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Possible role of EARLY FLOWERING 3 (ELF3) in clock-dependent floral regulation by SHORT VEGETATIVE PHASE (SVP) in *Arabidopsis thaliana*

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Summary

• Circadian clock proteins play key roles in adaptations of plants to diurnal environmental conditions. The photoperiodic flowering response is one of the mechanisms of adaptation to seasonal changes in the lengths of day and night.

• Double mutations in two clock genes, *LATE ELONGATED HYPOCOTYL* (*LHY*) and *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*), accelerated flowering under short days (SDs) but delayed flowering under continuous light (LL) in *Arabidopsis thaliana*. The mechanism underlying the late flowering of *lhy;cca1* mutants under LL was investigated here.

• Late flowering of plants with overexpression of *SHORT VEGETATIVE PHASE* (*SVP*) was much more pronounced under SDs and enhanced by *constans 2* (*co-2*) under long days (LDs), suggesting that SVP and CO act independently in the photoperiodic flowering pathway. However, how SVP and FLOWERING LOCUS C (FLC) mediated the effects of LHY/CCA1 and thus influenced flowering time was not completely clear. A mutant line *lhy;cca1* in the Landsberg *erecta* (L*er*) background was established, ethyl methanesulfonate (EMS)-mutagenized and used to screen suppressors of late flowering of *lhy;cca1* under LL. Mutations in the clock gene *EARLY FLOWERING 3* (*ELF3*) were identified as suppressors.

• Overexpression and loss-of-function of *ELF3* influenced SVP protein accumulation. Therefore, we propose that, as well as the classical GIGANTEA (GI)–CO pathway, LHY/CCA1 regulates a pathway negatively controlling *FLOWERING LOCUS T* (*FT*), possibly via ELF3-SVP/FLC.

Introduction

Plants are known to have the ability to adapt themselves to their environment. Diurnal and seasonal changes in temperature, humidity and the quality and quantity of light are known to affect the expression of genes, the activity of enzymes and the developmental processes of plants. Circadian clocks are endogenous time-keeping devices that provide temporal control of physiology in accordance with predicted daily changes in the environment (Yanovsky & Kay, 2002; Mizoguchi *et al.*, 2006). Therefore, circadian clocks in plants play key roles in adaptation to environmental day:night cycles. The photoperiodic control of flowering time by the clock is one of the most extensively studied examples of a clock-controlled process. Several other examples of clock-controlled processes allowing adaptation of plants to the environment, such as cold acclimation (Bieniawska *et al.*, 2008), freezing tolerance (Franklin & Whitelam, 2007) and the shade avoidance response (Salter *et al.*, 2003) have been reported. Regulation of general fitness of *Arabidopsis* plants by circadian clock has also been shown (Green *et al.*, 2002; Michael *et al.*, 2003).

The floral regulators GIGANTEA (GI), CONSTANS (CO) and FLOWERING LOCUS T (FT) play key roles in the photoperiodic flowering responses of the long-day (LD) plant *Arabidopsis thaliana* (Yanovsky & Kay, 2002; Mizoguchi

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et al., 2006). These three genes are highly conserved in many plant species and are thought to have similar roles in the control of flowering time (Boss *et al.*, 2004). The photoperiodic flowering of *A. thaliana* has been shown to be explained in part by the Bünning's external coincidence model in which clock-controlled expression of *CO* and stabilization of the CO protein by light have important roles (Suarez-Lopez *et al.*, 2001; Valverde *et al.*, 2004).

In *A. thaliana*, the MYB proteins LATE ELONGATED HYPOCOTYL (LHY) and CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) are very close relatives to each other and essential clock components (Mizoguchi *et al.*, 2002). These two clock proteins have redundant functions and play important roles in photoperiodic flowering by controlling the rhythmic expression of flowering-time genes (Carré & 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 LD and short day (SD) (Mizoguchi *et al.*, 2002, 2005; Más, 2005). Mutations in the *A. thaliana LHY* and *CCA1* genes (*lhy;cca1*) accelerated flowering under SDs and LDs (Mizoguchi *et al.*, 2002, 2005). Other *A. thaliana* clock genes, with mutations that also delay or accelerate flowering, have been identified (Más, 2005). For example, mutations in the *EARLY FLOWERING 3* (*ELF3*) gene accelerate flowering time under both LDs and SDs (Zagotta *et al.*, 1992). The *ELF3* gene of *A. thaliana* regulates not only flowering time but also plant morphology and circadian rhythms (Carré, 2002). *ELF3* encodes a novel protein with no significant sequence similarity to characterized proteins in the existing public databases. Therefore, biochemical roles of this clock protein have not been elucidated.

We have recently demonstrated that mutations in the circadian clock genes *LHY* and *CCA1* delay flowering of *A. thaliana* under continuous light (LL; Fujiwara *et al.*, 2008). Loss of function of either *SHORT VEGETATIVE PHASE* (*SVP*) (Hartman *et al.*, 2000) or *FLOWERING LOCUS C* (*FLC*) (Michaels & Amasino, 1999) suppressed the late flowering phenotype of *lhy;cca1* under LL. It was, however, still not clear how the *lhy;cca1* mutations delayed flowering of *A. thaliana* under LL, because the mRNA levels of *SVP* and *FLC* were not greatly affected by the *lhy;cca1* mutations (Fujiwara *et al.*, 2008), and protein–protein interactions between CCA1 and SVP or FLC were not detected in a yeast two-hybrid analysis (Fujiwara *et al.*, 2008). It was evident that the finding of a missing link between LHY/CCA1 and SVP/FLC would be an important step in elucidating how SVP and FLC delay flowering more strongly in *lhy;cca1* mutants than in wild-type plants under LL.

Several *lhy;cca1* alleles have been reported. However, *lhy-11*, *lhy-12* and *lhy-13* were identified in the Landsberg *erecta* (L*er*) background (Mizoguchi *et al.*, 2002) and *cca1-1* was identified in the Wassilewskija (Ws) background (Green & Tobin, 1999). Therefore, even after several rounds of introgression, the *lhy-11;cca1-1* (L*er*), *lhy-12;cca1-1* (L*er*), *lhy-13;cca1-1* (L*er*) have genomes from two accessions, L*er* and Ws (Mizoguchi *et al.*, 2002, 2005). The *lhy-11;cca1-1* (Columbia (Col)) (Niwa *et al.*, 2007) mutant has genomes from three accessions, L*er*, Ws and Col. For the mapping of suppressor/enhancer mutations of *lhy;cca1* after ethyl methanesulfonate (EMS)-mutagenesis and genetic analysis of the mutations, the *lhy;cca1* mutant as a progenitor line for mutagenesis in one particular accession has many advantages over mutants made by crossing two different accessions. In fact, we tried to identify enhancer mutations of *lhy-12;cca1-1* (L*er*/Ws) after EMS-mutagenesis, but experienced difficulties in mapping the mutations because of natural variations caused by the combination of the L*er*, Ws and Col genomes (Fujiwara *et al.*, 2005). To find mutations that suppress or enhance the phenotypes of the *lhy;cca1* mutant by EMS-mutagenesis, the *lhy;cca1* line in one particular accession such as L*er*, Ws or Col should be established.

Here we established the *lhy;cca1* mutant in the L*er* background by identification of a new allele of *cca1* as an enhancer of the early flowering induced by *lhy-12* under SDs. This *lhy;cca1* (L*er*) mutant was EMS-mutagenized and used to screen suppressors of the late-flowering phenotype of *lhy;cca1* under LL. In this screening, we identified mutations in the clock gene *ELF3* as suppressors. Accumulation of the SVP protein was affected by overexpression and loss of function of *ELF3*. These results suggest that ELF3 may mediate between LHY/CCA1 and SVP/FLC to repress *FT* expression.

Materials and Methods

Plant material, growth conditions and measurement of flowering time

The wild-type *Arabidopsis thaliana* L. Heynh Landsberg *erecta* (L*er*) ecotype was used unless otherwise specified. The *lhy-11* (L*er*) (Mizoguchi *et al.*, 2002), *lhy-12* (L*er*) (Mizoguchi *et al.*, 2002), *cca1-1* (L*er*) (Mizoguchi *et al.*, 2002), *lhy-11;cca1-1* (L*er*) (Mizoguchi *et al.*, 2002), *lhy-11;cca1-1* (Col) (Niwa *et al.*, 2007), *lhy-12;cca1-101* (Fujiwara *et al.*, 2008), *elf3-1* (Col) (Zagotta *et al.*, 1996) and *co-2* (Koornneef *et al.*, 1991) mutants have been described previously. Plants were grown on soil in controlled environment rooms or in a plant incubator (CF-305; Tomy, Tokyo, Japan) at 24°C under LDs (16 h light : 8 h dark), SDs (10 h light : 14 h dark), or LL (continuous light) unless otherwise specified. Flowering time was measured by scoring the number of rosette and cauline leaves on the main stem. Data are presented as mean ± SE.

