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# A Role for CHH Methylation in the Parent-of-Origin Effect on Altered Circadian Rhythms and Biomass Heterosis in *Arabidopsis* Intraspecific Hybrids[W][OPEN]

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**Hybrid plants and animals often show increased levels of growth and fitness, a phenomenon known as hybrid vigor or heterosis. Circadian rhythms optimize physiology and metabolism in plants and animals. In plant hybrids and polyploids, expression changes of the genes within the circadian regulatory network, such as *CIRCADIAN CLOCK ASSOCIATED1* (*CCA1*), lead to heterosis. However, the relationship between allelic *CCA1* expression and heterosis has remained elusive. Here, we show a parent-of-origin effect on altered circadian rhythms and heterosis in *Arabidopsis thaliana* F1 hybrids. This parent-of-origin effect on biomass heterosis correlates with altered *CCA1* expression amplitudes, which are associated with methylation levels of CHH (where H = A, T, or C) sites in the promoter region. The direction of rhythmic expression and hybrid vigor is reversed in reciprocal F1 crosses involving mutants that are defective in the RNA-directed DNA methylation pathway (*argonaute4* and *nuclear RNA polymerase D1a*) but not in the maintenance methylation pathway (*methyltransferase1* and *decrease in DNA methylation1*). This parent-of-origin effect on circadian regulation and heterosis is established during early embryogenesis and maintained throughout growth and development.**

## INTRODUCTION

Most living organisms have adapted to 24-h day/night cycles. Circadian rhythms are maintained by internal clocks and mediate physiology and metabolism in plants and animals (McClung, 2006; Wijnen and Young, 2006; Bass and Takahashi, 2010; Chen, 2010). In humans, energy intake and metabolism have a diurnal rhythm, and disturbance of the circadian rhythms leads to pathogenesis, including obesity, type 2 diabetes, and cardiovascular diseases (Prasai et al., 2008; Bass and Takahashi, 2010). *clock*−/− mutant mice lacking a diurnal feeding rhythm are hyperphagic and obese and develop a series of metabolic syndromes (Turek et al., 2005).

Accumulating evidence supports a role for the circadian clock in orchestrating carbohydrate metabolism, leading to hybrid vigor or heterosis. In *Arabidopsis thaliana*, circadian rhythms intrinsically regulate growth and development through diurnal changes in 30% or more of the transcriptome, including transcripts involved in photosynthesis and starch metabolism (Harmer et al., 2000; Smith et al., 2004; Covington et al., 2008). Transcriptional repressors including *CIRCADIAN CLOCK ASSOCIATED1* (*CCA1*), *LATE ELONGATED HYPOCOTYL* (*LHY*), and their reciprocal regulators *TIMING OF CAB EXPRESSION1* (*TOC1*) and *GIGANTEA* play critical roles in complex circadian regulatory networks in plants (McClung, 2006; Huang et al., 2012; Nagel and Kay, 2012). As a result, matching internal clock lengths with external light cycles increases $CO_2$ fixation, growth, and fitness (Dodd et al., 2005). This is consistent with the recent finding of the circadian regulation of gene expression and photosynthetic activities in chloroplasts (Noordally et al., 2013). Indeed, altering the expression of circadian regulators increases chlorophyll biosynthesis and sugar and starch metabolism in *Arabidopsis* hybrids and allopolyploids (Ni et al., 2009). Hybrids can synthesize more starch during the day and degrade more starch at night. Clearly, the more sugar and starch that accumulates during the day, the more these molecules can be utilized through degradation at night (Graf et al., 2010) to promote growth. In the clock and starch mutants, starch cannot be fully degraded to promote growth, leading to growth retardation (Graf and Smith, 2011). Thus, this positive correlation of daytime starch accumulation with growth vigor and the negative association of morning residual starch levels with growth are not contradictory (Chen, 2013). The growth vigor in *Arabidopsis* hybrids and allotetraploids is partly controlled by the epigenetic regulation of central circadian clock oscillator components, including *CCA1* (Ni et al., 2009). Notably, the association of increased biomass with *CCA1* repression during the day has been independently demonstrated in *Arabidopsis* intraspecific

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hybrids (Miller et al., 2012; Shen et al., 2012). In superhybrid rice (*Oryza sativa*), yield-related quantitative trait loci are also associated with gene expression changes in the circadian clock and light signaling pathways (Song et al., 2010). Moreover, down-regulation of *CCA1* expression during the day in *Arabidopsis* diploids leads to more starch at dusk and increased biomass, a phenotype similar to that seen in the hybrids (Ni et al., 2009).

The mechanism for regulating maternal and paternal alleles of clock genes that affect heterosis remains unknown. In *Arabidopsis*, de novo methylation of CHH and CHG sites (where H = A, T, or C) is established through the RNA-directed DNA methylation (RdDM) pathway (Wassenegger et al., 1994; Aufsatz et al., 2002). *NUCLEAR RNA POLYMERASE D1a* (*NRPD1a*) encodes a large subunit of RNA polymerase IV, which is essential for the biogenesis of 24-nucleotide small interfering RNAs (siRNAs) (Herr et al., 2005; Onodera et al., 2005). Double-stranded siRNAs are amplified through RNA-DEPENDENT RNA POLYMERASE2 and cleaved by the endoribonuclease DICER-LIKE3. These siRNAs are then loaded onto ARGONAUTE4 (AGO4), and AGO4-associated siRNAs are predicted to guide the de novo methyltransferase activity of DOMAINS REARRANGED METHYLASE2 (Zilberman et al., 2004; Gao et al., 2010), leading to RdDM (Haag and Pikaard, 2011; Law et al., 2013). The maintenance of DNA methylation requires *METHYLTRANSFERASE1* (*MET1*), which encodes a DNA methyltransferase, as well as *DECREASE IN DNA METHYLATION1* (*DDM1*), which is a SWI/SNF2-like chromatin-remodeling protein (Jeddeloh et al., 1999). DNA methylation affects the expression of circadian clock genes. For example, *CCA1* and *LHY* were upregulated in *Arabidopsis* DNA methylation mutants (Zhang et al., 2006; Kurihara et al., 2008) and in plants treated with 5′-aza-2′-deoxycytidine, which inhibits DNA methylation (Shen et al., 2012).

Changes in *CCA1* expression and DNA methylation were observed in *Arabidopsis* hybrids (Shen et al., 2012), but the relationship between them is unclear. The parent-of-origin effect on biomass vigor in reciprocal *Arabidopsis* hybrids suggests an epigenetic cause (Miller et al., 2012). In allotetraploids derived from *Arabidopsis* and *Arabidopsis arenosa*, the maternally transmitted *Arabidopsis CCA1* allele is more repressed than the paternally transmitted *A. arenosa* allele, which is associated with histone modifications (Ni et al., 2009). Here, we tested the hypothesis that the altered expression of circadian genes in hybrids is mediated by epigenetic factors such as DNA methylation (Chen, 2013). We investigated how and when the parent-of-origin effect on *CCA1* expression and growth vigor is established in *Arabidopsis* hybrids. The results support that *NRPD1a*- and *AGO4*-mediated changes in CHH methylation affect *CCA1* expression amplitudes and growth vigor in hybrids during vegetative and embryo development.

