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 HYOCOTYL (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 HYOCOTYL (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*.
Lines containing the lhy;cca1 mutations cause altered photoperiodic responses (Fujiiwara 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 (Fujiiwara 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 (Fujiiwara 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 (Fujiiwara 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 (dwarf4, Choe et al. 1998), deetiolated2 (det2, Li et al. 1996), constitutive photomorphogenic dwarf (cdp, 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;det2 and ref6;det2) 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 cdp. 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 dwarf4 (Choe et al. 1998) as pta5. A clear dwarf phenotype is present in lhy;cca1; dwarf4/pta5. pta5 did not enhance the late-flowering phenotype of 35S:SVP under LL. In addition, the early flowering phenotype of syp-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 (Ler) and Ws (Wassilewskija) were used as the wild type (WT). The mutants tested included lhy-12;cca1-101 (Ler, Fujiiwara et al. 2008), lhy-21;cca1-11 (Ws, Hall et al. 2003), lhy-11;cca1-1 (Col, Fujiiwara et al. 2008), dwarf4-101 (Ws, Nakamoto et al. 2006), 35S:SVP (Ler, Fujiiwara et al. 2008), and syp-3 (Ler, Fujiiwara 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 (Ler) 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 Na₂SO₄ and five times with distilled water for 30 min. M₁ seeds were collected in pools, with each pool containing ~20 M₁ plants. Approximately 13,000 M₂ seeds representing ~1,300 M₁ 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 (Ler) were isolated and named petanko 1–7 (pta1–7). The enhancer mutations in the lhy-12;cca1-101 (Ler) background were backcrossed to the parental line lhy-12;cca1-101 (Ler) at least once before phenotypic analysis. lhy-12;cca1-101;pta5 (Ler) was backcrossed with the WT (Ler), and the pta5 single mutant was obtained. In addition, an allelism test was performed between dwarf4-101 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 (Ler) and a polymorphic strain, lhy-11:cca1-1 (Col). F2 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 F2 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 (Fujisawa 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; 1µl 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
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 (*Ler*). 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 (*Ler*) was crossed with Columbia (*Col*) wild type. The F1 plants derived from this cross did not show the semi-dwarf phenotype under LL (Figure 2A), suggesting that *pta5* behaves as a recessive mutation.

F2 progeny of the cross between *pta5* (*Ler*) and Col wild type were grown under LL, and the hypocotyl and petiole lengths of the F2 progeny were measured and compared to those of *pta5* (*Ler*) and wild-type (Col and...
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 (Ler) and dwf4-101 (Ws, Nakamoto et al. 2006). F1 plants obtained from crosses of pta5 and dwf4-101 showed phenotypes similar to those of pta5 and dwf4-101 under LL. The controls, F1 plants obtained from crosses between the wild type (Ws) and pta5 or between the wild type (Ler) and dwf4-101, Ler) 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).

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Control of flowering by BR indicates that lhy;cca1 is not correlated with flowering time (Figure 3). This result grown for 28 days was similar in all of these lines and (Figure 3B). In contrast, the expression of FLC in plants and much lower than those in pta5 (Figure 3A), slightly lower than those in lhy-12;cca1-102;pta5 (Figure 3A) or between 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.

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 ssvp-3;pta5 were constructed, and their flowering times were compared to those of wild type, pta5, 35S:SVP, and ssvp-3 under LL (Figure 4A and B). No significant difference was detected between 35S:SVP;pta5 and 35S:SVP (Figure 4A) or between ssvp-3;pta5 and ssvp-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;pta5, 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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