EMS-mutagenesis and phenotypic screening for mutations that accelerate flowering of *lhy-12* under SDs

Approximately 20 000 *lhy-12* seeds were mutagenized by imbibition in 0.3% EMS (Sigma Aldrich, St Louis, MO,

USA) for 9 h, followed by washing with 0.1 M Na_2SO_3 (twice) and distilled water (five times). M2 seeds were collected in pools, with each pool containing ~20 M1 plants. Approximately 50 000 M2 seeds representing ~5000 M1 plants after mutagenesis of *lhy-12* seeds were sown on soil and screened for early-flowering mutants under SD conditions (10 h light : 14 h dark) in a glasshouse.

Mapping of *cca1-101*

To map the *cca1-101* mutation, we crossed *lhy-12;cca1-101* with Col wild type. F2 plants that produced more leaves than *lhy-12* plants in LL were used for mapping. Rough mapping located *cca1-101* at the lower arm of chromosome 2. Fine mapping of *cca1-101* showed that the mutation is tightly linked to the *CCA1* locus. To identify the molecular lesion in *cca1-101*, we amplified and sequenced a set of PCR fragments covering the *CCA1* region from *lhy-12;cca1-101*. Comparison of the mutant sequences with that of the wild type (L*er*) and the *A. thaliana* genome sequence revealed a base-pair change in *CCA1* (At2g46830).

Sequence analysis

Sequence analysis was performed using the CEQTM DTCS-Quick Start Kit (Beckman Coulter, Fullerton, CA, USA) following the manufacturer's instructions.

Semiquantitative RT-PCR analysis of gene expression

Plants were grown on soil for 10 or 14 d, and above-ground tissues were used for RNA preparation. RT-PCR of *FT* and *TUBULIN2* (*TUB*) was performed with specific primers as reported previously (Fujiwara *et al.*, 2005, 2008). For RT-PCR of *ELF3*, the following primers were used here: 5′-TGCAAGTGAAAAAGGTTGTGAATTG-3′ and 5′-GACCATAATATCCTCCATAATGCCC-3′. Signals were detected by Southern blot. We confirmed that the PCR amplification was not saturated with the number of PCR cycles used for the experiments (Fujiwara *et al.*, 2008). All RT-PCR analyses were performed at least twice with independent RNA samples.

Southern blot analysis

PCR products were separated on 1.5% agarose gels and transferred to Biodyne BMembranes (Nippon Genetics, Tokyo, Japan). RT-PCR products were cloned using pGEM-TEasy Vector System I (Promega, Madison, WI, USA), and plasmids were extracted for PCR templates to amplify DNA fragments. The fragments were radiolabeled to act as probes. Membranes were hybridized with the radioactive probe DNAs in hybridization solution that contained 5 × SSC, 0.1% SDS, 0.1% sarkosyl, 0.75% blocking reagent (Boehringer Mannheim, Mannheim, Germany), and 5% dextran sulfate sodium salt at 65°C for 16 h. The blot was washed first with 2 × SSC and 0.1% SDS for 20 min, and then with 0.5 × SSC and 0.1% SDS for 10 min at 65°C. The hybridization signal was visualized using a BioImaging Analyzer (BAS 5000; Fuji Photo Film, Tokyo, Japan); signal intensity was quantified with Science Lab 98 Image Gauge software (version 3.1; Fuji Photo Film).

EMS-mutagenesis and phenotypic screening for mutations that accelerate flowering of *lhy-12;cca1-101* under LL

Approximately 5000 *lhy-12;cca1-101* (L*er*) seeds were mutagenized by imbibition in 0.3% EMS (Sigma Aldrich) for 9 h, followed by washing with 0.1 M Na_2SO_3 (twice) and distilled water for 30 min (five times). M2 seeds were collected in pools, with each pool containing ~20 M1 plants. Approximately 13 000 M2 seeds representing ~1300 M1 plants after mutagenesis of *lhy-12;cca1-101* seeds were sown on soil and screened for the early-flowering mutants under LL.

TILLING assay

In a search for suppressor lines of the late-flowering phenotype of *lhy-12;cca1-101* under LL that have mutations in *ELF3*, 23 suppressor lines were picked up. RNA was extracted from one or two leaves of the 23 suppressor lines, the wild type (Col) and *elf3-1* (Col) (Zagotta *et al.*, 1996). For the first PCR, the following primers were used: region 1, CTTTTTGGGCT-CCACTTTTCCTATC (tilling-cold-F1) and AGATTCAG-CTCCATTATCAGTGTCC (tilling-cold-R1); region 2, GATCTCGAAAAATCAGCATCAAGTC (tilling-cold-F2) and AGGACTTGCTACCAGAGATTCCCTG (tilling-cold-R2). For the PCR reaction, Pyrobest DNA polymerase (TaKaRa, Shiga, Japan) was used following the manufacturer's instructions. PCR was performed at 98°C for 10 s followed by 30 cycles of 98°C for 10 s, 55°C for 30 s and 72°C for 90 s. The PCR products were separated on 1.5% agarose gels and bands amplified from cDNAs but not genomic DNA were collected and purified using Freeze 'N Squeeze DNA (Bio-Rad, Hercules, CA, USA). The purified cDNAs were used as templates in the TILLING assay. The TILLING assay was performed as described previously (Till *et al.*, 2003). For the second amplification, nested PCRs were performed using primers with fluorescent dye, or INFRARED DYE (IRD) (Till *et al.*, 2003). The primers used for nested PCR were as follows: region 1, TTGGGCTCCACTTTTCCTATCTCTT (TILLING F1, with IRD800) and CAGCTCCATTATCA-GTGTCCTTCAG (TILLING R1, with IRD700); region 2, TCTCGAAAAATCAGCATCAAGTCAT (TILLING F2, with IRD800) and ACTTGCTACCAGAGATTCCCTGTGG (TILLING R2, with IRD700).

Yeast two-hybrid assay

For the yeast two-hybrid assay, each gene was amplified by PCR and cloned into the pGBKT7 or pGADT7 vector (Clontech Laboratories, Mountain View, CA, USA) (Yoshida *et al.*, 2006). For interaction studies, plasmids containing fusion proteins were co-introduced into *Saccharomyces cerevisiae* AH109 and grown on medium lacking Leu, Trp and His in the presence of 0.5, 1, 5 and 10 mM 3-aminotriazole (3-AT). pGBKT7-53, which encodes a fusion between GAL4DNA-BD and murine p53, and pGADT7-T, which encodes a fusion between GAL4AD and the SV40 large T-antigen, were used as a positive control (53/T). Membrane filter assays for α-galactosidase activities were carried out according to the manufacturer's instructions (Matchmaker Two-Hybrid System 3; Clontech Laboratories).

Biochemical analysis

Western blot analysis was performed as described previously (Fujiwara *et al.*, 2008).

Results

Isolation of novel *cca1* alleles in L*er* as enhancer mutations of the early-flowering phenotype of *lhy-12* under SDs

The *lhy;cca1* double loss-of-function mutants flower much earlier than the *lhy* or *cca1* single loss-of-function mutants and lose free-running rhythms (FRRs) in clock-controlled gene (CCG) expression after a few cycles under LL (Mizoguchi *et al.*, 2002). The *lhy* or *cca1* mutation shortens periods of CCG expression under LL and accelerates flowering under SDs. In this sense, *cca1* enhances both circadian rhythm and flowering defects of the *lhy* mutants.