## RESULTS AND DISCUSSION

### Parent-of-Origin Effects on Starch and Biomass Heterosis in Hybrids

Biomass heterosis has been obviously observed in reciprocal F1 hybrids between *Arabidopsis* C24 and Landsberg *erecta* (L*er*) (Meyer et al., 2004; Groszmann et al., 2011; Shen et al., 2012). The reciprocal hybrids accumulated 50 and 100% more starch in rosette leaves at dusk (ZT15; Zeitgeber time 0 [ZT0] = dawn) than their parents C24 and L*er* (Figure 1A). Interestingly, the starch content was ~22% higher in the F1 (C24 × L*er*) hybrid (by convention, the maternal parent is listed prior to the paternal parent) than in the reciprocal F1 (L*er* × C24) hybrid ($P < 0.05$, Student's *t* test) (Figure 1B). The biomass (dry weight) was also significantly higher when C24 was the maternal parent in the reciprocal crosses (Figure 1C; $P < 0.05$). This parent-of-origin effect on biomass vigor was also observed in other reciprocal F1 hybrids between Columbia-0 (Col-0) and C24 or between Col-0 and L*er* (Miller et al., 2012), although the vigor level was low in the latter. The variable degree of biomass vigor among different hybrids may reflect genotypic effects (Meyer et al., 2004; Chen, 2010). Collectively, these data suggest a parent-of-origin effect on biomass heterosis. To minimize potential genotypic effects, unless noted otherwise, further analyses were performed in F1 crosses between C24 and L*er* ecotypes, including several mutants in the L*er* background.

### Parent-of-Origin Effects on Circadian Rhythms

Altered *CCA1* expression correlated with growth vigor in allopolyploids, hybrids, and diploids (Ni et al., 2009). Repressing *CCA1* peaks in *TOC1:cca1(RNAi)* transgenic plants during the day increases starch content and biomass, while overexpressing *TOC1:CCA1* in transgenic plants decreases starch content and biomass. These data suggest an important role for altered *CCA1* expression amplitudes in promoting growth vigor. In the hybrids between C24 and L*er*, expression levels of endogenous *CCA1* were 20 to 30% lower than the midparent value at ZT6 ($P < 0.05$) (Supplemental Figure 1A). RT-PCR analysis showed that *CCA1* expression peaks were lower in the cross when C24 was the maternal parent than in the reciprocal cross at ZT6, and lower *CCA1* expression levels were correlated with higher levels of starch and biomass in C24 × L*er* hybrids when C24 was the maternal parent (Figures 1B and 1C), suggesting an anticorrelation between endogenous *CCA1* expression levels and biomass vigor in hybrids.

RT-PCR analysis is limited to specific time points when the tissues can be collected for diurnal analysis. To overcome this caveat, we employed a stable L*er* (*CCA1:LUC* or *ProCCA1:LUC*) transgenic line (Salomé and McClung, 2005), which we designated L*er*C (Figure 1E; Supplemental Figures 1B and 1C). Using the reporter line, we tested how diurnal oscillation of *CCA1* expression in a period of 5 to 7 d is associated with biomass vigor in hybrids. Unless noted otherwise, bioluminescence assays included three biological replicates each with 24 to 32 seedlings, and resulting data points at ~1-h intervals were analyzed for statistical significance using paired Student's *t* tests between each comparison (e.g., reciprocal hybrids) (see Methods). In the control crosses, similar biomass levels (Figure 1D; Supplemental Figure 1B) correlated with equal expression levels of *CCA1:LUC* through the paternal or the maternal parent in reciprocal crosses between L*er* and L*er*C lines (Figure 1E). In reciprocal F1 hybrids between C24 and L*er*C (Supplemental Figure 1C), the *CCA1* expression peak was statistically significantly lower when *CCA1:LUC*

