered photoperiodic responses (Fujiwara et al. 2008). Under continuous light (LL), *lhy;cca1* mutants flower later than under SDs. This inverse response involves enhanced activity of the clock protein EARLY FLOWERING 3 (ELF3) and two floral repressors, SHORT VEGETATIVE PHASE (SVP) and FLOWERING LOCUS C (FLC), under LL (Fujiwara et al. 2008; Yoshida et al. 2009).

Understanding the molecular mechanisms underlying the control of the size of multicellular organisms such as fungi, insects, animals, and plants is an important and general issue. Brassinosteroid (BR) signaling has been proposed as a mechanism for controlling the size of plants by regulating hypocotyl and petiole elongation, which are reduced in light and promoted in darkness (Busov et al. 2008; Hardtke et al. 2007).

Under LL but not LD, *lhy;cca1* plants exhibit not only late flowering, but also semi-dwarf and dark-green curly leaf phenotypes (Fujiwara et al. 2008). These phenotypes are similar to those observed in plants with weak alleles of BR-deficient or -insensitive mutations. The late-flowering phenotype of *lhy;cca1* under LL was found to be largely suppressed by either *svp* or *elf3* (Fujiwara et al. 2008; Yoshida et al. 2009). However, the molecular mechanisms underlying the semi-dwarf phenotype of *lhy;cca1* have not been elucidated.

Two related genes, *EARLY FLOWERING 6* (*ELF6*) and *RELATIVE OF ELF6* (*REF6*), which encode jumonji proteins, were shown to be involved in BR signaling (Yu et al. 2008). The *elf6* and *ref6* mutants both show a semi-dwarf phenotype that is similar to that of *lhy;cca1*. The phenotypes of *elf6* and *ref6* are rather weak compared to those of the canonical BR dwarfs *brassinosteroid-insensitive 1* (*bri1*, Clouse et al. 1996), *dwarf4* (*dwf4*, Choe et al. 1998), *deetiolated2* (*det2*, Li et al. 1996), *constitutive photomorphogenic dwarf* (*cpd*, Szekeres et al. 1996), and *rotundifolia3* (*rot3*, Kim et al. 1998). However, if combined with a weak allele of *bri1* (*bri1–5*), the double mutants (*elf6;bri1* and *ref6;bri1*) show a clear dwarf phenotype (Yu et al. 2008). In addition, the *elf6* or *ref6* mutations have altered expression levels of BR-regulated genes. ELF6 and REF6 directly interact with BRI-EMS-SUPPRESSOR 1 (BES1, Yin et al. 2002), a positive regulator of BR signaling, clearly indicating that ELF6 and REF6 play important roles in the BR-dependent signaling pathway. This work demonstrates the importance of the detailed characterization of these novel proteins, even though phenotypes of the single mutants, *elf6* or *ref6*, are weak.

The phenotype of *lhy;cca1* is weaker than those of canonical BR dwarfs such as *det2* or *cpd*. In this work, we identified an enhancer, *petanko 5* (*pta5*), of the semi-dwarf and late-flowering phenotypes of *lhy;cca1* under LL. Genetic mapping, sequencing, and complementation tests identified a novel allele of *dwf4* (Choe et al. 1998) as *pta5*. A clear dwarf phenotype is present in *lhy;cca1;dwf4/pta5*. *pta5* did not enhance the late-flowering phenotype of *35S:SVP* under LL. In addition, the early flowering phenotype of *svp-3* was not affected by *pta5* under LL. These results suggest that LHY, CCA1, and SVP play key roles in the BR signaling pathway, helping to control flowering and organ elongation in *Arabidopsis*. Increases in the mRNA levels of the floral repressor *FLC* were proposed to be important in the delay of flowering in BR-related mutants (Domagalska et al. 2007; Yu et al. 2008). Our results, however, provide a novel mechanism for the BR-dependent control of flowering time, which does not require an increase in *FLC* expression.