To isolate novel genes that functionally interact with LHY and CCA1 to control circadian rhythms, EMS-mutagenesis was performed on *lhy-12* (L*er*) mutants and M2 populations were screened for enhancers of *lhy-12*. The rationale of this approach was that major classes of mutations would be (i) enhancer mutations of novel factors involved in the regulation of circadian rhythms together with LHY and CCA1, and (ii) new alleles of *cca1*. In total, 48 500 M2 seedlings were screened for individuals that flowered earlier than *lhy-12* and appeared similar to the *lhy;cca1* mutant under SDs. Of 596 candidate mutants recovered in 160 independent pools of M2 seedlings, 82 mutants isolated in the first screening were studied further (Fig. 1). These M2 plants were self-fertilized, and the M3 progeny were confirmed to be hygromycin resistant and kanamycin sensitive. Therefore, the 82 M2 seedlings that flowered quite early in SDs were derived from the *lhy-12* mutant and not from contamination of the *lhy;cca1* mutants that were generated previously and were kanamycin resistant. In the second screening, the plant height of the M3 plants was measured under SD conditions, because the *lhy;cca1* mutants have a shorter height phenotype (Fig. 1b–d,k; Mizoguchi *et al.*, 2002). Twelve out of 82 candidates had a shorter height than the wild type (L*er*). This phenotype was similar to that of the *lhy;cca1* mutant.

In the final screening, we tested whether these mutations enhanced the circadian rhythm defect of the *lhy-12* mutants (Fig. 2a–f). In wild-type plants, the *GI* gene showed a pattern of expression with peaks at approximately circadian time (CT) 8, CT 32 and CT 56 (Fig. 2g,h; Mizoguchi *et al.*, 2002). In the *lhy-12* and *cca1-1* single mutants, the *GI* gene cycled with a shorter period than in wild-type plants, so that under LL the second peak in expression of the *GI* gene occurred at *c*. CT 28 (Mizoguchi *et al.*, 2002; Fig. 2b,d). In the *lhy-12;cca1-1* double mutants, the first peak occurred at CT 4 and almost no clear rhythms in expression of the *GI* gene were detected after CT 8 (Mizoguchi *et al.*, 2002; Fig. 2c,d,g,h). We selected six time-points (CT 0, 4, 8, 28, 32 and 36) to examine the expression profiles of the *GI* gene under LL (Fig. 2a–f). Among these six time-points, there were two clear peaks at CT 8 and CT 32 in wild-type plants (Fig. 2a,d). The *lhy-12* progenitor line had a rather broad peak at *c*. CT 8 and CT 28 (Fig. 2b,d), whereas the *lhy;cca1* line showed a distinct expression profile, with a sharp peak at CT 4 (Fig. 2c,d). The rationale of the third screening was that (i) a major class of mutations isolated in the first screening would be enhancers that affect the flowering time of *lhy-12* through independent pathways affecting circadian rhythms, and (ii) mutations in genes whose products functionally interact with LHY and CCA1 would cause changes in *GI* expression (circadian rhythms) in the *lhy-12* background.

As expected, 10 out of 12 enhancer mutants showed quite similar profiles of *GI* gene expression to that of the *lhy-12* progenitor line (Fig. 2b,e). By contrast, two mutant lines, *70elf-1* and *38elf-1*, derived from independent pools showed a sharp peak of *GI* expression at CT 4 and gene expression was suppressed after CT 8 (Fig. 2f). These expression patterns were quite similar to those of the *lhy;cca1* double mutants constructed by crossing the *lhy-11*, *lhy-12* and *lhy-13* (L*er*) and *cca1-1* (Ws) mutants (Mizoguchi *et al.*, 2002). *70elf-1* and *38elf-1* were studied in detail.

The enhancer mutation of *lhy-12* in *70elf-1* was mapped to the lower arm of chromosome 2 (Materials and Methods). A circadian rhythm locus, *CCA1*, is located in this region. The *cca1* mutation enhances phenotypes of *lhy* (Mizoguchi *et al.*, 2002). Therefore, we then sequenced the *CCA1* gene of *70elf-1* and found a point mutation in the center of the seventh exon (Fig. 2i). This mutation causes a premature stop codon and truncates the CCA1 protein in the middle (Fig. 2i). We also found a point mutation at the end of the sixth exon of the *CCA1* gene in *38elf-1* (Fig. 2i). This mutation caused a mis-splicing of the *CCA1* mRNA and a premature stop codon (Fig. 2i,j).

We recently found that *38elf-1* possesses an additional mutation in *SVP* (*svp-3*; Fujiwara *et al.*, 2008), which accelerated flowering time of *38elf-1* under SDs. Therefore, *38elf-1* has three

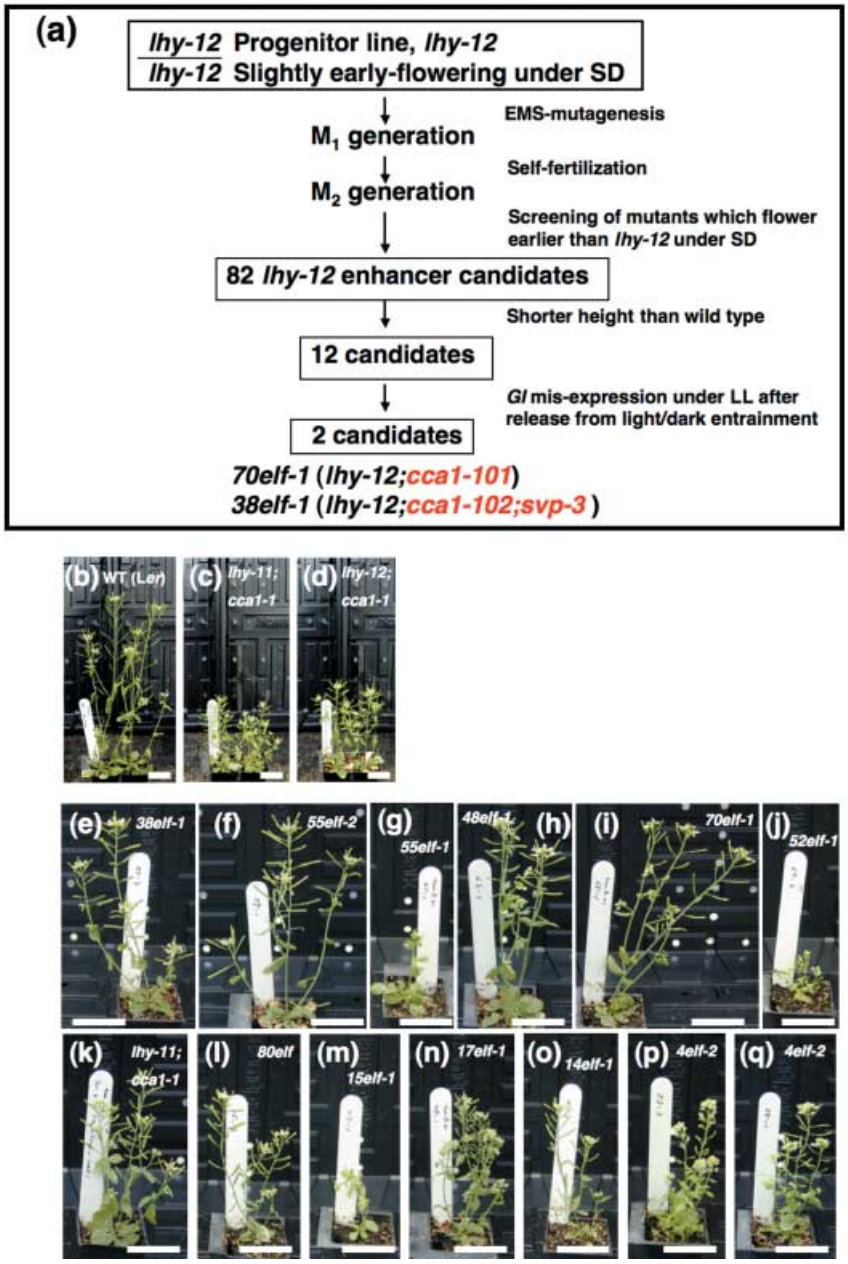

Fig. 1 Isolation of the *late elongated hypocotyl 12* (*lhy-12*) enhancer mutant lines that flowered earlier than the progenitor line *lhy-12* in short days (SDs). (a) The mutagenesis procedure used to isolate the *lhy-12* enhancer mutations. (b–q) Pictures of Landsberg *erecta* (L*er*) wild type (WT), and *lhy-11;cca1-1*, *lhy-12;cca1-1* and *lhy-12* enhancer lines (*cca*, *circadian clock associated*). *Arabidopsis thaliana* plants were grown for 4 wk under long days (LDs). *lhy-11;cca1-1* (c) and *lhy-12;cca1-1* (d) were shorter than L*er* WT (b). Twelve candidates for the *lhy-12* enhancer mutants also showed shorter heights than WT (e, *38elf-1*; f, *55elf-2*; g, *55elf-1*; h, *48elf-1*; i, *70elf-1*; j, *52elf-1*; k, *lhy-11;cca1-1* (control); l, *80elf*; m, *15elf-1*; n, *17elf-1*; o, *14elf-3*; p, *4elf-2*; q, *4elf-1*) (e–q). Bars, 3 cm.

mutations, *lhy-12*, *cca1* and *svp*. The *svp-3* mutation was separated from *38elf-1* by back-crossing with the L*er* wild type.