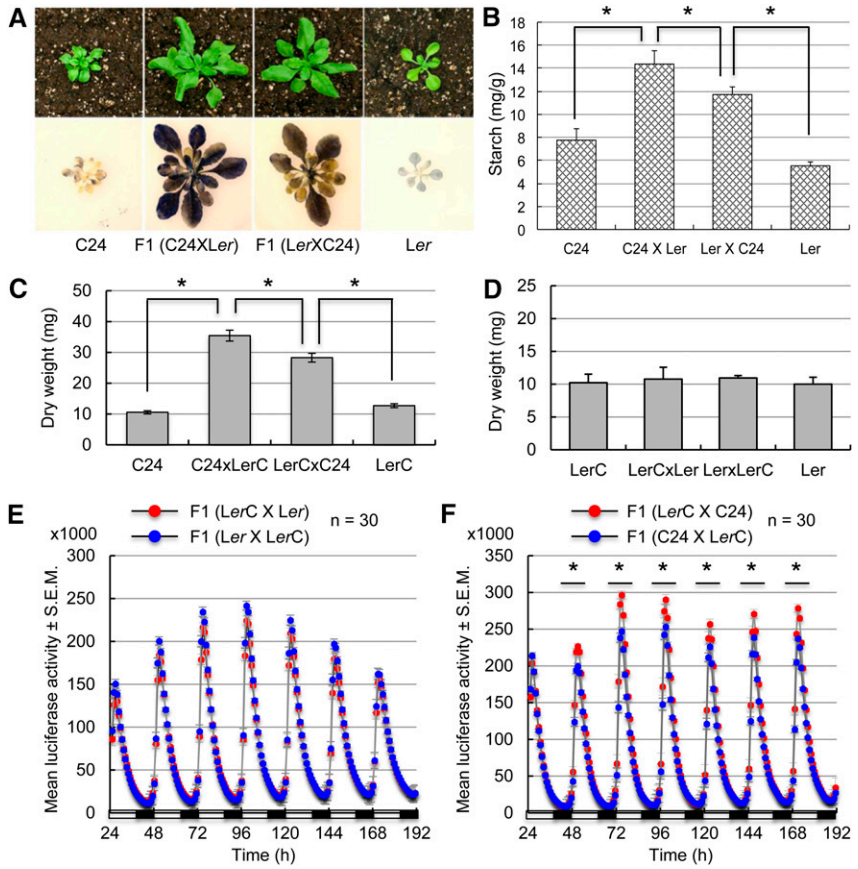


**Figure 1.** Parent-of-Origin Effects on *CCA1* Expression and Biomass Accumulation in F1 Hybrids.

**(A)** Larger size (top row) and higher starch-staining intensities at ZT15 (bottom row) in F1 (C24 × L*er*) than in F1 (L*er* × C24).
**(B)** Quantification of starch content at ZT15 in C24, L*er*, and C24 × L*er* and L*er* × C24 reciprocal F1 hybrids. Asterisks indicate a statistical significance level of 0.05 (Student's *t* test).
**(C)** Higher aerial dry weight ± SD (*y* axis) of 3-week-old seedlings in F1 (C24 × L*er*C) than in F1 (L*er*C × C24), relative to the parents C24 and L*er*C ($P < 0.05$; $n = 5$ in each of three replicates).
**(D)** No difference in aerial dry weight (*y* axis) in 3-week-old seedlings of reciprocal crosses between L*er* and L*er*C ($n = 5$ in each of three replicates).
**(E)** Mean values ± SE of bioluminescence counts (*y* axis; in thousands) in seedlings of the reciprocal F1 crosses L*er*C × L*er* (red) and L*er* × L*er*C (blue). Each data point was averaged from 24 plants with SE. The *x* axis shows hours with an alternating cycle of light (open bars) and dark (closed bars).
**(F)** Mean values ± SE of bioluminescence counts (*y* axis; in thousands) in seedlings of the reciprocal hybrids L*er*C × C24 (red) and C24 × L*er*C (blue). Specifics are as in **(E)**. Black lines with asterisks indicate the range of time points with statistically significant differences between the reciprocal crosses ($P < 0.05$, Student's *t* test).

was transmitted through the paternal parent (in C24 × L*er*C) than the maternal parent (in L*er*C × C24) (Figure 1F) ($P < 0.05$, Student's *t* test). Consistently, lower *CCA1* expression peaks in the C24 × L*er*C cross correlated with higher starch and biomass levels in the C24 × L*er*C hybrids than in the reciprocal cross (Figures 1B and 1C). These data indicate a parent-of-origin effect on the expression of transgene *CCA1:LUC* and endogenous *CCA1*, which negatively correlates with biomass heterosis.

## CHH Methylation and AGO4 Affect Parent-of-Origin Effects on Circadian Gene Expression

Parent-of-origin effects are often associated with paternal and maternal inheritance of DNA methylation patterns (Huh et al., 2008; Ferguson-Smith, 2011; Raissig et al., 2011), and methylation levels in promoter regions correlate with gene expression levels (Zilberman et al., 2007). To test this, we examined the methylation levels of CG, CHG, and CHH (where H = A, T, or C) sites in the *CCA1* promoter region using the bisulfite sequencing method (Gruntman et al., 2008). Degenerate primers flanking the *CCA1* promoter region (−382 to −39, relative to the transcription start site of +1) were used to amplify bisulfite-treated DNA, which was subsequently cloned and sequenced. Methylation levels of CHH, CHG, and CG sites were calculated and compared between reciprocal hybrids. In addition to this larger promoter fragment (−382 to −39), methylation analyses were also performed on a smaller region including a motif domain (−280 to −230) that contains a G-box and a *CCA1 HIKING*

*EXPEDITION* (*CHE*), a class I TCP protein, binding site (Pruneda-Paz et al., 2009), which is named GTBS (Figure 2A; Supplemental Figure 2A). This GTBS region was selected for the methylation analysis because CHE binds there to mediate *CCA1* expression (Pruneda-Paz et al., 2009). In reciprocal F1 hybrids between C24 and L*er* (wild type), within the GTBS region, CHH methylation levels were statistically significantly higher in C24 × L*er* hybrids than in L*er* × C24 hybrids (Figures 2B and 2C) ($P < 0.05$, Student's *t* test). CG and CHG methylation levels showed large variation, and this variability could result from a few CG and CHG sites, compared with CHH sites, in the promoter regions analyzed (Figure 2B). The differences in CHH methylation in the GTBS region between the reciprocal F1 hybrids of C24 and L*er* were correlated negatively with the endogenous *CCA1* expression and positively with biomass and starch content in F1 hybrids between C24 and L*er* (Figure 1B; Supplemental Figure 1A). In the larger *CCA1* promoter region, methylation levels of CHH sites were also higher in the C24 × L*er* hybrids than in the L*er* × C24 hybrids, although such differences were statistically insignificant (Supplemental Figure 1D).

We further analyzed DNA methylation in reciprocal F1 plants between L*er*C (transgenic line) and C24 or L*er* (Figures 2B and 2D; Supplemental Figures 1E and 2). In the hybrids between C24 and L*er*C, methylation levels at CHH sites in the larger *CCA1* promoter region were significantly higher in C24 × L*er*C hybrids when C24 was the maternal parent than in L*er*C × C24 hybrids (Supplemental Figure 2C). Within the GTBS, the trend of higher CHH methylation in C24 × L*er*C hybrids remained, but the difference between the reciprocal crosses was statistically insignificant (Figure 2D). However, total and CG methylation levels were statistically significantly higher in the C24 × L*er*C cross than in the reciprocal cross (Figure 2D). This is probably because of an increased methylation level in the CG sites of the transgene promoter (Supplemental Figure 3), which may obscure the difference in CHH methylation. In control crosses between L*er*C and L*er*, methylation levels of all sites were similar in the larger *CCA1* promoter region (Supplemental Figures 2B and 2D) or within the GTBS (Supplemental Figure 1E).

Higher methylation levels in the transgenic *CCA1:LUC* locus than in the endogenous *CCA1* locus were also observed in the 5′ untranslated region (UTR) (Supplemental Figure 3A), in which endogenous and transgene loci could be discriminated in the F1 hybrids (Supplemental Figures 3B and 3C). However, the methylation level differences between the reciprocal hybrids were not significant (Supplemental Figures 3D to 3F). These data suggest that the transgene is highly methylated, especially at CG and CHG sites, and that the methylation changes in the 5′ UTR are not correlated with the transgene or endogenous *CCA1* expression in the reciprocal hybrids. Instead, CHH methylation changes in the GTBS region are correlated with lower *CCA1* expression when C24 is the maternal parent in the hybrids.

In the RdDM pathway (Law and Jacobsen, 2010; Haag and Pikaard, 2011), AGO4 controls locus-specific methylation of CHH and CHG sites (Zilberman et al., 2004; Gao et al., 2010). We first tested if *CCA1:LUC* expression is altered in the *ago4-1* mutant. Indeed, *CCA1:LUC* expression levels were statistically significantly higher in the *ago4-1* homozygous mutant (red) than in the wild type (blue) (Figure 3A) ($P < 0.05$, Student's *t* test). We

