Original Paper

Isolation of novel gain- and loss-of-function alleles of the circadian clock gene *LATE ELONGATED HYPOCOTYL (LHY)* in *Arabidopsis*

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Abstract In *Arabidopsis*, two myb-related proteins, LATE ELONGATED HYPOCOTYL (LHY) and CIRCADIAN CLOCK ASSOCIATED 1 (CCA1), play key roles in the control of circadian rhythms. Photoperiodic flowering and elongation of hypocotyls are regulated by a circadian clock. Here, we report the isolation and characterization of a novel deletion allele (*lhy-14*) and a gain-of-function allele (*lhy-2*) of *lhy*. Based on long hypocotyl and late flowering phenotypes under long days, *lhy-2* was isolated as an intragenic suppressor of *lhy-12*, one of the loss-of-function alleles of *lhy*. Although the *lhy-12* mRNA has a 19-bp truncation, we found that this was not caused by a deletion in the *LHY* but by a point mutation. We propose a model explaining the loss-of-function of *LHY* in *lhy-12* and the partial suppression of the *lhy-12* phenotype by *lhy-2*, by the abnormal splicing of *LHY* mRNA.

Key words: *Arabidopsis*, CCA1, circadian rhythms, LHY, splicing.

Feedback regulation of the transcription of clock-component genes is one of the most common and important mechanisms that control clock functions in animals, fungi, and plants (Dunlap 1999, McClung 2001, Mas 2005). At least six genes are putative central oscillators of a circadian clock in *Arabidopsis* (Mizuno and Nakamichi 2005). Two of these, *LATE ELONGATED HYPOCOTYL* (*LHY*) and *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*), encode closely related transcription factors of the MYB family (Schaffer et al. 1998; Wang and Tobin 1998). The second class includes four related genes, *TIMING OF CAB EXPRESSION 1* (*TOC1*)/*PSEUDO-RESPONSE REGULATOR 1* (*PRR1*), *PRR5*, *PRR7*, and *PRR9* (Strayer et al. 2000; Mizuno and Nakamichi 2005). These genes encode proteins containing a sequence related to the receiver domain of two-component signaling (Strayer et al. 2000, Mizuno and Nakamichi 2005). An initial model of the *Arabidopsis* circadian clock consisted of a single negative feedback loop comprised of the morning-expressed *LHY* and *CCA1* and the evening-expressed *TOC1* (Alabadi et al. 2001). The negative regulation of *TOC1* by LHY and CCA1 was proposed based on two observations. First, *TOC1* expression is dampened in *LHY*- or *CCA1*-overexpressing plants (Alabadi et al. 2001). Second, LHY and CCA1 bind to the evening element (EE) of the *TOC1* promoter *in vitro* (Alabadi et al. 2001). *TOC1* has been assumed to function as a positive effector of *LHY* and *CCA1*, because *LHY* and *CCA1* expression is lower in the loss-of-function mutant *toc1-2* (Alabadi et al. 2001). However, reduced *LHY* and *CCA1* expression is observed in both *TOC1*-overexpressing plants and the *zeitlupe* (*ztl*) mutant, which is defective in TOC1 protein turnover (Somers et al. 2000; Makino et al. 2002; Mas et al. 2003). Other genes, such as *GIGANTEA* (*GI*), *PRR3*, *EARLY FLOWERING 3* (*ELF3*), *EARLY FLOWERING 4* (*ELF4*), *LUX ARRHYTHMO* (*LUX*), *TIME FOR COFFEE* (*TIC*), and *LOV KELCH PROTEIN 2* (*LKP2*) have also been proposed to be involved in the regulation of the *Arabidopsis* circadian clock (Hicks et al. 2001; Schultz et al. 2001; Doyle et al. 2002; Hall et al. 2003; Hazen et al. 2005, Mizoguchi et al. 2005; Mizuno and Nakamichi 2005). The initial negative feedback model composed of only LHY, CCA1, and TOC1 has been modified and improved with the addition of some of these clock-

Abbreviations: *CCA1, CIRCADIAN CLOCK ASSOCIATED 1*; *CO*, *CONSTANS*; *FT*, *FLOWERING LOCUS T*; *GI, GIGANTEA*; LD, long day; *LHY*, *LATE ELONGATED HYPOCOTYL*; SD, short day.
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associated genes (Locke et al. 2005).

Photoperiodic flowering responses are crucial for plants to adapt to changing seasonal day lengths. Long-day (LD) plants flower earlier when the day length exceeds a certain critical threshold, whereas short-day (SD) plants flower earlier when the day length is shorter than a critical amount of time. As *Arabidopsis* is a quantitative (or facultative) LD plant, a seasonal change in the day length is essential for the determination of flowering time (Koornneef et al. 1998). A change in the daily light cycle affects the central oscillators of a circadian clock, and contributes to the timing of the transition from the vegetative phase to the reproductive phase (Searle and Coupland 2004; Imaizumi and Kay 2006). Circadian defects cause early flowering under non-inductive SD conditions, resulting in flowering being promoted in the *lhy*, *cca1*, and *toc1* mutants under short days (Somers et al. 1998; Mizoguchi et al. 2002). The loss of function of both *LHY* and *CCA1* causes extremely early flowering under short days (Mizoguchi et al. 2002). *GI* mediates the circadian clock and the control of flowering time in *Arabidopsis*. A phase shift of *GI* appears to cause the phase shift of downstream gene *CONSTANS* (*CO*), and the activation of the further downstream gene *FLOWERING LOCUS T* (*FT*) and quite early flowering in the *lhy cca1* double mutant (Mizoguchi et al. 2005). The flowering pathway containing the *GI*, *CO*, and *FT* genes is preserved in rice, a SD plant (Hayama and Coupland 2004). The rice genes orthologous to *CO* and *FT* are *Hd1* and *Hd3a*, respectively. The reverse responses to daylength in *Arabidopsis* and rice are mainly due to the different functions of *CO* in *Arabidopsis* and *Hd1* in rice (Hayama and Coupland 2004).