To determine whether the phenotypes of *70elf-1* and *38elf-1* without *svp-3* were caused by *lhy;cca1* mutations, *70elf-1* and *38elf-1* without *svp-3* were crossed with *lhy-12;cca1-11* and the flowering time of the F_1 plants was examined under SDs. The flowering time of the F_1 plants was similar to those of *lhy-12;cca1-11*, *70elf-1* and *38elf-1* without *svp-3*, indicating that *70elf-1* and *38elf-1* without *svp-3* have novel alleles of *cca1*. These are the first alleles of *cca1* in the L*er* background, and were named *cca1-101* and *cca1-102*, respectively (Fig. 2i). To examine the circadian rhythms of *70elf-1* (*lhy-12;cca1-101*) and *38elf-1* (*lhy-12;cca1-102;svp-3*) for longer periods, the mutant seedlings were first grown under light:dark cycles and then shifted to continuous light (LL; Fig. 2g,h). L*er* wild-type and *lhy-12;cca1-1* plants were used as controls. Seedlings of each genotype were harvested at CT 0, and then each 4 h for 72 h under LL conditions. RNA gel blots were made and hybridized with a *GI* gene probe (Fig. 2g,h).

Under LDs, in wild-type plants a peak in *GI* mRNA abundance occurred at zeitgeber time 8 (ZT 8) or ZT 12, as previously reported (Fig. 2g,h; Mizoguchi *et al.*, 2002). Under LL, in wild-type plants a circadian rhythm in *GI* mRNA abundance occurred, with peaks in abundance at *c.* 8, 32 and 56 h as reported previously (Fig. 2g,h; Mizoguchi *et al.*, 2002). In *70elf-1* (*lhy-12;cca1-101*) and *38elf-1* (*lhy-12;cca1-102;svp-3*),

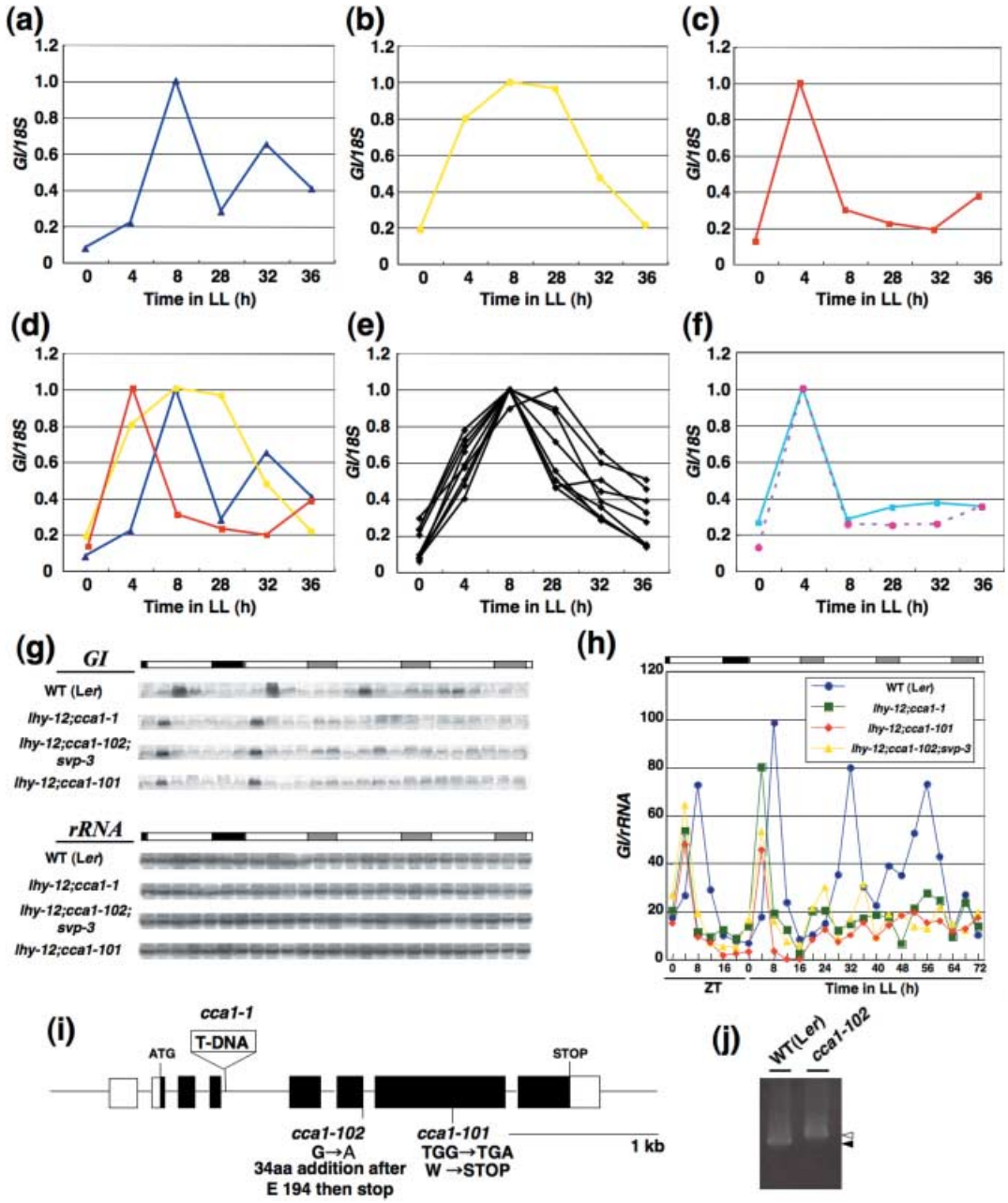

Fig. 2 Characterization of *70elf-1* (*lhy-12;cca1-101*) and *38elf-1* (*lhy-12;cca1-102;svp-3*) as the *lhy-12* enhancer mutant lines in short days (SDs) (*elf*, *early flowering*; *lhy*, *late elongated hypocotyl*; *cca*, *circadian clock associated*; *svp*, *short vegetative phase*). (a–f) The second screening of the *lhy-12* enhancer mutants based on mis-expression of *GIGANTEA* (*GI*) in continuous light (LL). Landsberg *erecta* (L*er*) wild type (WT), *lhy-12*, *lhy-12;cca1-1* and the 12 candidates for the *lhy-12* enhancers were grown in long days (LDs) for 10 d and then transferred to LL. *GI* expression levels at time 0, 4, 8, 28, 32 and 36 h were determined by northern blot analysis with 18S RNA as the control. *GI* expression profiles of L*er* WT (a), *lhy-12* (b) and *lhy-12;cca1-1* (c) were different and could be distinguished (d). *GI* expression profiles of *70elf-1* (blue) and *38elf-1* (pink) (f) were similar to that of *lhy-12;cca1-1* (c). By contrast, *GI* expression profiles of the other candidates (e) were similar to that of *lhy-12* (b). (g, h) Northern blot analysis of *GI* and the abundance of rRNA in L*er* WT, *lhy-12;cca1-1*, *lhy-12;cca1-102;svp-3* (*38elf-1*) and *lhy-12;cca1-101* (*70elf-1*). Plants were entrained under LD conditions (16 h light : 8 h dark) for 10 d and then transferred to LL conditions. The analysis is shown from the time 24 h before transferring to LL. Open and black boxes indicate light and dark periods, respectively. Gray boxes indicate subjective nights. Quantification (h) was performed with Science Lab 98 Image Gauge software as described in the Materials and Methods. Blue, green, red and yellow lines show *GI* expression in L*er* WT, *lhy-12;cca1-1*, *lhy-12;cca1-101* (*70elf-1*) and *lhy-12;cca1-102;svp-3* (*38elf-1*), respectively. (i) *CCA1* alleles in *Arabidopsis thaliana*. *cca1-1* is the first allele of the *cca1* mutation and was caused by a T-DNA insertion in the Wassilewskija (Ws) background. Both *cca1-101* and *cca1-102* were produced by point mutations. *cca1-101* in *70elf-1* was identified by mapping and sequencing. *cca1-102* was identified by sequencing. (j) *cca1-102* caused a mis-splicing and a 83-bp addition in the *CCA1* transcripts. Black and white triangles indicate the *CCA1* transcripts in wild-type and *cca1-102* plants, respectively.