Materials and methods

Plant materials and growth conditions

Plants of the *Arabidopsis thaliana* L. Heynh (*Arabidopsis*) accessions Landsberg *erecta* (L*er*) and Ws (Wassilewskija) were used as the wild type (WT). The mutants tested included *lhy-12;cca1-101* (L*er*, Fujiwara et al. 2008), *lhy-21;cca1-11* (Ws, Hall et al. 2003), *lhy-11;cca1-1* (Col, Fujiwara et al. 2008), *dwf4-101* (Ws, Nakamoto et al. 2006), *35S:SVP* (L*er*, Fujiwara et al. 2008), and *svp-3* (L*er*, Fujiwara et al. 2008). Seeds were stratified and cold-treated at 4°C for 3 days in darkness before germination. Plants were grown in soil in controlled environment rooms and plant cultivation chambers (Biotron; NKsystems, Japan) at 22°C. The light conditions were LL (continuous white light) with a photon flux density of about 40 μmol m^{-2} s^{-1}.

EMS mutagenesis and screening for enhancer mutations of lhy-12;cca1-101

Approximately 5,000 *lhy-12;cca1-101* (L*er*) seeds were mutagenized by imbibition in 0.3% EMS (Sigma-Aldrich, St Louis, MO) for 9 h, followed by washing twice with 0.1 M Na_2SO_3 and five times with distilled water for 30 min. M_2 seeds were collected in pools, with each pool containing ~20 M_1 plants. Approximately 13,000 M2 seeds representing ~1,300 M_1 plants after mutagenesis of *lhy-12;cca1-101* seeds were sown on soil and screened for plants with shorter petioles than those of the progenitor line *lhy-12;cca1-101* grown under LL. Seven enhancer lines of *lhy-12;cca1-101* (L*er*) were isolated and named *petanko1-7* (*pta1–7*). The enhancer mutations in the *lhy-12;cca1-101* (L*er*) background were backcrossed to the parental line *lhy-12;cca1-101* (L*er*) at least once before phenotypic analysis. *lhy-12;cca1-101;pta5* (L*er*) was backcrossed with the WT (L*er*), and the *pta5* single mutant was obtained. In addition, an allelism test was performed between *dwf4-101* and *pta5*.

Measurement of flowering time

Flowering time was scored by growing plants in soil under LL; the numbers of rosette and cauline leaves on the main stem were counted after bolting. The data are presented as means±SE (n≧10). Flowering time data were gathered at least

twice, with similar results.

Analysis of hypocotyl and leaf length

Hypocotyl and leaf lengths were measured in plants grown for 2 and 4 weeks under LL, respectively (Niinuma et al. 2008). The data are presented as means±SE (n≧10). Each experiment was performed at least twice with similar results. Student's t-test (p<0.01) was used for both analyses to determine whether the differences were statistically significant.

Genetic mapping

Crosses were performed between *lhy-12;cca1-101;pta5* (L*er*) and a polymorphic strain, *lhy-11;cca1-1* (Col). F_2 plants that produced leaves with shorter petioles than those of WT plants grown in LL were used for mapping. Simple Sequence Length Polymorphism (SSLP) markers were also used for mapping. Twelve markers that cover the entire genome were used to analyze the pooled DNAs for initial linkage analyses. Once the linkage between the mutations and genetic markers was established, 70 to 300 F_2 mutant plants were further analyzed by PCR with flanking markers to determine the fine-scale genetic linkage, ideally narrowing the field to the region of the mutation. All of the markers used for fine mapping were described by Nefissi et al. (2011). The PCR products were separated on 3-3.5% agarose gels, and the recombination value was calculated based on the band pattern.