One gain-of-function allele (*lhy-1*) (Schaffer et al. 1998) and four loss-of-function alleles (*lhy-11*, *lhy-12*, *lhy-13*, and *lhy-21*) (Mizoguchi et al. 2002; Hall et al. 2003) of *lhy* and two loss-of-function alleles of *cca1* (*cca1-1* and *cca1-11*) (Green and Tobin 1999; Hall et al. 2003) have been identified and characterized. All of these mutations are associated with transgenes with antibiotic markers such as kanamycin and hygromycin resistance genes. Although many clock-associated genes have been isolated recently, the roles of these clock proteins in clock-controlled biological events, such as photoperiodic flowering, leaf movement, and hypocotyl elongation, are largely unknown. To test the genetic interactions and epistatic relations among the clock-associated genes, it is necessary to establish *Arabidopsis* mutants with many combinations of loss- and gain-of-function mutations. For this analysis, novel loss-of-function alleles free of antibiotic markers, and more gain-of-function alleles of *lhy* and *cca1*, would be useful.

In this report, we present the isolation and characterization of a novel deletion allele (*lhy-14*) and a gain-of-function allele (*lhy-2*) of *lhy* in the L*er* background. We propose a model to explain the loss-of-function of *LHY* in *lhy-12* and the partial suppression of the *lhy-12* phenotype by *lhy-2*, based on the abnormal splicing of the *LHY* mRNA.

Materials and methods

Plant materials and growth conditions

The Landsberg *erecta* (L*er*) wild-type accession of *Arabidopsis thaliana* was used unless otherwise indicated. The *lhy-1* (L*er*, Schaffer et al. 1998), *lhy-11*, *lhy-12*, and *lhy-13* (L*er*, Mizoguchi et al. 2002) mutants were described previously. The *cca1-1* mutant was originally isolated in the Ws background (Green and Tobin 1999). F3 populations introgressed twice into L*er* were selected for *cca1-1*-homozygous lines based on kanamycin resistance (Mizoguchi et al. 2002). The *lhy-2* and *lhy-14* mutants were backcrossed once or twice to L*er* before phenotypic analysis.

Plants were grown in soil in controlled environment rooms under either LDs (16 h light/8 h dark) or SDs (10 h light/14 h dark), as described (Mizoguchi et al. 2005). For continuous-light (LL) experiments, plants were grown on soil under LDs (16 h light/8 h dark) for eight days and then transferred to LL at dawn, as described (Mizoguchi et al. 2002).

Mutagenesis and phenotypic screening for mutations that cause long hypocotyls in lhy-12

Approximately 20,000 *lhy-12* seeds were mutagenized by imbibition in 0.3% ethyl methanesulfonate (EMS; Sigma) for 9 h, followed by washing with 0.1 M Na_2SO_3 (twice) and distilled water (five times). M_2 seeds were collected in pools, with each pool containing ~20 M_1 plants. Approximately 40,000 M2 seeds representing ~2,000 M1 mutagenized *lhy-12* seeds were sown on agar plates and grown under LD, and the seedlings were screened for long hypocotyls.

Isolation of lhy-14

Arabidopsis mutant plants that flowered earlier than wild-type plants were isolated (Reeves et al. 2002), and one of these mutants possessed two mutations, *early in short days 4* (*esd4*) (Reeves et al. 2002; Murtas et al. 2003) and *lhy-14*.

Measurement of flowering time

Flowering time was measured by scoring the number of rosette and cauline leaves on the main stem. The number of days from sowing until flower buds were visible by eye at the center of the rosette was also recorded. Data are presented as mean +/− SD.

Luminescence measurement and analysis of circadian rhythms using CAB2:luciferase (LUC) transgenic plants

lhy-2, *lhy-1*, *lhy-11*, *lhy-11*, and *cca1-1* mutant plants containing the *CAB2 : LUC* gene were generated by crossing and subjected to luminescence analysis (Mizoguchi et al. 2005). Seven-day-old plants grown on agar in LD were transferred into agar-filled wells of 96-well opaque microtiter plates and treated with 20 μL of 5 mM D-luciferin per plant

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(n=24). Starting the next day, the luminescence of individual seedlings was measured by counting in a Packard TopCount scintillation counter (Packard, Meriden, CT). The average luminescence for each genotype at each time point was calculated from the luminescence normalized for each emitting individual.

RNA analysis

RNA (10 μg) was separated on denaturing formaldehyde gels of 1.2% agarose and transferred to Hybond NX nylon membrane. Hybridization with radioactively labeled probes was performed overnight at 65 °C in 0.3 M sodium phosphate buffer, pH 7.0, 7% SDS, 1 mM EDTA, and 1% bovine serum albumin. The blot was washed twice for 20 min at 65 °C with 0.2X SSC, 0.1% SDS. Full-length *CCA1* and *LHY* cDNAs were used as probes. Images were visualized and the intensities were quantified; the values are represented relative to the lowest value of the wild-type samples after normalization to the level of the 18 S RNA control.