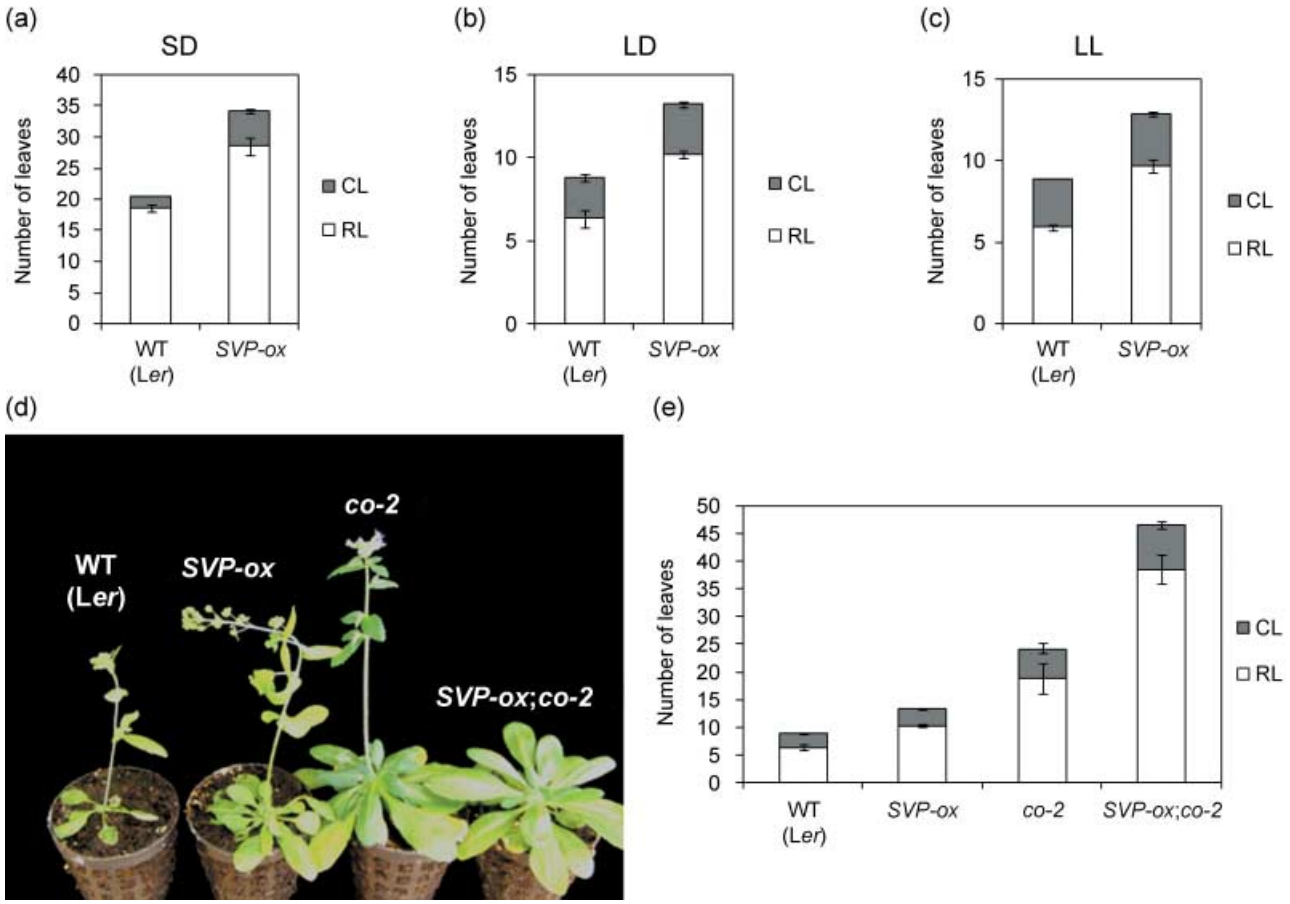

Fig. 3 Plants with overexpression of *SHORT VEGETATIVE PHASE* (*SVP-ox*) delayed flowering in the CONSTANS (CO)-independent pathway. (a–c) Flowering times of *Arabidopsis thaliana* wild-type (Landsberg *erecta* (L*er*)) and *SVP-ox* plants under different photoperiod conditions. Plants were grown under short days (SDs; a), long days (LDs; b) and continuous light (LL; c). CL and RL represent cauline and rosette leaves, respectively. (d, e) Flowering times of wild-type (L*er*), *SVP-ox*, *co-2* and *SVP-ox;co-2* plants under LDs. (d) A picture of WT (L*er*), *SVP-ox*, *co-2* and *SVP-ox;co-2* grown under LDs. (e) Flowering times of plants shown in (d). CL and RL represent cauline and rosette leaves, respectively.

the peaks in the abundance of *GI* mRNA occurred earlier than in wild-type plants under LDs and within 24 h under LL. In contrast to the wild type, no rhythms in the expression of the *GI* gene were detected in *70elf-1* (*lhy-12;cca1-101*) and *38elf-1* (*lhy-12;cca1-102;svp-3*) after CT 40 in LL (Fig. 2g,h). These *GI* expression patterns in *70elf-1* (*lhy-12;cca1-101*) and *38elf-1* (*lhy-12;cca1-102;svp-3*) were similar to that in *lhy-12;cca1-1* (Fig. 2g,h; Mizoguchi *et al.*, 2002). These results indicated that the mis-expression of *GI* was caused by *lhy;cca1* mutations in *70elf-1* (*lhy-12;cca1-101*) and *38elf-1* (*lhy-12;cca1-102;svp-3*).

Delay of flowering time in *A. thaliana* plants with overexpression of *SHORT VEGETATIVE PHASE* (*SVP-ox*) was much more pronounced under SDs and the late-flowering phenotype of *SVP-ox* was enhanced by *co-2* under LDs

Under LDs, wild-type *A. thaliana* (L*er*) flowered after producing a total of *c.* 9 leaves (Fig. 3b). Under LL the total number of leaves produced was *c.* 9 (Fig. 3c), and this number increased to approx. 21 under noninductive SD conditions (Fig. 3a). *SVP-ox* plants produced more leaves than the wild type under three photoperiodic conditions, LD (Fig. 3b), LL (Fig. 3c) and SD (Fig. 3a). Differences in total leaf numbers between wild-type and *SVP-ox* plants were much more pronounced under SDs (Fig. 3a). These results also suggest that *SVP-ox* delayed flowering time in a pathway independent of CO.

In order to test this possibility, a *SVP-ox;co-2* double mutant was constructed and the flowering time of *SVP-ox;co-2* was compared with those of the wild type, *SVP-ox* and *co-2* under LDs (Fig. 3d,e). Either *SVP-ox* or *co-2* delayed flowering time under these conditions, as previously reported (Fujiwara *et al.*, 2008). The late-flowering phenotype of *SVP-ox;co-2* was additive and *SVP-ox* plants produced much more leaves than either *SVP-ox* or *co-2*. These results indicate that SVP and CO act independently in the photoperiodic flowering pathway and support our idea that the *lhy;cca1* may delay flowering in the CO-independent pathway (Fujiwara *et al.*, 2008).