Gene expression analysis

Plants were grown on soil under LL for 14, 21, or 28 days. The aerial parts of plants were harvested and used for RNA extraction using an RNeasy Plant Mini kit (Qiagen, Courtabuf, France). To synthesize cDNA, one ng of each RNA sample was reverse-transcribed with the SuperScript First-Strand Synthesis System for reverse transcription-PCR (RT-PCR; Invitrogen, Carlsbad, CA, USA). cDNA was subjected to PCR amplification using ExTaq (TaKaRa, Shiga, Japan). The primer sequences used for *FT*, *SOC1*, and *TUBULIN2* (*TUB2*) were reported previously (Fujiwara et al. 2008). The primer set for *FLC* was described by Ratcliffe et al. (2003). The PCR products were separated on 1% agarose gels, and expression was quantified using a Bio-Rad Molecular Imager (Molecular Imager Fx, 1998 Bio-Rad Laboratories Inc.). The data are presented relative to the value of the *TUB2* control. All RT-PCR analyses were performed at least twice with independent RNA samples, and similar results were obtained from the two experiments.

The same cDNA was used for quantitative RT-PCR; 1 μl of the diluted cDNA was amplified with SYBR Premix ExTaq II (TaKaRa, Japan) and a primer set using the Thermal Cycler Dice Real Time System TP800 (TaKaRa, Japan). The level of *TUB2* mRNA was used as the internal control. The primer sets used for *FT*, *SOC1*, and *TUB2* were described by Endo et al. (2005). The primer set used to amplify *FLC* was described by Nefissi et al. (2011). All of the primer sets included at least one primer that spanned an exon–exon junction. The following standard thermal cycling was used for all PCRs: 95°C for