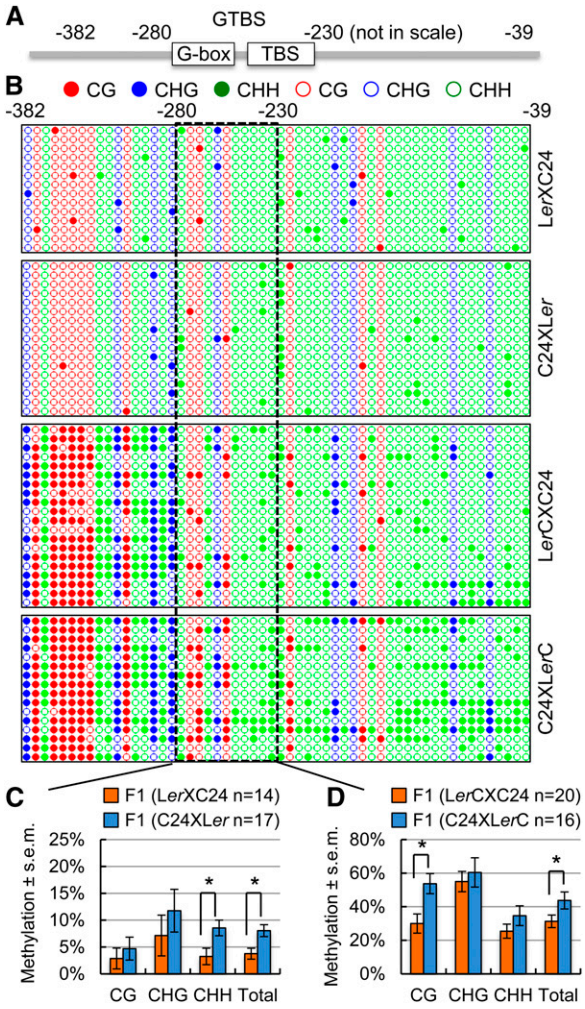


**Figure 2.** Bisulfite Sequencing Analysis of DNA Methylation in Reciprocal Hybrids.

**(A)** Diagram of a larger promoter region (−382 to −39) of *CCA1* and a motif region (−280 to −230) that contains a G-box and CHE with a class I TCP protein binding site, named GTBS.

**(B)** Dot-plot analysis of CG, CHG, and CHH methylation changes in L*er* × C24 (top), C24 × L*er* (second), L*er*C × C24 (third), and C24 × L*er*C (bottom) in the large promoter region and the GTBS region (boxed). A total of 14 to 20 individual promoter fragments were sequenced and analyzed in each sample. Red, blue, and green circles indicate CG, CHG, and CHH methylation (closed) or no methylation (open).

**(C)** Percentage of methylation changes ± SE in the GTBS region between reciprocal hybrids of L*er* × C24 (orange) and C24 × L*er* (blue). *n* = number of clones sequenced in each replicate. Asterisks indicate a statistical significance level of 0.05 (Student's *t* test).

**(D)** Percentage of methylation changes ± SE in the GTBS region between reciprocal hybrids of L*er*C × C24 (orange) and C24 × L*er*C (blue). *n* = number of clones sequenced in each replicate. Asterisks indicate a statistical significance level of 0.05 (Student's *t* test).

then tested if *CCA1:LUC* expression is altered in reciprocal F1 crosses involving the *ago4-1* mutant. Remarkably, in the F1 crosses between the *ago4-1* (L*er*) mutant and L*er*C, the *CCA1:LUC* expression peak was statistically significantly higher when

the *ago4-1* mutation was carried on the maternal genome (blue) than on the paternal genome (red) (Figure 3B), suggesting gene silencing by the RdDM pathway (Wassenegger et al., 1994; Aufsatz et al., 2002) through the action of maternal siRNAs in leaves as in the endosperm (Mosher et al., 2009; Lu et al., 2012). These siRNAs induce RdDM (Law and Jacobsen, 2010; Haag and Pikaard, 2011), and consistent with this notion, the methylation levels of all sites were lower in the *ago4-1* × L*er*C cross than in the L*er*C × *ago4-1* cross in both the larger promoter region ($P < 0.05$, Student's *t* test) (Supplemental Figure 5A) and the GTBS region (Figure 3C). The biomass was significantly higher in the L*er*C × *ago4-1* cross than in the *ago4-1* × L*er*C cross ($P = 0.05$, Student's *t* test) (Figure 3D; Supplemental Figure 6A). In reciprocal F1 hybrids between C24 and *ago4-1* (L*er*), the biomass in F1 (*ago4-1* × C24) was increased, making it similar to but not higher than that in F1 (C24 × *ago4-1*) (Supplemental Figures 6C and 6E). Notably, siRNAs and RdDM pathways may also regulate other traits, such as seed size. For example, seed size was dramatically increased when the maternal siRNAs were reduced (Lu et al., 2012) or when the demethylation lines were the maternal parent in the genetic crosses (Adams et al., 2000; Xiao et al., 2006). In addition, biomass accumulation is affected by other factors, such as quantitative trait loci, that regulate metabolism during the early stages of seedling development (Lisec et al., 2009; Meyer et al., 2012).

Consistent with the role for the RdDM pathway in circadian gene expression, the expression direction of *CCA1:LUC* was also altered in the reciprocal F1 crosses ColC (Col-0 [*CCA1:LUC* or *ProCCA1:LUC*] transgenic line) × *nrpd1a* and *nrpd1a* × ColC (Supplemental Figure 7B), whereas *CCA1:LUC* was equally expressed in the control crosses (Supplemental Figure 7A). Note that Col-0 was used in this study because the *nrpd1a* mutant and ColC or Col-0 (*ProCCA1:LUC*) would be in the same genetic background. The altered direction of expression was different from the crosses involving *ago4-1*, which could be associated with different genotypes (Col-0 versus L*er*) and/or different steps of NRPD1a and *AGO4* involved in the RdDM pathway (Law and Jacobsen, 2010; Haag and Pikaard, 2011).