RT-PCR analysis

RT-PCR was performed with 1 μg of total RNA using a SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA). For the detection of misspliced *LHY*, the following primers were used: 5′-GATGCAAA-ACTTGTTTCATCGGCCT-3′ and 5′-GAGGTCTTACTTGT-TTCAATGTCG-3′. The products were separated on 2.5% agarose gels and stained with ethidium bromide.

Results

Isolation of an intragenic suppressor mutation of lhy-12 based on the long-hypocotyl phenotype

The *lhy-1* mutation is a dominant allele from which the *LHY* mRNA is overexpressed, due to the insertion of a transposon in the *LHY* 5’-untranslated leader (Schaffer et al. 1998). As well as disrupting circadian rhythms, the *lhy-1* mutation causes elongated hypocotyls and late flowering under LDs (Schaffer et al. 1998). To isolate loss-of-function mutations in *LHY*, EMS mutagenesis was performed on *lhy-1*, and M2 populations were screened for suppressors of the *lhy-1* phenotype. *lhy-12* was isolated as an intragenic suppressor that inactivates the *LHY* gene (Mizoguchi et al. 2002). *lhy-12* shortens circadian rhythms and causes early flowering under SDs, but the hypocotyl lengths of *lhy-12* plants are similar to those of wild-type plants (Mizoguchi et al. 2002).

To isolate factors that functionally interact with LHY/CCA1, EMS mutagenesis was performed on *lhy-12*, and M2 populations were screened for mutants that showed long hypocotyls on agar plates under LDs. The circadian clock controls hypocotyl length in plants, and mutations of clock-component genes often cause hypocotyls longer or shorter than those in wild-type plants (Mizoguchi and Coupland 2000). We expected that second mutations in genes with clock-associated functions in the *lhy-12* background would cause severe defects in circadian rhythms and, therefore, the long-hypocotyl phenotype.

In total, 40,000 M2 seedlings from 100 independent pools were screened for individuals with a longer hypocotyl than *lhy-12* seedlings (Figure 1A). Of eleven candidate mutants recovered in eight independent pools of M2 seedlings, one line (*101/105LH-1*) was studied in detail, because this plant showed not only long hypocotyls but also late flowering and long petioles under LDs (Figure 1B, C). All of these characteristics are rather similar to those of *lhy-1* (Schaffer et al. 1998). The *101/105LH-1* plant was self-fertilized, and the M3 progeny were confirmed as carrying the transposon in the 5′-untranslated leader of the *LHY* gene, based on resistance to hygromycin. Although all of the M3 progeny were hygromycin-resistant, the hypocotyl lengths of the M3 progeny segregated into a 3 : 1 ratio (long: short hypocotyls). All of the segregants with long hypocotyls flowered later than *lhy-12* and wild-type plants under LDs. In contrast, the flowering times of the segregants with short hypocotyls were similar to those of *lhy-12* and wild-type plants under LDs.

The *101/105LH-1* mutant plant from the M2 population was backcrossed to the L*er* wild type, and the F1 progeny were examined for hygromycin resistance, hypocotyl length, and flowering time. All of the F1 seedlings were resistant to hygromycin. Approximately half of the F1 plants showed the long-hypocotyl phenotype, and all of these plants flowered later than *lhy-12* and wild-type plants under LDs. Hygromycin resistance, long hypocotyls, and late flowering co-segregated in the next generation. These results indicate either that *101/105LH-1* contains an intragenic suppressor mutation in the *LHY* gene that behaves as a dominant mutation in the *lhy-12* background, or that *101/105LH-1* was a revertant of the *lhy-12* mutation. The mutation present in the *101/105LH-1* line was preliminarily assigned the name *lhy-2*.

lhy-2 and lhy-1 have similar effects on clock-controlled genes under LL

The clock-regulated *CAB2 : LUC* reporter was used to test the circadian rhythm in *lhy-2* (Figure 1F). *CAB2 : LUC* in *lhy-12*, *lhy-12 cca1-1*, *lhy-1*, and the wild type were also tested as controls. *lhy-12* and *lhy-12 cca1-1* showed short-period and arrhythmic phenotypes, respectively, as reported (Mizoguchi et al. 2002). *lhy-2* showed an arrhythmic phenotype similar to that of *lhy-1* under LL. A similar arrhythmic phenotype was also observed in the expression of *CCA1* in northern blot analysis (Figure 1G).