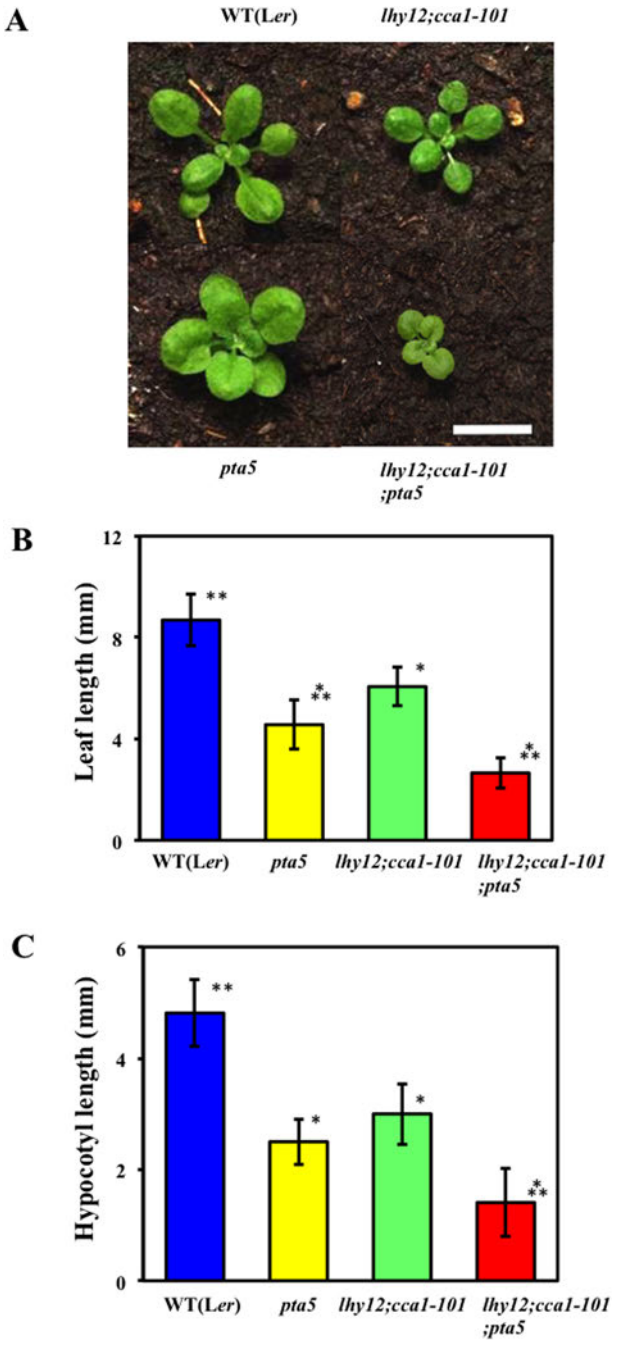

Figure 1. Effects of an enhancer mutation on organ elongation in *lhy;cca1* in LL. (A) Images of wild-type (WT, L*er*), *lhy-12;cca1-101*, *pta5*, and *lhy-12;cca1-101;pta5* grown under LL for 2 weeks. Scale bar=1 cm. (B) Lengths of leaves of WT (L*er*), *lhy-12;cca1-101*, *pta5*, and *lhy-12;cca1-101;pta5* grown under LL for 4 weeks. Single and double asterisks denote statistical significance in comparison with the WT (L*er*) and *lhy-12;cca1-101*, respectively (Student's t-test, p<0.001). Means±SE are shown (n≧10). (C) Lengths of hypocotyls of WT (L*er*), *lhy-12;cca1-101*, *pta5*, and *lhy-12;cca1-101;pta5* grown under LL for 2 weeks; data are means of 10 plants±SE Single and double asterisks denote statistical significance in comparison with the values for WT (L*er*) and *lhy-12;cca1-101*, respectively (Student's t-test, p<0.001). Each experiment was performed at least twice with similar results.

10 s, 40 cycles of 95°C for 5 s, and 60°C for 30 s. The data were analyzed using the TaKaRa TP800 software.

Results

Screen for an enhancer mutant of the semi-dwarf phenotype of lhy;cca1 under LL

Genetic screening was performed for enhancers of the short-hypocotyl and short-petiole phenotypes of *lhy-12;cca1-101* using EMS-mutagenized *lhy-12;cca1-101* seeds under LL. Enhancer candidates were identified, and seven mutant lines with severe phenotypes were chosen for further analysis. The rosette leaves of these mutants were flat and therefore named *petanko* (*pta*; flat in Japanese). One mutant line with a severe dwarf phenotype, *pta#5* (*lhy-12;cca1-101;pta5*), was subjected to further analysis (Figure 1). The *pta5* mutation enhanced the short-leaf and short-petiole phenotypes of *lhy-12;cca1-101* (Figure 1B and C).

To separate the enhancer mutation from the *lhy-12;cca1-101* mutations, *pta#5* was backcrossed with the wild type (L*er*). Segregants were obtained without the *lhy;cca1* mutations but with short-hypocotyl/petiole and normal-flowering phenotypes under LL (Figure 1).

pta5 as a novel allele of dwf4

To map and identify the *pta5* mutation and to determine whether it was recessive or dominant, a mutant plant of the *pta5* candidate line (L*er*) was crossed with Columbia (Col) wild type. The F_1 plants derived from this cross did not show the semi-dwarf phenotype under LL (Figure 2A), suggesting that *pta5* behaves as a recessive mutation.

F_2 progeny of the cross between *pta5* (L*er*) and Col wild type were grown under LL, and the hypocotyl and petiole lengths of the F_2 progeny were measured and compared to those of *pta5* (L*er*) and wild-type (Col and