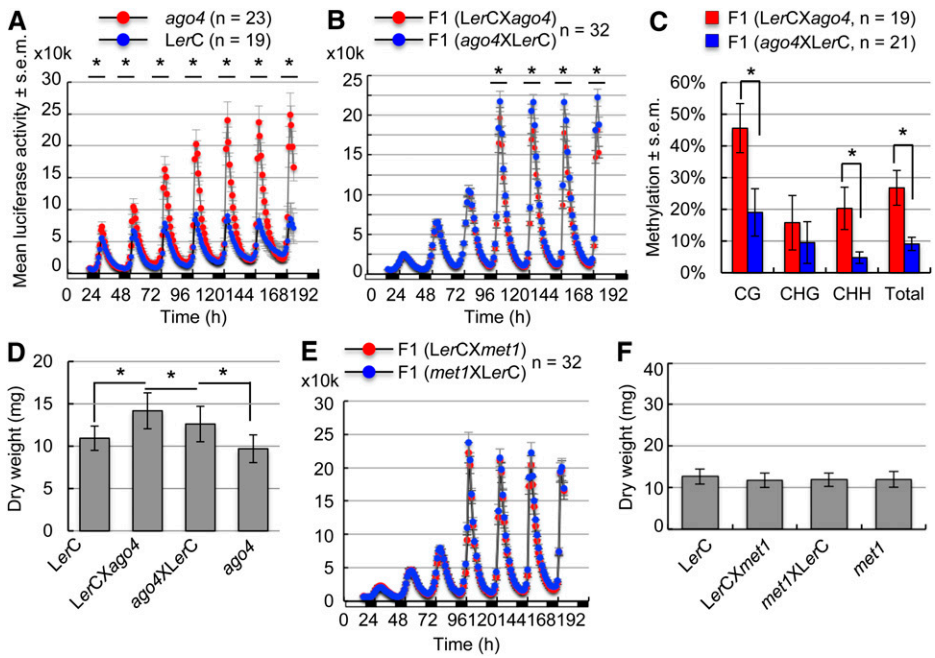


**Figure 3.** Parent-of-Origin Effects on *CCA1* Expression Depend on CHH Methylation and *AGO4* in Reciprocal Hybrids.

**(A)** Mean values ± SE of bioluminescence counts (in ten thousands [10k]; *y* axis) for *CCA1:LUC* expression in seedlings of the wild-type (L*er*C) (blue) and the *ago4-1* homozygous mutant (*ago4*) (red). Each data point was averaged from 19 and 23 plants, respectively. Black lines with asterisks indicate the peak time points with statistically significant differences between the reciprocal F1 crosses ($P < 0.05$, Student's *t* test). The *x* axis shows hours with an alternating cycle of light (open bars) and dark (closed bars).

**(B)** Mean values ± SE of bioluminescence counts (in ten thousands [10k]; *y* axis) in seedlings of the reciprocal F1 crosses between L*er*C × *ago4-1* (red) and *ago4-1* × L*er*C (blue). Specifics are as in **(A)**.

**(C)** Percentage of methylation changes in the GTBS region between reciprocal F1 crosses of L*er*C × *ago4-1* (red) and *ago4-1* × L*er*C (blue). Asterisks indicate statistical significance ($P < 0.05$, Student's *t* test).

**(D)** Aerial dry weight comparison (*y* axis) between 3-week-old seedlings in L*er*C × *ago4-1* and in *ago4-1* × L*er*C crosses ($n = 15$; $P = 0.05$, Student's *t* test) and between the reciprocal F1 seedlings and the *ago4-1* or L*er*C parent ($P < 0.05$).

**(E)** Mean values ± SE of bioluminescence counts (in ten thousands [10k]; *y* axis) in seedlings of the reciprocal F1 crosses between L*er*C × *met1-1* (red) and *met1-1* × L*er*C (blue). Each data point was averaged from 32 plants with SE.

**(F)** No difference in aerial dry weight (*y* axis) in 3-week-old seedlings of reciprocal F1 crosses between *met1-1* and L*er*C ($P = 0.7$, Student's *t* test).

To test if the maintenance of DNA methylation affects *CCA1* expression, we made reciprocal F1 crosses between L*er*C and *ddm1-2* (L*er*) (Jeddeloh et al., 1998) or *met1-1* (L*er*) (Kankel et al., 2003). In the *met1-1* mutant, *CCA1:LUC* expression remained unchanged relative to that in the wild type (Supplemental Figure 5C). Consequently, no alteration of the parent-of-origin effect on *CCA1* expression amplitudes was found in F1 crosses between L*er*C and the *met1-1* mutant (Figure 3E), which had similar biomass (Figure 3F; Supplemental Figure 5D). Similarly, in the reciprocal crosses between L*er*C and the *ddm1-2* mutant, *CCA1* expression amplitudes were equal in the F1 crosses regardless of whether L*er*C was used as the maternal or the paternal parent (Supplemental Figure 7C). In reciprocal F1 crosses between C24 and *ddm1-2*, the parent-of-origin effect on biomass remained unchanged, namely, higher in C24 × *ddm1-2* than in *ddm1-2* × C24 (Supplemental Figures 6D and 6F). Methylation analysis showed that CG and CHG methylation levels but not CHH methylation were lower in *ddm1-2* × L*er*C than in L*er*C × *ddm1-2* in the larger promoter fragment (Supplemental Figure 5B) or in the GTBS region (Supplemental Figure 7D). However, only CG methylation was significantly different ($P < 0.05$, Student's *t* test) between the reciprocal F1 plants, and the direction for the parent-of-origin effect on *CCA1:LUC* expression remained unchanged. These data suggest that *DDM1* and *MET1* affect mainly CG and CHG methylation but do not alter CHH methylation or parent-of-origin effects on *CCA1* expression and biomass.

### Parent-of-Origin Effects on *CCA1* Expression during Early Stages of Embryo Development

A key question is, when is the parent-of-origin effect on circadian rhythms established? Plant cells are totipotent and contain cell-autonomous multiple-loop clocks (McClung, 2006; Harmer, 2009; Nagel and Kay, 2012). The circadian clock is obviously present in leaves but also in roots and shoots (James et al., 2008) and germinating seeds (Penfield and Hall, 2009). This suggests that the clock may function during embryo and seed development and that preferential expression of the maternal *CCA1* allele may be established during these stages. We first examined the expression of *CCA1*, *LHY*, *TOC1*, and *CHE* in developing siliques 5 d after pollination in two ecotypes (L*er* and Col-0). The mRNA abundance of two morning-phased genes, *CCA1* and *LHY*, peaked at ZT0, rapidly decreased toward a minimum at ZT12, and then rapidly increased toward ZT24 (Supplemental Figures 8A to 8D). The evening-phased genes, *TOC1* and *CHE*, exhibited antiphasic diurnal expression patterns compared with those of *CCA1* and *LHY* (Supplemental Figures 8E to 8H). These data suggest that a robust clock is maintained in developing siliques as in leaves, roots (James et al., 2008), and germinating seeds (Penfield and Hall, 2009).

In addition to an embryo, a typical seed contains an endosperm and a seed coat, which are maternal tissues that do not transmit genetic information to the next generation. To test if the clock is functional in developing embryos, we dissected embryos 10 d after pollination (see Methods), when the embryos could grow in culture medium (Figures 4A to 4C; Supplemental Figures 9A and 9B). After 4 d on culture medium, embryos were subjected to bioluminescence assays (Figure 4D; Supplemental

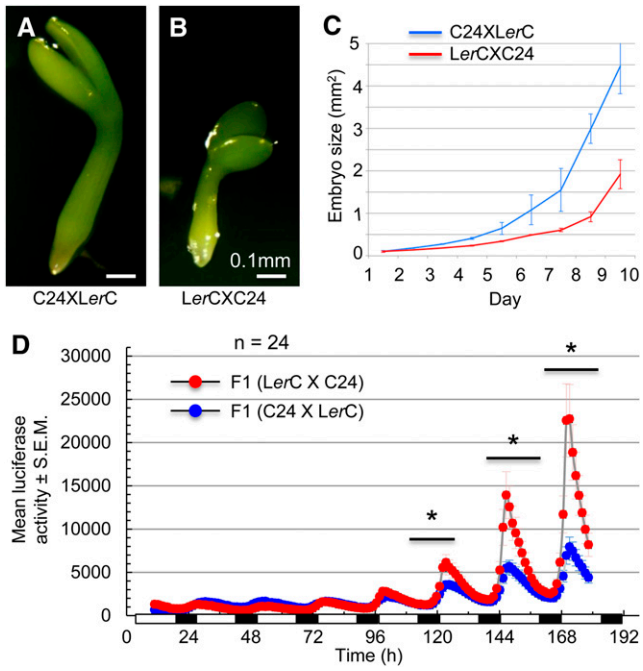


**Figure 4.** Parent-of-Origin Effect on Circadian Rhythms and Embryo Growth Rates in Hybrids.

**(A)** and **(B)** Embryos of C24 × L*er*C **(A)** and L*er*C × C24 **(B)** were dissected 10 d after pollination and cultured in medium for 1 d (day 1). After 4 d (day 4) in culture, C24 × L*er*C **(A)** and L*er*C × C24 **(B)** embryos were subjected to bioluminescence assays. Bars = 0.1 mm.