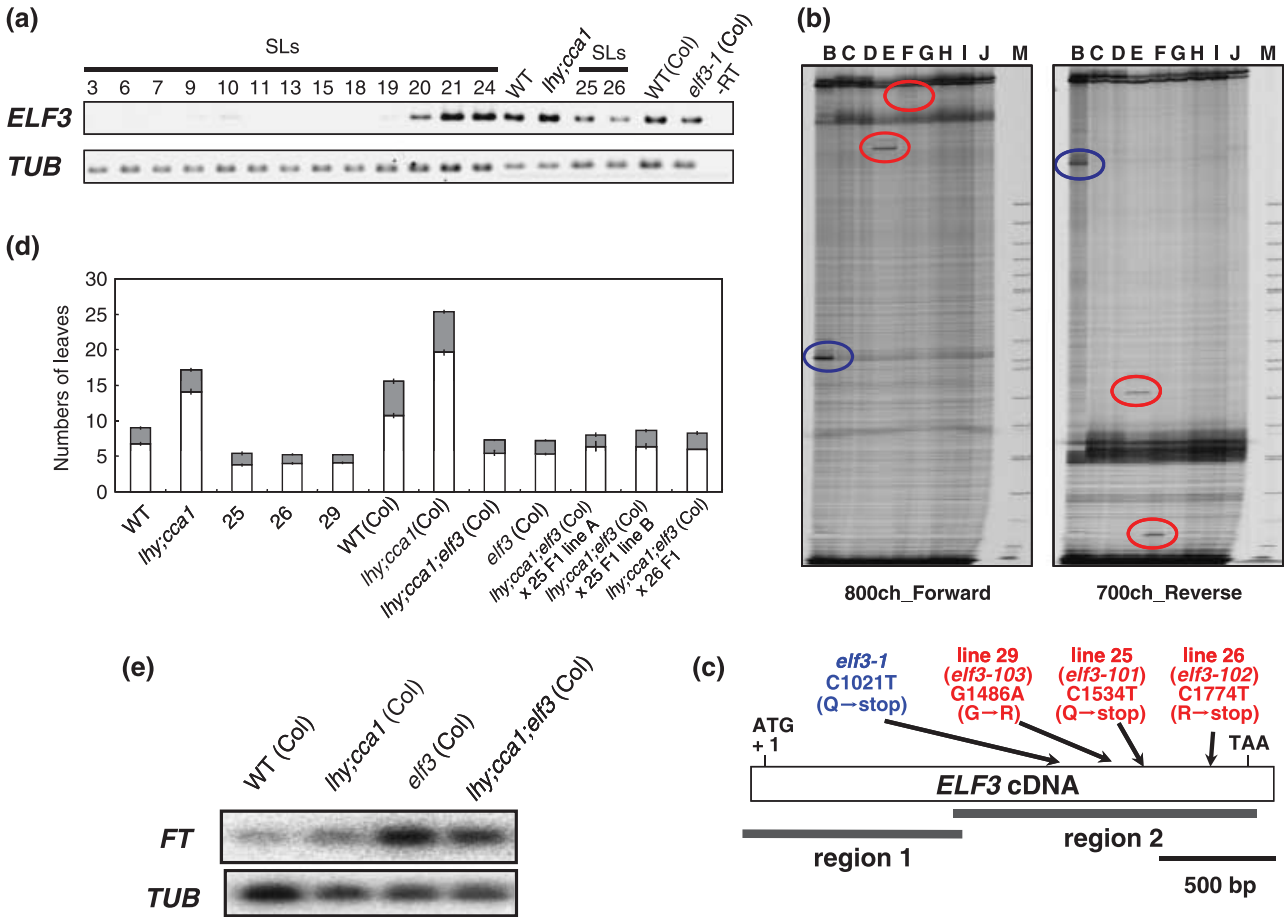

Fig. 4 Suppression of the late-flowering phenotype of *lhy;cca1* by *elf3* (*elf*, *early flowering*; *lhy*, *late elongated hypocotyl*; *cca*, *circadian clock associated*). (a) The expression level of *ELF3* was lowered in the suppressor lines (SLs) of the late-flowering phenotype of *lhy-12;cca1-101* under LL. *TUBULIN2* (*TUB*) levels are represented as controls. WT, *lhy;cca1*, WT (Col), *elf3-1* (Col) and –RT represent *Arabidopsis thaliana* wild-type ((Landsberg *erecta* (Ler)), *lhy-12;cca1-101* (Ler), wild-type (Columbia (Col)), the *elf3-1* mutant (Col) and a control without reverse transcriptase, respectively. Line numbers are shown above each lane. (b) Identification of the *lhy;cca1* suppressor mutations by TILLING (Till *et al*., 2003). *Cel*I-cleaved heteroduplexes appear as dark bands (circled in blue and red) and indicate the presence of a mutation. B, C–I, J and M indicate *elf3-1*, suppressor lines, *lhy;cca1* and marker, respectively. (c) The *elf3* mutations in the suppressor lines 25 (*elf3-101*), 26 (*elf3-102*) and 29 (*elf3-103*), and *elf3-1* were confirmed by sequencing. (d) Complementation analysis between *lhy-11;cca1-1;elf3-1* (Col) and suppressor lines (Ler). (e) *FLOWERING LOCUS T* (*FT*) expression in *lhy-11;cca1-1;elf3-1* under LL. All the experiments represented in this figure were performed at least twice with similar results.

Isolation of *elf3* as the third suppressor of the late-flowering phenotype of *lhy;cca1* under LL

The precise mechanisms underlying the negative regulation of flowering in *lhy;cca1* under LL were still not clear, because *lhy;cca1* mutations did not greatly affect the mRNA levels of *SVP* or *FLC* (Fujiwara *et al*., 2008), and we did not detect protein–protein interactions between CCA1 and SVP or FLC (Fujiwara *et al*., 2008). Therefore, how SVP and FLC delayed flowering more strongly in *lhy;cca1* mutants than in wild-type plants under LL was unknown. To find a missing link between LHY/CCA1 and SVP/FLC, we screened for more mutations that caused *lhy;cca1* to flower earlier than wild-type plants under LL (Fig. 4). To isolate other potent suppressor candidates quickly, RT-PCR screening of the M2 plants was performed. For further analysis, 43 early-flowering lines were picked. We found that 23 of the resulting suppressor lines reduced the level of *ELF3* mRNA as compared with wild-type plants (Fig. 4a). These data suggest that these lines contain mutations in *ELF3* or in a regulator gene(s) of *ELF3*.

ELF3, a potential missing link between LHY/CCA1 and SVP/FLC to control flowering

For rapid identification of possible point mutations in *ELF3* in the suppressor lines, two regions in the *ELF3* cDNA were analyzed by TILLING (Fig. 4b,c; Till *et al*., 2003). Point mutations were detected in the coding region of *ELF3* in three of the 23 suppressor lines (Fig. 4c). To determine whether these *elf3* mutations were responsible for the suppression of the

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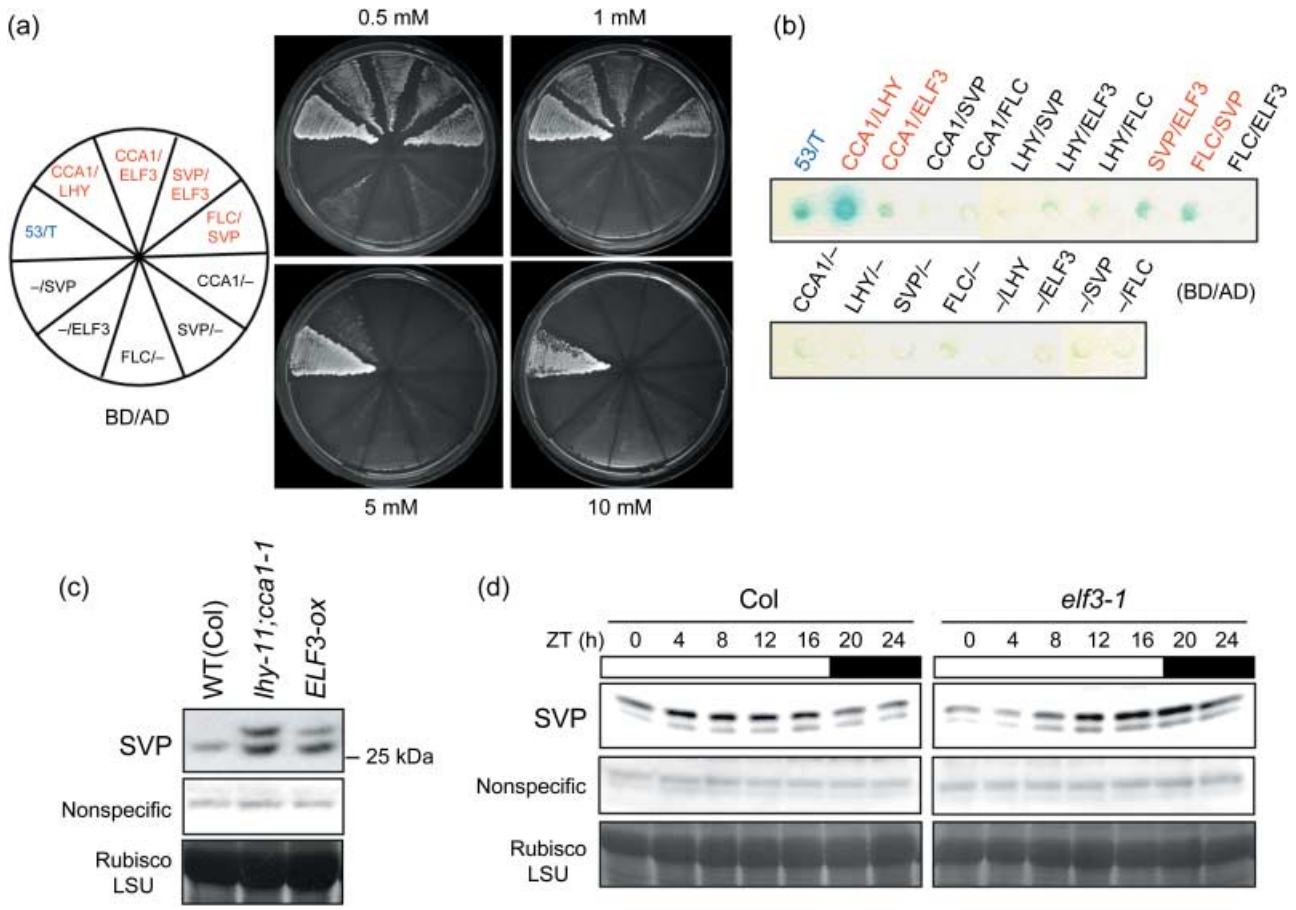