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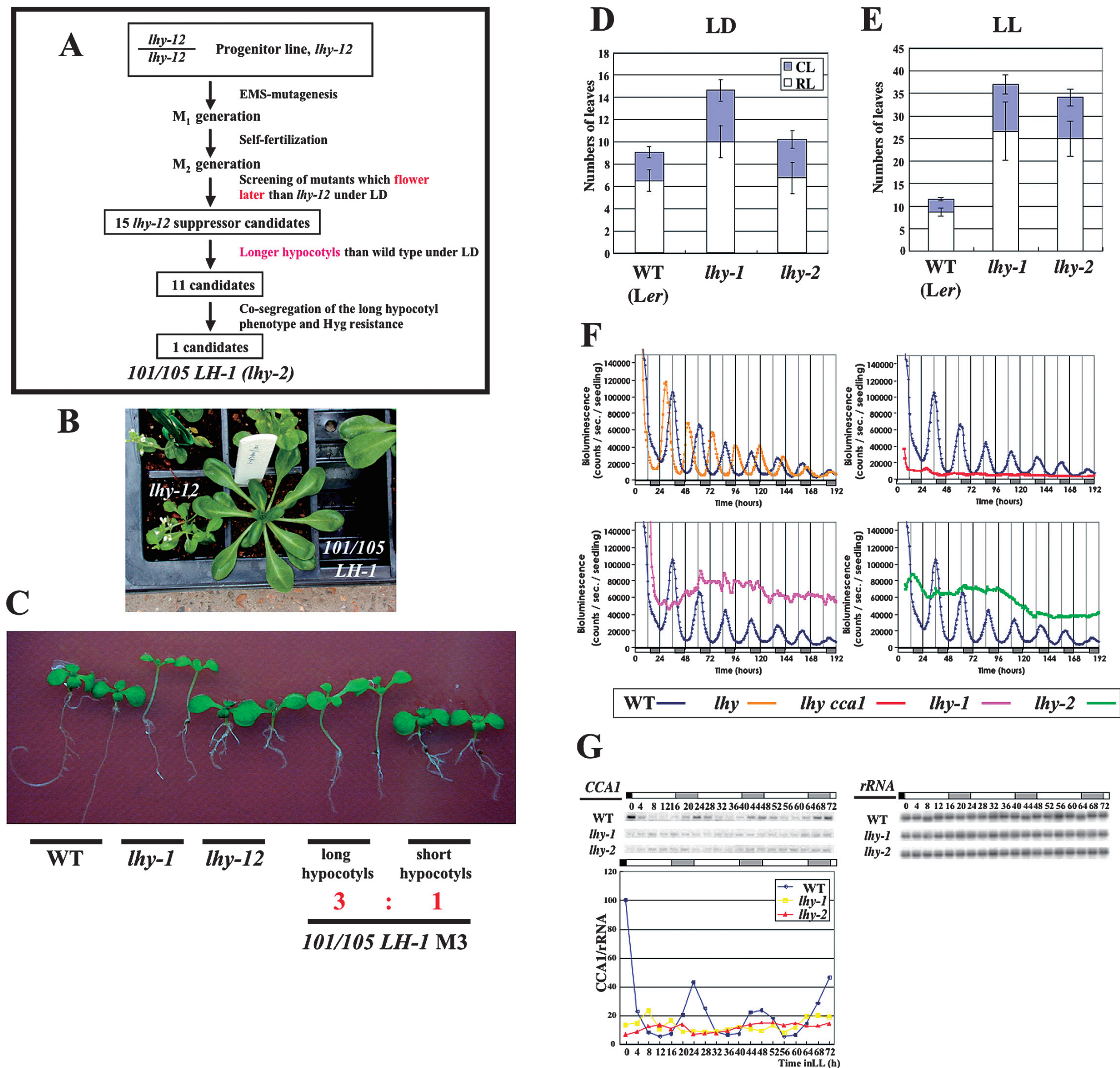

Figure 1. Isolation and characterization of *lhy-2*. (A) Mutagenesis procedure used to isolate *lhy-2*. The progenitor line carried the recessive *lhy-12* mutation. (B) Late-flowering phenotype of the *101/105 LH-1* mutant (M2) under LDs. *lhy-12* and *101/105 LH-1* M2 plants were grown under LD for four weeks. (C) *lhy-2* is a dominant allele that causes long hypocotyls. A *101/105LH-1* M2 plant was self-fertilized, and the M3 progeny segregated into a 3 : 1 ratio (long: short hypocotyls). Wild-type, *lhy-12*, and *lhy-1* plants are shown as controls. (D, E) Late-flowering phenotype of *lhy-2* under LDs (D) and LL (E). Wild-type and *lhy-1* plants are shown as controls. CL and RL represent cauline and rosette leaves, respectively. Means are shown +/− standard deviation. (F) *CAB2 : LUC* expression in wild-type (L*er*), *lhy-11*, *lhy-11 cca1-1*, *lhy-1*, and *lhy-2* plants under LL. The expression of the clock-controlled gene *CAB2* was followed by the luminescence of transgenic plants carrying the *CAB2 : LUC* transgene and grown under LL. The results are presented as normalized luminescence. Data are the means of the luminescence of 20 individual seedlings. Numbers on the horizontal axis represent the time in hours after the start of the LL treatment. Light and dark boxes on the horizontal axis represent subjective day and subjective night, respectively. Each experiment was performed at least twice, with similar results. (G) *CCA1* expression in wild-type (L*er*), *lhy-1*, and *lhy-2* plants under LL. Plants were entrained to LD cycles (16 h light/ 8 h dark) for eight days and then released into LL. The mRNA abundance was analyzed by RNA gel blotting (Materials and methods). The results are presented as a proportion of the highest value after standardization with respect to the 18S RNA (rRNA) levels. Light and dark boxes on the horizontal axis represent subjective day and subjective night, respectively. Each experiment was performed at least twice, with similar results.