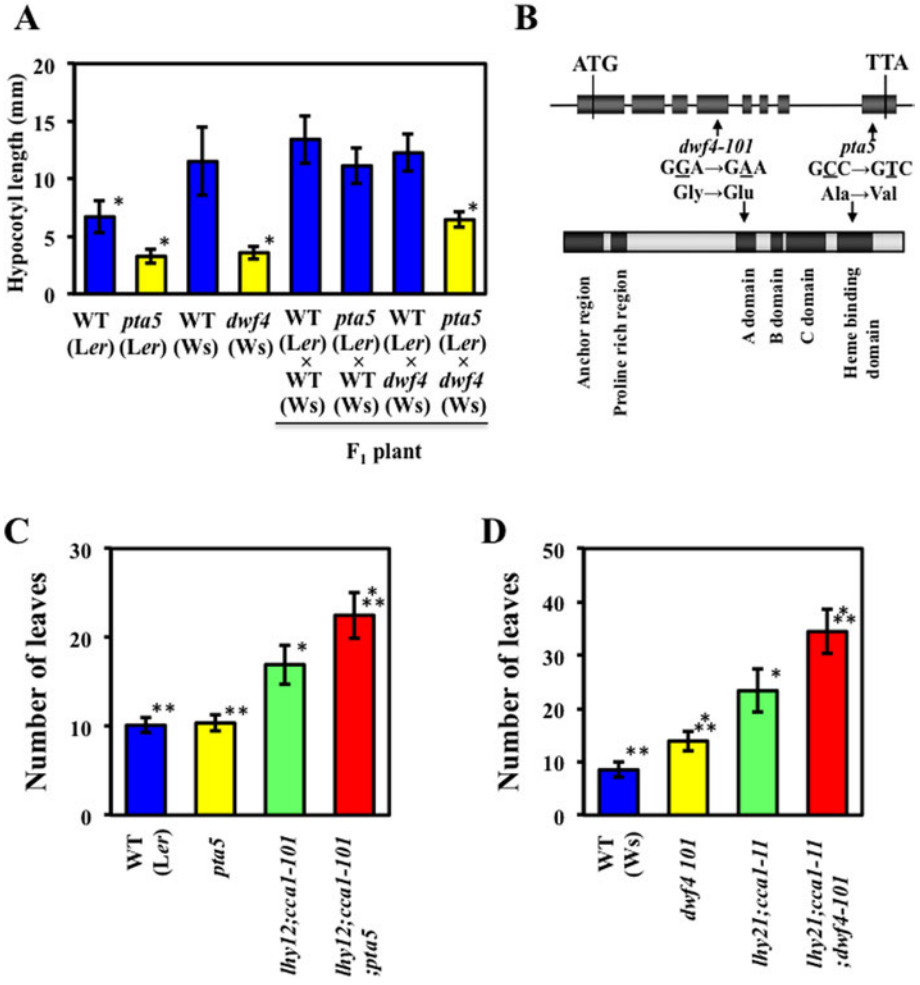

Figure 2. Identification of *pta5* as a new missense allele of *dwf4*. (A) Allelism test between *dwf4-101* (Ws) and *pta5* (L*er*). Asterisks denote statistical significance in comparison with the values for WT (L*er*)×WT (Ws) (Student's *t*-test, $p<0.01$). (B) Gene and protein structure of DWF4. A C-to-T substitution point mutation occurred in the eighth exon of *DWF4*. (C) Flowering times of WT (L*er*), *pta5*, *lhy-12;cca1-101*, and *lhy-12;cca1-101;pta5* plants grown under LL. (D) Flowering times of WT (Ws), *dwf4-101*, *lhy-21;cca1-11*, and *lhy-21;cca1-11;dwf4-101* plants grown under LL. Flowering times were scored by counting the total numbers of rosette and cauline leaves on the main stem after bolting. Means are shown±SE ($n\geqq10$). Each experiment was performed at least twice with similar results. Single and double asterisks denote statistical significance in comparison with the WT (L*er* or Ws) and *lhy;cca1* (L*er* or Ws) (Student's *t*-test, $p<0.01$).