Fig. 5 Yeast two-hybrid analysis and SHORT VEGETATIVE PHASE (SVP) protein accumulation. (a, b) Yeast two-hybrid assay showing direct interaction between CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and EARLY FLOWERING 3 (ELF3) and between ELF3 and SVP. A His test (a) and filter β-gal assay (b) are shown. The concentration of 3-aminotriazole (3-AT) used in this test is shown in mM. CCA1/ELF3 indicates that CCA1 and ELF3 are fused to the Gal4 activation domain and DNA-binding domain, respectively. As a positive control, 53/T was used. (c) SVP protein level in wild type (Columbia (Col)) and plants with overexpression of *ELF3* (*ELF3-ox*) (Col) under continuous light (LL). (d) SVP protein level in wild type (Col) and *elf3-1* (Col) under long days (LDs) (16 h light : 8 h dark). Leaf extracts from each plant (30 μg) were subjected to SDS-PAGE, and immunoblotted using SVP-specific antibody. Equal protein loading was confirmed by staining of Rubisco Large Subunit (LSU) with zinc stain (Bio-Rad). All the experiments represented in this figure were performed at least twice with similar results.

late-flowering phenotype of *lhy;cca1* in LL, complementation tests between *lhy-11;cca1-1;elf3-1* and the suppressor candidates (lines 25 and 26) were performed (Fig. 4d). The F_1 progeny of these crosses showed the same characteristic *lhy;cca1;elf3* phenotype as the parents (Fig. 4d), indicating that the suppressor mutations contained in these lines were indeed *elf3* mutant alleles. These alleles were named *elf3-101* and *elf3-102*. The *elf3* mutation in line 29 was named *elf3-103*.

The *FT* mRNA level in *lhy-11;cca1-1;elf3-1* (Col) was substantially higher than in *lhy-11;cca1-1* (Col) under LL (Fig. 4e). Previously *elf3* mutants were shown to be early flowering, while overexpression of *ELF3* was demonstrated to cause late flowering and dark-green/curled leaves, phenotypes similar to those observed in *lhy;cca1* plants under LL (Covington *et al.*, 2001). Although *elf3* mutations have been reported to affect circadian rhythms, flowering and response to light, the precise biochemical function of ELF3 has remained unknown. In this work, we found that ELF3 interacted with both the clock protein CCA1 and the floral repressor SVP in the yeast two-hybrid assay (Fig. 5a,b). These interactions may be an important indication of possible *in planta* interactions, which support our hypothesis of an important link between LHY/CCA1 and SVP/FLC functions. However, false-positive interactions are relatively common in the yeast two-hybrid assay and the interactions should be verified using different methods before firm conclusions are drawn.

Accumulation of the SVP protein in *ELF3-ox* plants under LL and phase shift of SVP protein level by *elf3-1* under LDs

To elucidate the molecular mechanism underlying the delay of flowering time under LL, we performed western blots to

look for changes in SVP protein levels in *ELF3-ox* and control plants under LL. We detected accumulation of the SVP protein in *ELF3-ox* (Fig. 5c). The *lhy;cca1* and SVP-*ox* increased SVP protein levels in LL (Fujiwara *et al.*, 2008). This result is consistent with the delayed flowering of *lhy;cca1* and *ELF3-ox*, and can explain why *svp* and *elf3* mutations suppressed the late-flowering phenotype of *lhy;cca1* under LL.

To determine whether the accumulation of the SVP protein was affected by *elf3-1*, SVP protein abundance was examined in *elf3-1* and wild-type (Col) plants under LDs. Seedlings of *elf3-1* and wild-type plants were harvested at dawn (ZT 0) and then every 4 h for 24 h. SVP protein accumulation in the wild type (Col) showed a diurnal change under LDs (Fig. 5d, Supporting Information Fig. S1) as reported in the wild type (L*er*) (Fujiwara *et al.*, 2008). The SVP protein abundance was at trough levels at ZT 20 and 24 in the wild type (Col). Higher accumulation of the SVP protein was detected at ZT 4, 8, 12 and 16 in the wild type (Col). The *elf3-1* mutation delayed the phase of the SVP protein abundance by *c.* 8 h. These results also indicate that both LHY/CCA1 and ELF3 play key roles in the control of SVP protein accumulation, consistent with the genetic and two-hybrid analysis (Figs 4, 5).

Discussion

Recently we have shown that clock proteins LHY and CCA1 can regulate flowering independently of their role in regulating the established photoperiodic response pathway through the transcription of *GI-CO-FT* (Fujiwara *et al.*, 2008). We have proposed that LHY and CCA1 both activate the photoperiodic response pathway that promotes flowering and repress inhibitors of flowering such as two MADS box proteins, SVP and FLC (Fujiwara *et al.*, 2008; Mizoguchi & Yoshida, 2009). However, the molecular mechanism of the regulation of flowering time by LHY and CCA1 via SVP/FLC has not been elucidated. In this work, we found that (i) the *elf3* mutation suppressed the late-flowering phenotype of *lhy;cca1* under LL; (ii) ELF3 interacted with both CCA1 and SVP in the yeast two-hybrid assay, and (iii) *ELF3-ox* and *elf3* influenced SVP accumulation. These results indicate that the roles of LHY, CCA1 and ELF3 in regulating SVP accumulation are interrelated. These results also suggest that ELF3 may have a role as a missing link between the clock proteins LHY/CCA1 and the floral repressors SVP/FLC in the control of photoperiodic flowering in *A. thaliana*. These are discussed in more detail in the following sections.

A hypothetical model showing a possible role of ELF3 in the control of SVP by LHY and CCA1

The circadian clock has been shown to be divided into three components: input, central oscillator and output (Dunlap, 1999; Fig. 6). Our genetic studies together with our recent work (Fujiwara *et al.*, 2008) indicate that the change of flowering response in *lhy;cca1* involves enhanced activity of the clock protein ELF3 and two floral repressors SVP and FLC under LL. The yeast two-hybrid analysis suggested that ELF3 might interact directly with both CCA1 and SVP, which are part of the central oscillator and the output pathway of the *A. thaliana* circadian clock, respectively (Fig. 6). Although we used other independent methods such as the pull-down assay and Biomolecular Fluorescence Complementation (BiFC), the yeast two-hybrid results could not be confirmed using an independent method. Nevertheless, these interactions may still be an important indication of possible *in planta* interactions, which support our hypothesis of an important link between LHY/CCA1 and SVP/FLC functions (Fig. 6).

ELF3 has been shown to interact with the photoreceptor phytochrome B (phyB), which plays key roles in the input pathway (Liu *et al.*, 2001). This suggests that ELF3 may function as a scaffold to form a clock protein complex in *A. thaliana*. We propose that the circadian clock controls pathways that promote and repress flowering, and altering the balance between these pathways can switch the photoperiodic response type of a single species.

The blue-light receptor CRYPTOCHROME 2 (CRY2), the E3 ubiquitin-ligase CONSTITUTIVE PHOTOMORPHOGENESIS 1 (COP1) and the clock-associated proteins ELF3 and GI have been shown to be involved in photoperiodic flowering. Recently, ELF3 has been shown to interact directly with both COP1 and GI *in vivo* (Yu *et al.*, 2008). These data suggest that COP1 modulates the light input signal to the central oscillator of the circadian clock via targeted destabilization of the clock-associated protein GI under light:dark cycles. ELF3 appears to act as a substrate adaptor for COP1 to regulate GI. Blue light may regulate the stability of the CO protein in the GI-independent pathway (Valverde *et al.*, 2004; Yu *et al.*, 2008).