A 19-bp truncation in the LHY mRNA in lhy-12 may be caused by missplicing

To examine whether the *lhy-2* mutation was an intragenic suppressor or a revertant of *lhy-12*, genomic DNA was isolated from *101/105LH-1*, *lhy-12*, and wild-type plants, and the *LHY* gene sequences in these lines were determined. The *LHY* cDNA in *lhy-12* has a 19-bp truncation in the seventh exon (Mizoguchi et al. 2002). To our surprise, there was no deletion in the *lhy-12* genomic DNA, but there was a point mutation (G to A) near the end of the sixth intron (Figure 2A, D). This result suggests that the 19-bp truncation in the *LHY* gene

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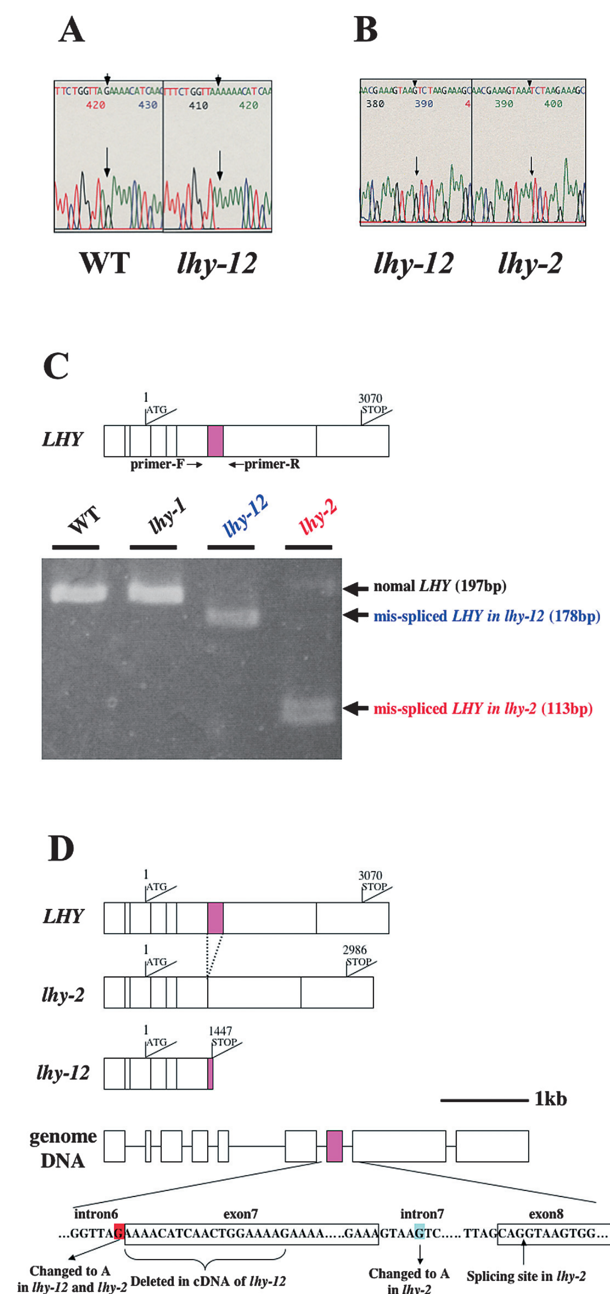

Figure 2. Identification of the *lhy-2* mutation. (A) A G-to-A substitution at the end of the sixth intron in *lhy-12*. (B) A G-to-A substitution in the seventh exon in *lhy-2*. (C) Missplicing of *LHY* in *lhy-12* and *lhy-2*. Exons are represented by open boxes. Primers that hybridize at the locations shown were used to amplify transcripts of the *LHY* gene by RT-PCR from wild-type (L*er*), *lhy-1*, *lhy-12*, and *lhy-2*. The different sizes of the RT-PCR products are indicated by arrowheads. (D) *lhy-12* and *lhy-2/lhy-12* contain truncation of a 19-bp region and a large portion, respectively, of the seventh exon of the *LHY* transcript. A point mutation (G1414 to A) was found at the end of the sixth intron of *LHY* in *lhy-12* (Figure 2A). The final G at the end of an intron is essential for the proper splicing of transcripts (Matsushima et al. 2004). The *lhy-12* mutation appears to cause the missplicing of *LHY*, the truncation of the first 19 nucleotides of the seventh exon, and a premature stop codon. A point mutation (G1500 to A) was found near the beginning of the seventh intron of *LHY* in *lhy-2* (Figure 2B). The first three nucleotides of the eighth exon together with the seventh exon are truncated in *lhy-2*.

in *lhy-12* is caused by missplicing of the *LHY* mRNA.

The lhy-2 mutation causes a truncation of the seventh exon of the LHY gene in the lhy-12 background

We next amplified exons 6–8, including the region containing the truncation in *lhy-12*, by RT-PCR using cDNAs as templates, and compared the sizes of the PCR products (Figure 2C). The size of this region in *lhy-12* was smaller than that in wild-type plants, as expected. The size of the major PCR product in *101/105LH-1* was much smaller than that of *lhy-12*, indicating that *lhy-2* is not a revertant of the *lhy-12* mutation, but is, instead, a second mutation in the *lhy-12* background. Comparing the sequence from *101/105LH-1* to that from *lhy-12* identified a change from G to A at position +1500 in the seventh intron (Figure 2B, D). A truncation of the seventh exon and the first three nucleotides of the eighth exon in the *LHY* cDNA from the *101/105LH-1* line was confirmed, but the truncation did not cause a premature stop codon in the *LHY* transcript (Figure 2D).