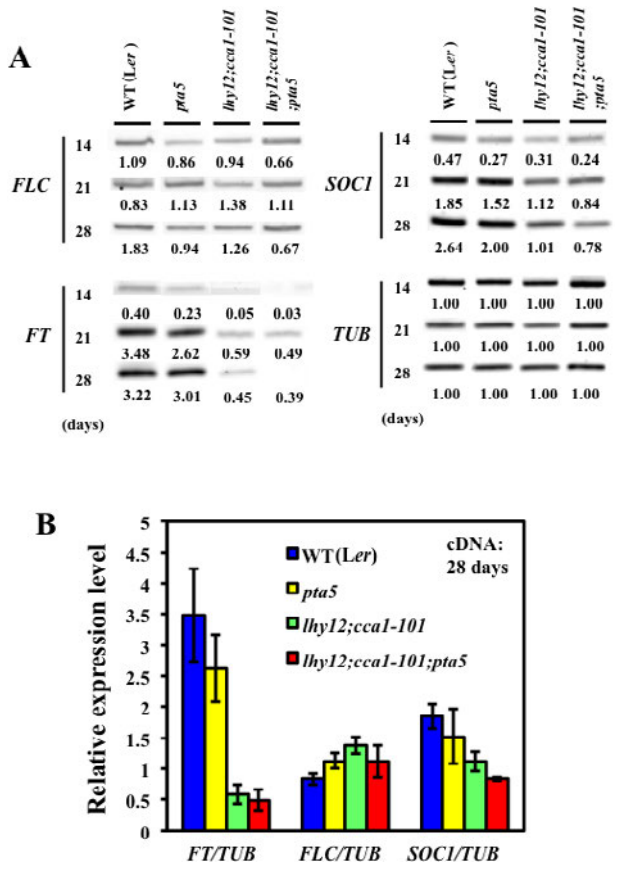

Figure 3. Expression levels of the floral activator genes *FT* and *SOC1* and the repressor gene *FLC* in plants grown under LL for 14, 21, or 28 days. The levels of the *FT*, *SOC1*, and *FLC* mRNAs in WT (L*er*), *pta5*, *lhy-12;cca1-101*, and *lhy-12;cca1-101;pta5* relative to *TUB2* were measured by semi-quantitative (A) and quantitative (B) RT-PCR. Numbers below the bands in (A) indicate relative expression levels compared to *TUB2*. For quantitative RT-PCR (B), cDNA prepared from plants grown under LL for 28 days was used. The *FT*, *FLC*, and *SOC1* transcript levels were normalized to the expression of *TUB2* measured in the same RNA samples. Data are the mean±SD of three independent RNA samples.

L*er*) plants. The *pta5* mutation was mapped to the bottom of chromosome 3 based on the semi-dwarf phenotype. The *DWF4* locus is located in this region. DWF4 is one of the key enzymes involved in the biosynthesis of brassinosteroid (Choe et al. 1998). *dwf4* mutant plants exhibit dwarf or semi-dwarf phenotypes similar to that of *pta5* (Choe et al. 1998). Therefore, we sequenced the *DWF4* gene of *pta5*, and found a point mutation (C3767 to T) that causes an amino acid substitution from alanine to valine (A466 to V) in the DWF4 protein (Figure 2B).

To determine whether the *pta5* mutation was responsible for the semi-dwarf phenotype, a complementation test was performed between *pta5* (L*er*) and *dwf4-101* (Ws, Nakamoto et al. 2006). F_1 plants obtained from crosses of *pta5* and *dwf4-101* showed phenotypes similar to those of *pta5* and *dwf4-101* under LL. The controls, F_1 plants obtained from crosses between the wild type (Ws) and *pta5* or between the wild type (L*er*) and *dwf4-101*,