The late-flowering phenotype of the *ELF3-ox* plants can be explained by both increased accumulation of the floral repressor SVP and decreased levels of the floral activator GI. Consistent with this idea, loss of function of *ELF3* delayed the phase of SVP accumulation which did not overlap with the timing of increase of *FT* expression, and the *elf3* mutation increased the level of GI protein under light:dark cycles. If COP1 is involved in the ELF3-, LHY- and CCA1-dependent control of the SVP protein, ELF3 might act as a negative regulator for the COP1-dependent degradation of the SVP protein.

Roles of LHY and CCA1 under LL and light:dark cycles

Why does the effect of LHY/CCA1 on the acceleration of flowering time appear to be so much more important in LL than in light:dark cycles? The ELF3 protein was shown to be unstable in darkness (Liu *et al.*, 2001), suggesting that the ELF3 protein may be more abundant in LL than LDs. The ELF3 protein peaks around dusk but is still expressed at dawn (Liu *et al.*, 2001). The LHY and CCA1 proteins peak around

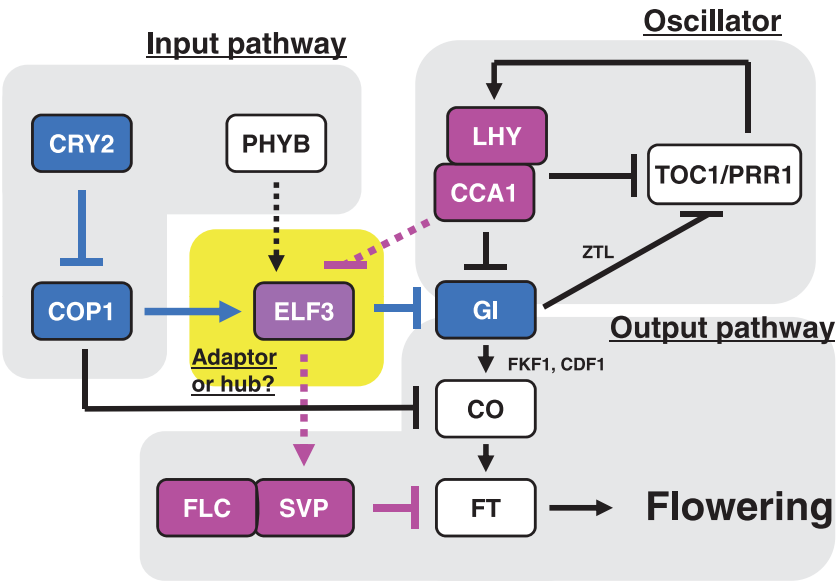

Fig. 6 A hypothetical model showing possible roles of EARLY FLOWERING 3 (ELF3) in the photoperiodic control of flowering time by the circadian clock in *Arabidopsis thaliana*. The circadian clock is composed of three components, the input pathway, the central oscillator and the output pathway (Dunlap, 1999). The input pathway includes photoreceptors such as phytochromes and cryptochromes (Mizoguchi *et al*., 2006). CONSTITUTIVE PHOTOMORPHOGENESIS 1 (COP1) also plays important roles in this process (Yu *et al*., 2008). LATE ELONGATED HYPOCOTYL (LHY), CIRCADIAN CLOCK ASSOCIATED 1 (CCA1), TIMING OF CAB EXPRESSION 1 (TOC1)/PSEUDO-RESPONSE REGULATOR1 (PRR1) and several other clock proteins are part of the central oscillator (Mizoguchi *et al*., 2006). The three floral activators GIGANTEA (GI), CONSTANS (CO) and FLOWERING LOCUS T (FT) play key roles in the photoperiodic induction of flowering under long days (LDs). Mutations in the *LHY* and *CCA1* genes (*lhy;cca1*) shift the phase of *GI* expression and accelerate flowering under short days (SDs) and LDs (Mizoguchi *et al*., 2002; Mizoguchi *et al*., 2005). By contrast, *lhy;cca1* appears to delay flowering in the GI–CO-independent pathway under continuous light (LL) and this phenotype is suppressed by mutations in the MADS box gene *SHORT VEGETATIVE PHASE* (*SVP*) or *FLOWERING LOCUS C* (*FLC*) (Fujiwara *et al*., 2008; Mizoguchi & Yoshida, 2009). Protein–protein interactions between LHY and CCA1 and between SVP and FLC have been shown in the yeast two-hybrid system (Fujiwara *et al*., 2008; Mizoguchi & Yoshida, 2009). In this work, we have identified *elf3* as the third suppressor mutation of the late-flowering phenotype of *lhy;cca1* under LL. Overexpression of *ELF3* increased the SVP protein level under LL and *elf3* delayed the phase of diurnal accumulation of the SVP protein under LDs. Yeast two-hybrid analysis suggests that ELF3 may interact both with CCA1 and with SVP. These results suggest that ELF3 may have an important role in the control of SVP protein accumulation by LHY and CCA1, and this is shown in pink. It has recently been demonstrated that the E3 ubiquitin-ligase COP1 of the input pathway and ELF3 coordinately regulate the central oscillator and one of the output pathways, flowering time. COP1 and ELF3 modulates the activity of the clock associated protein GI possibly in the blue light and CRY2 dependent pathway (Yu *et al*., 2008). During the night, levels of COP1 and ELF3 proteins increase in the nucleus (Liu *et al*., 2001; von Arnim & Deng, 1994; Yu *et al*., 2008), where they interact and bind to GI to promote its degradation (Yu *et al*., 2008). These processes are shown in blue in this figure. Blue-light-enhanced formation of GI–ZEITLUPE (ZTL) and GI–FLAVIN-BINDING, KELCH REPEAT, F-BOX 1 (FKF1) complexes has been demonstrated (Kim *et al*., 2007; Sawa *et al*., 2007). GI–ZTL plays a key role in the protein degradation of TOC1/PRR1 and affects circadian rhythms (Kim *et al*., 2007), whereas GI–FKF1 is involved in the control of a transcription factor, CYCLING DOF FACTOR 1 (CDF1), that negatively regulates the *CO* mRNA level (Sawa *et al*., 2007). ELF3 appears to be a multifunctional protein and may act as an important hub or adaptor protein to transduce a variety of inputs into the outputs in the complex signaling network. GI may have a similar role as a hub or adaptor protein.

dawn (Wang & Tobin, 1998) and may prevent SVP and FLC from forming a complex that represses *FT* expression at dawn in LDs. By contrast, in LL, the ELF3 protein level may be higher than that in LDs, but constant expression of the CCA1 protein may negatively regulate ELF3 function. The coincidence of a higher level of LHY/CCA1 with a higher level of ELF3 in LL than LDs may explain in part why the effect of CCA1 on ELF3 appears to be more important in LL than LDs. Nuclear localization of COP1 is controlled by light and dark (von Arnim & Deng, 1994). COP1 plays key roles in the degradation of many regulatory proteins in the dark period. The protein–protein interaction between COP1 and ELF3 (Yu *et al*., 2008) suggests that COP1 may have an important role in the opposite flowering phenotypes of *lhy;cca1* under LL and SDs.

Change of day-length response by *lhy;cca1*

Under SDs, *lhy;cca1* plants flowered earlier than under LL. Moreover, *lhy;cca1* plants flowered earlier as the dark period was extended (Fujiwara *et al*., 2008), indicating that mutations in circadian clock components appeared to change the photoperiodic response type of *A. thaliana* from a facultative LD plant to one with unique characteristics of an SD plant.

Under light:dark cycles, the promotion of flowering in *lhy;cca1* mutants through the photoperiodic pathway predominates and early flowering occurs. However, when these mutants are grown under LL, the repression of flowering may occur through interactions with two MADS box proteins, SVP and FLC, and a clock protein, ELF3. In wild-type plants, the balance in activity between these pathways appears to be different from that in *lhy;cca1* mutants, so that even in LL the promotion of flowering by the photoperiodic pathway may overcome the effect of SVP and FLC. We propose that the *lhy;cca1* double mutants exhibit unique characteristics of SD plants, flowering earlier under SDs than under LL, possibly through alteration of the balance between these pathways.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 SHORT VEGETATIVE PHASE (SVP) protein accumulation in wild-type (Col) and *early flowering 3-1* (*elf3-1*) (Col) under long days (LDs).

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