Isolation of a deletion allele of lhy (lhy-14) in the Ler background

A mutant line that flowered much earlier than wild-type plants under SDs was previously isolated and named *early flowering in short-days 4* (*esd4*) (Reeves et al. 2002). The *ESD4* gene was isolated using map-based cloning (Reeves et al. 2002, Murtas et al. 2003). We found that this mutant line showed a short-period phenotype using the *CAB2 : LUC* reporter under continuous-light conditions (data not shown). The extreme early-flowering phenotype was complemented by transformation of the *ESD4* gene, but the short-period phenotype was not (data not shown). These results indicate that a mutation distinct from *esd4* is responsible for the clock phenotype.

The short-period mutation was separated from the *esd4* mutation and mapped near the circadian-rhythm locus *LHY* on the top of chromosome 1 (data not shown). Loss of function of *LHY* (*lhy-11, lhy-12, and lhy-13*) shortens circadian rhythms in *Arabidopsis* (Mizoguchi et al. 2002). Therefore, we sequenced the *LHY* gene of the short-period mutant and found a 649-bp deletion from the fifth intron to eighth exon (Figure 3A). This mutation causes a premature stop codon and truncates the LHY protein in the middle (Figure 3B). The mutant exhibited an early-flowering phenotype similar to those of *lhy-11, lhy-12,* and *lhy-13* under SDs but not LDs (Figure 3C, D). To test whether the early-flowering phenotype of the mutant was caused by the *lhy* mutation, the short-period mutant was crossed with *lhy-12* and the flowering time of the F1 plants under SDs was examined. The flowering time of the F1 plants was similar to those of the short-period mutant and *lhy-12* plants (data not shown),

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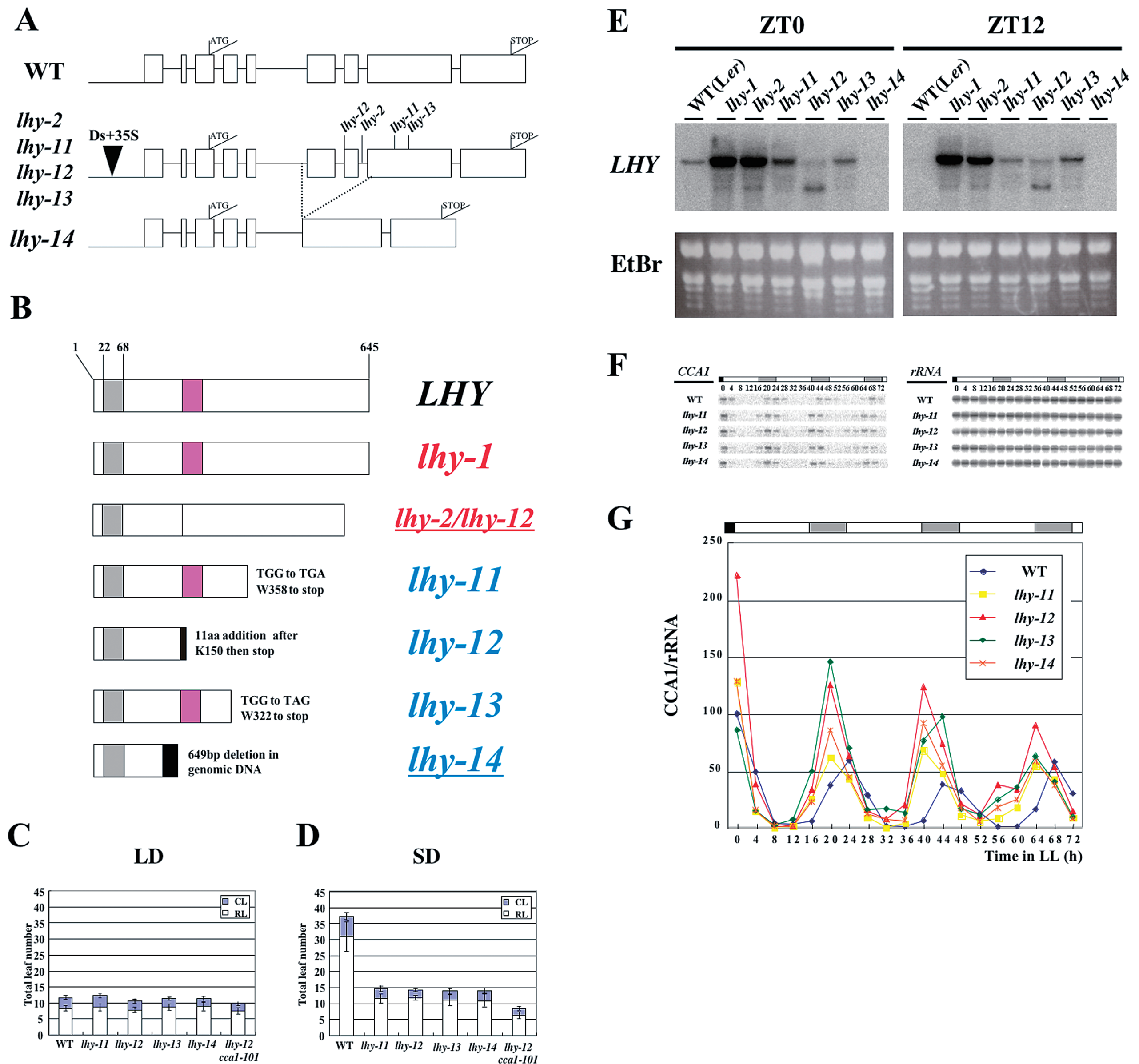