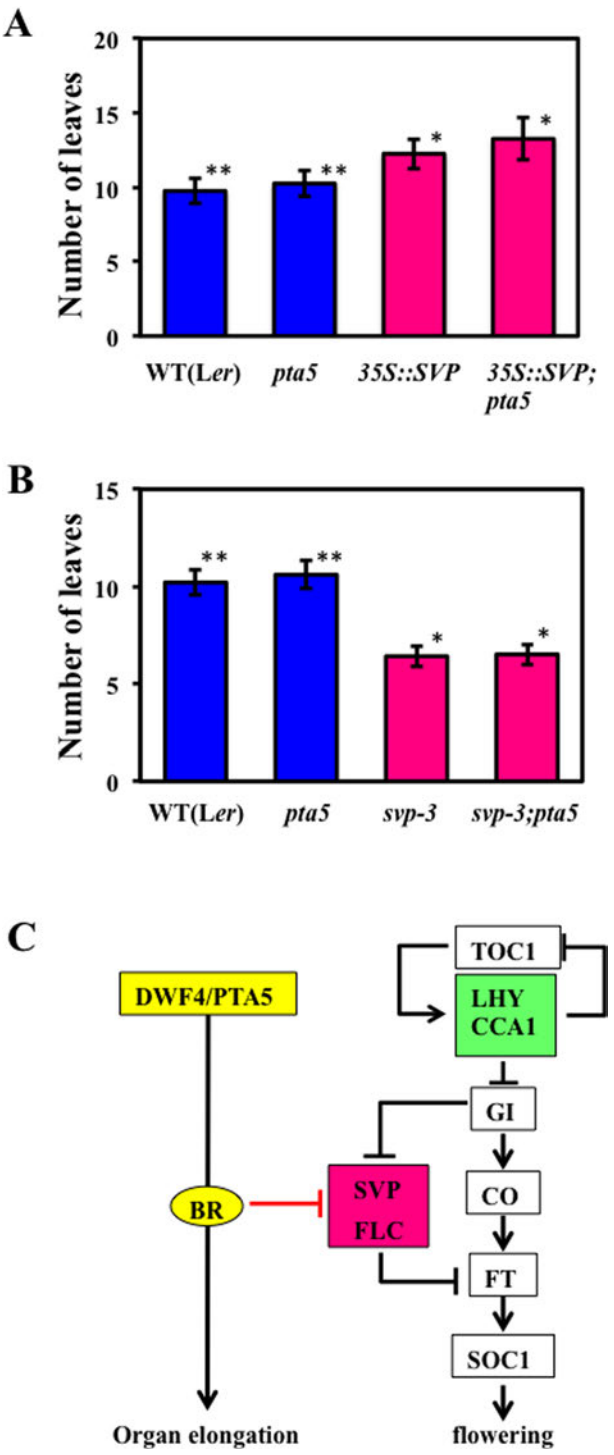

Figure 4. Genetic analysis of *35S:SVP;pta5* and *svp-3;pta5*, and a hypothetical model. (A) Flowering times of WT (L*er*), *pta5*, *35S:SVP*, and *35S:SVP;pta5* grown under LL. Single and double asterisks denote statistical significance in comparison with the WT (L*er*) and *35S:SVP*, respectively (Student's *t*-test, $p<0.01$). (B) Flowering times of WT (L*er*), *pta5*, *svp-3*, and *svp-3;pta5* grown under LL. Single and double asterisks denote statistical significance in comparison with the WT (L*er*) and *svp-3*, respectively (Student's *t*-test, $p<0.01$). Means are shown±SE ($n=10$). Each experiment was performed at least twice with similar results. (C) A hypothetical model of the regulation of organ elongation and flowering by LHY, CCA1, SVP, and BR.

showed phenotypes similar to those of the wild type (Ws and L*er*) under LL (Figure 2A). These results indicate that the semi-dwarf mutation present in the *pta5* line is indeed the *dwf4* mutant allele.

To confirm that *dwf4* enhances the late-flowering phenotype of *lhy;cca1* under LL, *lhy-21;cca1-11;dwf4-101* (Ws) was constructed. *pta5* (L*er*) delayed the flowering time of *lhy-12;cca1-101* (L*er*, Figure 2C). *dwf4-101* enhanced the late-flowering phenotype of *lhy-21;cca1-11* (Ws), similar to *pta5* enhancing that of *lhy-12;cca1* (Figure 2D).