**(C)** Changes in embryo growth rates between C24 × L*er*C (blue) and L*er*C × C24 (red) crosses were determined by measuring embryo surface areas (means ± SD) (*y* axis) during 10 d in culture (*x* axis). $n = 5$ in each of three replicates.

**(D)** Mean values ± SE of bioluminescence counts (*y* axis) in cultured embryos of the reciprocal F1 hybrids L*er*C × C24 (red) and C24 × L*er*C (blue).

Figures 9C to 9E). Consistent with circadian rhythms in seedling leaves, *CCA1:LUC* expression amplitudes were statistically significantly higher when the transgene was transmitted through the maternal parent (in L*er*C × C24) than through the paternal parent (in C24 × L*er*C) (Figure 4D), which correlated negatively with embryo growth rates (Figure 4C). In control crosses between L*er*C and L*er*, the embryos were of similar size (Supplemental Figure 9A), and no *CCA1:LUC* expression difference was observed in the embryos of reciprocal F1 hybrids (Supplemental Figure 9C). The stronger maternal expression of *CCA1* was also found in the embryos of another pair of reciprocal F1 hybrids between L*er* and C24 (*CCA1:LUC*) or the C24 (*CCA1:LUC* or *ProCCA1:LUC*) transgenic line (C24C) (Supplemental Figures 9B and 9E), while in embryos of the control crosses, *CCA1:LUC* expression levels were equal (Supplemental Figure 9D).

### A Model for Parent-of-Origin Effects on Altered Circadian Rhythms and Growth Vigor in Hybrids

The available data support a model that explains how changes in *CCA1* expression mediate growth vigor in hybrids (Figure 5).

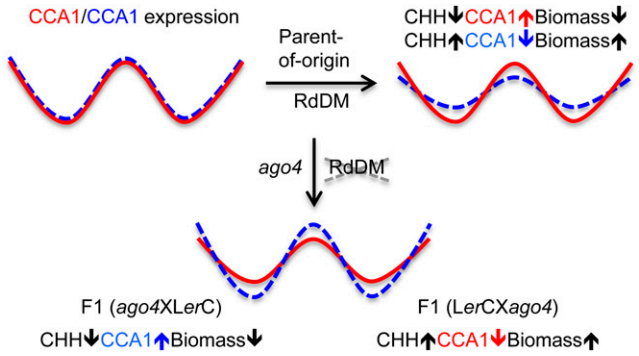


**Figure 5.** A Model for the Parent-of-Origin Effect on Circadian Rhythms and Growth Vigor in Hybrids.

At top left, maternal (red, solid line) and paternal (blue, dashed line) *CCA1* alleles are equally expressed in reciprocal crosses in the same genotype. At top right, in reciprocal F1 hybrids, when CHH methylation levels are low in the promoter of the maternal *CCA1* allele, its expression is high, whereas when CHH methylation levels are high in the promoter of the paternal *CCA1* allele, its expression level is low. This parent-of-origin effect on *CCA1* expression is anticorrelated with biomass accumulation. At bottom, in F1 crosses involving the *ago4* mutant, disruption of RdDM leads to lower CHH methylation levels in the promoter of the paternal allele than that of the maternal allele. As a result, paternal *CCA1* expression is increased and biomass is decreased (left). In the reciprocal cross, maternal *CCA1* expression is lower than that of the paternal allele (right). The reversal of the parent-of-origin effect on *CCA1* expression leads to increased levels of biomass accumulation.

Changes in circadian expression amplitude (or phase) without altering the clock period can have significant consequences for clock-controlled metabolic rhythms in animals (Nakahata et al., 2009) as well as in plants (Ni et al., 2009). Epigenetic repression of the maternal *CCA1* allele is correlated with increased starch content and biomass in *Arabidopsis* hybrids and allotetraploids (Ni et al., 2009). Correlation of *CCA1* repression with growth vigor is confirmed in *Arabidopsis* hybrids (Miller et al., 2012; Shen et al., 2012). This repression of the *CCA1* expression peak is established during embryo development, which requires *AGO4* and other components such as *NRPD1a* in RdDM (Gao et al., 2010; Law and Jacobsen, 2010). Quantitative variation of CHH methylation levels in the regulatory motif (GTBS) of the *CCA1* promoter region correlates with *CCA1* expression amplitudes and depends on the *NRPD1a*- and *AGO4*-mediated pathway, possibly through interactions with 24-nucleotide siRNAs. There is evidence for maternal transmission of 24-nucleotide siRNAs in endosperm (Mosher et al., 2009; Lu et al., 2012) and an overall increase of 24-nucleotide siRNAs in *Arabidopsis* hybrids relative to the parents (Groszmann et al., 2011; Shen et al., 2012). These data suggest a role for siRNAs in the parent-of-origin effect on *CCA1* expression. AGO4 could recognize maternal and paternal siRNAs and guide parent-of-origin effects on DNA methylation. This discrimination mechanism may not depend solely on the primary DNA sequence, because promoter sequences between two ecotypes are the same. The promoters could be associated with differential modifications of chromatin, including histone acetylation and methylation. For example, H3K27me3 could induce CHG methylation through the action of CMT3 (Cao et al., 2003) or CHH methylation through the action of CMT2 (Zemach et al., 2013). As a result, methylation of the *CCA1* promoter region inhibits the binding of CHE and other proteins to GTBS, altering *CCA1* expression. When the CHH methylation level in the promoter is high, *CCA1* is repressed and biomass is increased (Figure 5, right). Disruption of *AGO4* in the F1 crosses reverses CHH methylation levels in the promoter of paternal and maternal *CCA1* alleles and their expression directions, leading to altered biomass accumulation (Figure 5, bottom). When CHH methylation levels in the promoter are reduced, the paternal *CCA1* is increased and biomass is also decreased (Figure 5, bottom left). *AGO4* is required for changes in CHH methylation but not sufficient to alter biomass vigor at a statistically significant level. In plants, biomass vigor could be affected by many other epigenetic factors and metabolic pathways (Chen, 2013). For example, disruption of the RdDM pathway could alter the expression of other genes in stress response and metabolic pathways, which in turn alter growth vigor. The opposite is true in the reciprocal cross (Figure 5, bottom right). However, disruption of the maintenance of DNA methylation (mainly CG) through *ddm1* or *met1* mutations does not change the parent-of-origin effect on *CCA1* expression and biomass.