Figure 3. Isolation of a deletion allele of *lhy*. (A) Summary of the *LHY* genomic structure in *lhy* alleles. The presence of a transposon (Ds) with the *35S* constitutive promoter is indicated by black triangles. Exons are represented by open boxes. (B) LHY structure in *lhy* alleles. Dark and pink boxes represent myb DNA-binding domains and the truncated region in *lhy-2/lhy-12*, respectively. In *lhy-14*, a 649-bp deletion of the genomic DNA sequence causes a premature stop codon and truncates the LHY protein. (C, D) Flowering times of *lhy-14* under LDs (C) and SDs (D). Wild-type, *lhy-11*, *lhy-12*, *lhy-13*, and *lhy-12 cca1-101* (Fujiwara et al. submitted) were also used as controls. CL and RL represent cauline and rosette leaves, respectively. Means are shown +/− standard deviation. (E) *LHY* expression in loss- and gain-of-function mutants of *LHY* at ZT0 and ZT12 under LDs. *lhy-11, lhy-12, lhy-13*, and *lhy-14* are loss-of-function alleles, and *lhy-1* and *lhy-2* are gain-of-function alleles. The abundance of the *LHY* mRNA peaks around dawn (ZT0) and is quite low in the evening (ZT12). The mRNA abundance was analyzed by RNA gel blotting (Materials and methods). rRNAs stained with ethidium bromide are shown as a loading control. (F, G) *CCA1* expression in wild-type (L*er*), *lhy-14*, *lhy-11*, *lhy-12*, and *lhy-13* plants under LL. Plants were entrained to LD cycles (16 h light/8 h dark) for eight days and then released into LL. The mRNA abundance was analyzed by RNA gel blotting. The results are presented as a proportion of the highest value after standardization with respect to the 18S RNA (rRNA) levels (G). Black, white, and dark boxes on the horizontal axis represent night, subjective day, and subjective night, respectively. Each experiment was performed at least twice, with similar results.

indicating that the mutation is a novel allele of *lhy*. This is the first allele of *lhy* not caused by the insertion of a transgene (such as a T-DNA or a transposon). The mutation was named *lhy-14*.

Next, we tested the expression levels of *LHY* in the *lhy* alleles and wild-type plants (Figure 3E). LD-grown seedlings of each genotype were harvested at Zeitgeber Time (ZT) 0 and ZT12. RNA gel blots were prepared and hybridized with a full-length *LHY* probe. The endogenous *LHY* expression in wild-type plants peaks at dawn (ZT0), decreases gradually, and is at trough level at ZT12 (Schaffer et al. 1998; Figure 3E). *LHY* was highly expressed in the two gain-of-function alleles *lhy-1* and *lhy-2* at both ZT0 and ZT12. Constitutive levels of *LHY*

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transcripts in *lhy-11*, *lhy-12*, and *lhy-13* were observed at ZT0 and ZT12 (Figure 3E). The expression levels in *lhy-11*, *lhy-12*, and *lhy-13* were higher than those in the wild-type but lower than that in *lhy-1*/*lhy-2* at ZT12 (Figure 3E). The size of the major transcript of *LHY* in *lhy-12* is much smaller than those of the wild-type and other *lhy* alleles (Figure 3E). In *lhy-14*, the expression of *LHY* was undetectable using this method (Figure 3E). Point mutations, missplicing, and large or small truncation in the *LHY* transcripts may affect the stability of the mutant forms of the *LHY* mRNAs through a mechanism called the nonsense-mediated mRNA decay (NMD) (Isken and Maquat 2007, Figure 3A, B, E).

Comparison of circadian rhythms in loss-of-function alleles of lhy (lhy-14, lhy-11, lhy-12, and lhy-13) under LL

The circadian rhythms of *lhy-12* have been extensively analyzed and reported, but those of *lhy-11* and *lhy-13* have not. To compare the circadian rhythms of the novel deletion allele of *lhy*, *lhy-14*, to those of *lhy-11*, *lhy-12*, and *lhy-13*, the mutant seedlings were first grown under light/dark (L/D) cycles and then shifted to continuous light LL (Figure 3F, G). Seedlings of each genotype were harvested at circadian time 0 (CT 0; Methods) and then at 4-h time points for 72 h under LL. RNA gel blots were prepared and hybridized with a *CCA1* gene probe (Figure 3F, G).

In wild-type plants under LL, a circadian rhythm in the abundance of the *CCA1* mRNA was observed, with peaks in abundance at subjective dawn (Green and Tobin 1999, Mizoguchi et al. 2002). In *lhy-14*, *lhy-11*, *lhy-12*, and *lhy-13*, circadian rhythms in the *CCA1* mRNA abundance were still detected, but the peaks in the mRNA abundance occurred earlier than in wild-type plants (Figure 3F, G). Therefore, at this resolution, the *lhy-14* mutation in the L*er* background appears to shorten the period of circadian rhythms in LL, as seen in *lhy-11*, *lhy-12*, and *lhy-13*.