Expression of the FT, SOC1, and FLC genes

The impact of the *pta5* mutation on the mRNA levels of the floral activator genes *FT* and *SOC1* and the floral repressor gene *FLC* was tested by semi-quantitative (Figure 3A and B) and quantitative (Figure 3C) RT-PCR in *lhy-12;cca1-102;pta5* plants grown for 14, 21, or 28 days under LL (Figure 3). Consistent with the flowering times under LL, the levels of *FT* and *SOC1* mRNAs in *lhy-12;cca1-102;pta5* plants grown for 28 days were slightly lower than those in *lhy-12;cca1-102* (Figure 3A), and much lower than those in *pta5* and the wild type (Figure 3B). In contrast, the expression of *FLC* in plants grown for 28 days was similar in all of these lines and not correlated with flowering time (Figure 3). This result indicates that *lhy;cca1* and *lhy;cca1;pta5* exhibit delayed flowering independent of *FLC* expression.

SVP and DWF4/PTA5 act in a single linear genetic pathway that controls flowering time under LL

To determine whether SVP and DWF4/PTA5 act in distinct flowering pathways, *35S:SVP;pta5* and *svp-3;pta5* were constructed, and their flowering times were compared to those of wild type, *pta5*, *35S:SVP*, and *svp-3* under LL (Figure 4A and B). No significant difference was detected between *35S:SVP;pta5* and *35S:SVP* (Figure 4A) or between *svp-3;pta5* and *svp-3* (Figure 4B). These results suggest that SVP plays a key role in the BR-dependent pathway that controls flowering time in *lhy;cca1* under LL (Figure 4C).

Discussion

In this work, we show that a mutation in the *DWF4*/*PTA5* gene, which encodes a BR biosynthesis enzyme, enhances the late-flowering and weak semi-dwarf phenotypes of *lhy;cca1* under LL (Figures 1 and 2). This result suggests that LHY, CCA1, and DWF4/PTA5 play key roles in the BR-dependent pathway that controls both flowering time and organ elongation under LL.

Alternatively, the enhanced phenotypes of *lhy;cca1* by *dwf4*/*pta5* might be due to the roles of LHY-CCA1 and DWF4/PTA5 in distinct pathways. However, this is unlikely, because i) SVP plays a key role in the late-flowering phenotype of *lhy;cca1* under LL (Fujiwara et al. 2008), and ii) SVP and DWF4/PTA5 appear to act in a single genetic pathway (Figure 4A and B).

Although the BR-dependent control of flowering time was suggested long ago, the molecular mechanisms underlying it have not been elucidated. Two groups (Domagalska et al. 2007; Yu et al. 2008) pointed out that an increased level of *FLC*, which encodes a major floral repressor, appears to play a key role in BR-dependent flowering. In our work, however, we have elucidated a novel mechanism completely different from the works by Domagalska et al. and Yu et al. We demonstrated that *lhy;cca1;pta5* and *lhy;cca1* affect the BR-dependent control of flowering time without affecting the *FLC* mRNA level, indicating that at least two distinct modes are involved in BR-dependent flowering.

Recently, we have identified seven enhancers (*pta1–7*) of *lhy;cca1* under LL. It is worth testing whether other *pta* mutations are present in genes encoding proteins involved in the biosynthesis, accumulation/degradation, or signaling of BR. Protein–protein interaction between FLC and SVP and a partial redundant role to delay flowering time were reported (Fujiwara et al. 2008; Figure 4C). An upcoming challenge will involve understanding the molecular mechanisms of how LHY, CCA1, and SVP could control the BR signaling pathway. Exogenous application of BR on the delayed flowering of *lhy;cca1* under LL would also be useful. Microarray analysis of *lhy;cca1* and *lhy;cca1;svp*, and yeast two-hybrid analysis to identify interactors of LHY, CCA1, and SVP are underway. These may be helpful to elucidate the molecular mechanisms underlying a connection between the circadian clock and BR-related control of flowering time

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