Methylation and small RNA changes were previously observed in the same hybrids between C24 and L*er* (Groszmann et al., 2011; Shen et al., 2012), but correlation of *CCA1* and *LHY* repression with specific methylation sites was not obvious. It is possible that genome-wide assays could not provide in-depth analysis of these loci in specific regions because the read coverage ranges from 2 to 47% of the genome (Shen et al., 2012). We predict that siRNA and RdDM pathways may also affect other targets or regulators such as *LHY*, *TOC1*, and *CHE* in the circadian feedback loop and related networks, which in turn mediate *CCA1* expression (McClung, 2006; Harmer, 2009; Nagel and Kay, 2012). Reduction in *CCA1* expression amplitudes or transcript levels promotes the expression of downstream genes that are negatively regulated by CCA1 abundance, as shown in *Arabidopsis* diploids and allopolyploids (Ni et al., 2009). This altered circadian regulation could affect photosynthetic and metabolic pathways that are altered in F1 hybrids (Fujimoto et al., 2012; Meyer et al., 2012) as well as overall regulatory networks related to growth and development (Birchler et al., 2010).

This model of the parent-of-origin effect on circadian rhythms and growth vigor is consistent with the parental conflict theory for imprinting in mammals and flowering plants, which predicts that the maternal genome provides factors that inhibit growth whereas the paternal genome carries the factors that promote growth (Moore and Haig, 1991; Ferguson-Smith, 2011; Raissig et al., 2011; Haig, 2013). This parental conflict theory could apply to the maternal effect of the clock function on growth vigor during the early stages of embryo development in hybrids and sexually reproducing organisms. When the maternal *CCA1* is repressed, growth vigor is increased. When the maternal *CCA1* expression is upregulated, growth vigor is reduced. The parent-of-origin effect on an early mouse embryo phenotype was also reported (Han et al., 2008). This parent-of-origin effect on circadian rhythms and growth vigor in embryos is likely a general

phenomenon to regulate growth and development in plant hybrids and allopolyploids as well as in sexually reproducing organisms including mammals.

## METHODS

### Plant Materials

Plant materials included three *Arabidopsis thaliana* ecotypes, C24, L*er*, and Col-0, three mutants in DNA methylation genes, *met1-1* (L*er*) (Kankel et al., 2003), *ddm1-2* (L*er*) (Jeddeloh et al., 1998), and *ago4-1* (CS6364; L*er*), and a small RNA biogenesis mutant, *nprd1a-4* (CS66151). *met1-1* and *ddm1-2* mutant seeds were kindly provided by Eric Richards at the Boyce Thompson Institute for Plant Research, and *ago4-1* and *nprd1a-4* were obtained from the ABRC. For comparison in F1 hybrids and crosses, manual pollination was used to produce seeds in both parents and reciprocal hybrids. For gene expression and starch analyses in vegetative tissues, plants were grown for 3 weeks in 16/8-h light/dark cycles at 22/18°C and harvested at ZT0 (dawn). Rosette leaves were harvested from a pool of 6 to 12 plants as one biological replicate and used immediately or frozen in liquid nitrogen for future use. Leaves were collected prior to bolting to minimize developmental variation among genotypes. Except where noted otherwise, three replicates were used for each experiment.

For gene expression analysis in developing siliques, manual pollination was performed 1 d after emasculation. Young siliques at 5 d after pollination were harvested every 3 h for a period of 24 h for diurnal expression analysis.

### Transgenic Plants Expressing Luciferase Reporter

The *ProCCA1:LUC* construct was transformed into L*er* as described previously (Salomé and McClung, 2005) to generate L*er*C stable transgenic plants for this study. To generate the ColC and C24C lines (Supplemental Figures 7 and 9), a *CCA1* promoter (from −715 to −1 bp, relative to the transcription start site plus full 5′ UTR) was amplified by PCR and cloned into the plasmid between the restriction sites *Xho*I and *Nco*I. A *ProCCA1:LUC* plasmid construct was generated by inserting the luciferase gene between the restriction enzyme sites *Nco*I and *Bam*HI in the pFAMIR plasmid that was modified from pFGC5941 (McGinnis et al., 2005). The construct was introduced into *Arabidopsis* (Col-0 or C24) plants using *Agrobacterium tumefaciens*–mediated transformation (strain GV3101) with the floral dip method (Clough and Bent, 1998). Primary transformants (seedlings) were screened on Murashige and Skoog (MS) agar medium (M9274; Sigma-Aldrich) (Murashige and Skoog, 1962) supplemented with 7.5 μg/mL Basta (Sigma-Aldrich). Stable transgenic plants (T2 and later) with uniform herbicide resistance were used for the expression assays and for making crosses. For hybrid crosses involving L*er* and C24 ecotypes, either the L*er*C or C24C reporter line was used. For crosses involving the *nrpd1a* mutant, ColC was used such that the mutant and the reporter lines are in the same ecotype background.

### Embryo Dissection and Culture

Siliques at 10 d after pollination were harvested and rinsed with 70% ethanol and soaked in 100% Clorox for 2 min. After rinsing with autoclaved water twice, the siliques were kept in sterilized liquid MS medium (Murashige and Skoog, 1962) in a Petri dish. Embryos were dissected using an optical microscope (SMZ445; Nikon) and transferred to a plate containing the agar embryo culture medium, which contained 40% Suc, 0.5× MS salts, 0.9 mg/L thiamine, 0.5 g/L MES (Sigma-Aldrich), 8 g/L agar, and 0.69 g of Leu-DO amino acid supplements (Clontech). Final pH was adjusted to 5.9 with KOH. Forty to 50 embryos from each genotype were transferred to one agar plate and cultured in an incubator at 22°C (16/8-h light/dark cycles) for 2 d. A total of 24 healthy embryos (no brown spots or any visible damage) from each genotype were transferred to a 96-well microtiter plate (Nagle Nunc International) containing 40% Suc, 0.5× MS salts (Murashige and Skoog, 1962), 0.9 mg/L thiamine, 0.5 g/L MES (Sigma-Aldrich), 8 g/L agar, and 0.69 g of Leu-DO amino acid supplements. After adding luciferin to a final concentration of 2.5 mM, the plate was subjected to luciferase assays over a period of 5 to 7 d (see below).