Discussion

lhy and cca1 mutant alleles in Arabidopsis

At least five loss-of-function and two gain-of-function alleles of *lhy* exist. Three loss-of-function alleles (*lhy-11*, *lhy-12*, and *lhy-13*) were isolated as intragenic suppressors of *lhy-1* in the L*er* background (Mizoguchi et al. 2002). They therefore contain a transposon insertion with the *35S* promoter and the hygromycin resistance gene in their 5'-untranslated regions (Figure 3A). *lhy-21* is a T-DNA insertional mutant with a kanamycin resistance gene in the Ws accession (Hall et al. 2003). *lhy-14*, isolated in this work, is the first deletion allele of *lhy* in the L*er* accession with no selection marker. In contrast, two loss-of-function alleles of *cca1* are known. *cca1-1* and *cca1-11* are T-DNA insertional mutants with the kanamycin resistance gene in the Ws accession (Green and Tobin 1999; Hall et al. 2003). We recently isolated two loss-of-function alleles, *cca1-101* and *cca1-102*, in the L*er* accession that lack selection markers (Fujiwara et al. submitted). *lhy-14*, *cca1-101*, and *cca1-102* will be useful in generating *lhy cca1* double mutants in the L*er* accession. Three selection markers, the kanamycin, hygromycin, and BASTA resistance genes, are most often used for transformation of *Arabidopsis. lhy cca1* double mutants free of selection markers will be useful for the transformation of more than two constructs. Most of the important flowering-time mutants are in the L*er* accession. *lhy-14*, *cca1-101*, and *cca1-102* will be useful in generating and analyzing multiple mutants of the flowering/clock pathways, and in avoiding natural variations caused by crossing two different accessions. All of the loss-of-function alleles of *lhy* and *cca1* isolated so far are nonsense alleles or T-DNA insertional mutants. *LHY* and *CCA1* encode proteins with a single myb domain and long C-terminal regions (Schaffer et al. 1998; Wang and Tobin 1998). The myb domains function as DNA-binding domains that recognize the CCA1 binding sequence (CBS) and the EE, which are key *cis* elements for clock-controlled expression (Wang et al. 1997; Harmer et al. 2000). In contrast, the functions of the long C-terminal domains have not been fully elucidated. The end of the C-terminal region of CCA1 has been shown to be required for interaction with the β subunit of casein kinase II (CK2) (Sugano et al. 1998; Mizoguchi et al., 2006). LHY and CCA1 have 6 conserved regions including the myb domain and these 6 regions are highly conserved in LHY/CCA1 related proteins from other plant species (Miwa et al. 2006). The seventh exon is located in the center of *LHY* coding sequence and outside of the conserved regions and, at least, is dispensable for the long hypocotyl, late flowering, and arrhythmic phenotypes under LL in *LHY*-overexpressing plants (Figures 1 and 2). Isolation of missense alleles of *lhy* and *cca1* will be useful in understanding the detailed functions of each domain. These mutations that create missense alleles may have much weaker phenotypes than those that lead to nonsense alleles or T-DNA insertional mutants. Screening for such missense alleles based on the EMS mutagenesis of wild-type seeds may be difficult. We have isolated two *cca1* alleles, *cca1-101* and *cca1-102*, in L*er* as enhancers of the early flowering of *lhy-12* under SDs (Fujiwara et al. submitted). This sensitized system can be used to identify missense alleles of *cca1* with subtle phenotypes when separated from the *lhy-12* mutation. Conversely, EMS mutagenesis of *cca1* can be used to screen for missense alleles of *lhy*.

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lhy-12 and lhy-2 cause missplicing of the LHY mRNA

We have reported that the *lhy-12* cDNA has a 19-bp truncation that leads to a premature stop codon (Mizoguchi et al. 2002). In this work, we found the point mutation near the border of the sixth intron and seventh exon of *LHY* in *lhy-12* (Figure 2D). The single nucleotide change in *lhy-12* is likely to cause the truncation in the *LHY* mRNA (Figures 2C and 3A). AG sequences at the ends of introns, such as that at the end of the sixth intron, are important for the proper splicing of an mRNA (Matsushima et al. 2004). The next AG sequence 19 bp downstream of the *lhy-12* mutation appears to be recognized as a splicing site. Furthermore, the *lhy-12* and *lhy-2* mutations are likely to cause the truncation of a large portion of the seventh exon and the first three nucleotides of the eighth exon of the *LHY* transcript (Figures 2D and 3A). The *lhy-12* and *lhy-2* mutations might change the conformation of premature *LHY* mRNA. The molecular mechanism responsible for the missplicing is unclear.

lhy-2 had the same effects on the circadian clock as *lhy-1* (Figure 1F, G), suggesting that the seventh exon does not have an essential role in clock regulation. However, the late-flowering phenotype in *lhy-2* was weaker than that of *lhy-1* under LDs but not under LL (Figure 1D, E). The LHY protein level is controlled by proteosomal degradation, and a diurnal rhythm of LHY is seen in *lhy-1*, suggesting that the rhythmic phenotype of *lhy-1* under light-dark cycles is caused by the diurnal oscillation of the LHY protein level (Kim et al. 2003; Song et al. 2005). The stability of LHY under LD cycles may be different in *lhy-1* and *lhy-2*. Alternatively, LHY may have two distinct roles in the control of general circadian rhythms and flowering. Overproduction of LHY proteins with or without the seventh exon had different effects on flowering under LDs, suggesting that a domain encoded by the seventh exon is required for protein-protein interaction with a key molecule to control flowering under these conditions. Screening for mutants that suppress the late-flowering phenotype of *lhy-1* under LD and yeast two-hybrid screening of proteins that interact with LHY, but not that produced from mRNAs without the seventh exon, will be useful in testing this possibility.

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