### Luciferase Assays and Data Analysis

Stable transgenic embryos or seedlings containing *ProCCA1:LUC* constructs were analyzed using a TopCount NXT luminometer and scintillation counter (Perkin-Elmer). For seedlings, seeds were sterilized and plated on 1% (w/v) agar MS medium (Murashige and Skoog, 1962) plus 30 g/L Suc. Seeds were stratified for 2 d in the dark at 4°C and then transferred to a 16-h-light/8-h-dark cycle for 8 d at 22°C. Seedlings were transferred to white microtiter plates (Nagle Nunc International) containing agar MS medium plus 30 g/L Suc, and then 30 μL of 0.5 mM luciferin (Gold Biotechnology) was added to each well. Microtiter plates were covered with clear plastic MicroAmp sealing film (Applied Biosystems), in which holes were placed above each well for seedling gas exchange. Plates were moved to the TopCount device and interleaved with three clear plates to allow light diffusion to the seedlings. Luciferase activity was measured approximately every 1 h by integrating photons emitted by seedlings during a 10-s sampling period. Data were analyzed by fast Fourier transform-nonlinear least squares (Plautz et al., 1997) using the Biological Rhythms Analysis Software System Excel macros (available from http://millar.bio.ed.ac.uk/PEBrown/BRASS/BrassPage.htm).

All values are presented as means ± SE. Expression amplitudes are shown as bioluminescence counts. Unless noted otherwise, each data point was averaged from 24 to 32 plants in each experiment, and graphic data from one of three replicated experiments are shown.

### RNA Preparation and RT-PCR

Total RNA was extracted using Plant RNA reagent (Invitrogen). First-strand cDNA synthesis was performed using RT SuperScript III (Invitrogen). For RT-PCR, the total RNA obtained from siliques was treated with RNase-free DNase (Promega) for 30 min. The reaction was terminated by adding phenol:chloroform:isoamyl alcohol (25:24:1) solution, and the total RNA was precipitated by the addition of ethanol. An aliquot (1:100) of cDNA was used for quantitative RT-PCR analysis using the primer pairs listed in Supplemental Table 1 and SYBR Green in an ABI7500 machine (Applied Biosystems). Amplification of *ACT7* served as a control to estimate relative expression levels.

### Genomic DNA Extraction and Bisulfite Sequencing

Genomic DNA was extracted from 3-week-old seedlings (100 mg) using the DNeasy Plant Mini Kit (Qiagen). About 500 to 800 ng of genomic DNA was then used for bisulfite conversion using the EpiTect Bisulfite Kit (Qiagen) according to the manufacturer's instructions. Bisulfite-treated DNA (5 μL) was then amplified by PCR in a 25-μL reaction using ZymoTaq DNA polymerase (ZYMO Research) and degenerate primers (Supplemental Table 1) targeting the −382 to −39 *CCA1* promoter region containing the G-box and the CHE binding site (a motif region spanning −280 to −230). Bisulfite sequencing was also performed using a 5′ forward primer targeting the 5′ UTR of *CCA1* and a gene-specific 3′ primer targeting either the endogenous *CCA1* coding region or the transgenic *LUC* coding region (Supplemental Figure 3A). PCR products were then resolved on a 1% agarose gel, excised, purified using the UltraClean DNA Purification Kit (MO BIO Laboratories), and cloned into a pGEM-T vector (Promega) for

sequencing. For each plant genotype, 14 to 20 independent top-strand clones were sequenced. Bisulfite DNA sequences and the levels of DNA methylation at the *CCA1* promoter were analyzed using the online Kismeth program (Gruntman et al., 2008). For each genotype, the percentage of cytosine methylation in each context (CG, CHH, or CHG) was calculated, and the difference in DNA methylation between two genotypes was analyzed using Student's *t* test. In Supplemental Figure 2, an integrative genome browser was used to display methylation levels at the single nucleotide level (Robinson et al., 2011). Methylation at the *ASA1* locus was used as a control for the bisulfite conversion (~95%) (Jeddeloh et al., 1998), and the methylation levels of the endogenous *ASA1* locus were similar between the L*er* × C24 and L*er*C × C24 reciprocal hybrids (Supplemental Figure 4).

### Starch and Biomass Analysis

Starch content was measured in rosette leaves from a pool of five to six plants (~100 to 300 mg fresh weight) as one biological replication according to a published protocol (Ni et al., 2009) (http://www.nature.com/protocolexchange/protocols/521; doi:10.1038/nprot.2009.12). Three replicates were used in each assay. In each replicate, total starch was quantified using 30 μL of the insoluble carbohydrate fraction using a kit from Boehringer Mannheim (R-Biopharm).

Whole rosettes from hybrids and parents were harvested at ~3 weeks of age (before bolting) and placed in Lawson #217 hybridization bags (Lawson Bags). The weight from aerial rosette leaves was determined after drying the plants at 80°C for 24 h. Aerial rosettes of 6 to 15 plants in three biological replicates were weighed individually, and the average was used to calculate SD (Miller et al., 2012).

### Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL databases under the following accession numbers: *CCA1* (*At2g46830*), *LHY* (*At1g01060*), *TOC1* (*At5g61380*), *CHE* (*At5g08330*), *ACT7* (*At5g09810*), and *ASA1* (*At1g19920*).

### Supplemental Data

The following materials are available in the online version of this article.

**Supplemental Figure 1.** *CCA1* Expression, Plant Size, and Bisulfite Sequencing Analysis of DNA Methylation in Reciprocal Hybrids.

**Supplemental Figure 2.** Bisulfite Sequencing Analysis of DNA Methylation in Reciprocal Hybrids.

**Supplemental Figure 3.** Bisulfite Sequencing Analysis of DNA Methylation at the 5′ UTR of *CCA1*.

**Supplemental Figure 4.** Bisulfite Sequencing Analysis of DNA Methylation at *ASA1*.

**Supplemental Figure 5.** Changes in DNA Methylation and Circadian Gene Expression.

**Supplemental Figure 6.** Biomass Analysis in Reciprocal Hybrids and Their Parents.

**Supplemental Figure 7.** Analyses of *CCA1* Expression and DNA Methylation in Reciprocal Hybrids.

**Supplemental Figure 8.** Diurnal Expression of Clock Regulators in Developing Siliques in *Arabidopsis* (Col-0 and L*er*).

**Supplemental Figure 9.** Parent-of-Origin Effects of *ProCCA1:LUC* Expression in Embryos of Reciprocal Hybrids.

**Supplemental Table 1.** Primers for Quantitative RT-PCR, PCR with CAPS, and Methylation Assays.


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### AUTHOR CONTRIBUTIONS

Z.J.C., M.M., D.W.-K.N., H.H.Y., and C.R.M. designed experiments. D.W.-K.N., M.M., H.H.Y., E.-D.K., T.-Y.H., J.L., and Q.X. performed experiments. D.W.-K.N., M.M., H.H.Y., Q.X., C.R.M., and Z.J.C. analyzed data. Z.J.C., D.W.-K.N., M.M., H.H.Y., and C.R.M. wrote the article.

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