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TIME FOR COFFEE Encodes a Nuclear Regulator in the *Arabidopsis thaliana* Circadian Clock[W]

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The plant circadian clock is required for daily anticipation of the diurnal environment. Mutation in *Arabidopsis thaliana TIME FOR COFFEE* (*TIC*) affects free-running circadian rhythms. To investigate how *TIC* functions within the circadian system, we introduced markers for the evening and morning phases of the clock into *tic* and measured evident rhythms. The phases of evening clock genes in *tic* were all advanced under light/dark cycles without major expression level defects. With regard to morning-acting genes, we unexpectedly found that *TIC* has a closer relationship with *LATE ELONGATED HYPOCOTYL* (*LHY*) than with *CIRCADIAN CLOCK ASSOCIATED1*, as *tic* has a specific *LHY* expression level defect. Epistasis analysis demonstrated that there were no clear rhythms in double mutants of *tic* and evening-acting clock genes, although double mutants of *tic* and morning-acting genes exhibited a similar free-running period as *tic*. We isolated *TIC* and found that its mRNA expression is continuously present over the diurnal cycle, and the encoded protein appears to be strictly localized to the nucleus. Neither its abundance nor its cellular distribution was found to be clock regulated. We suggest that *TIC* encodes a nucleus-acting clock regulator working close to the central oscillator.

INTRODUCTION

The rotation of the earth generates predictable daily changes in the light and temperature environment, and many organisms have evolved molecular oscillators to adapt to these anticipated changes (Harmer et al., 2001; Young and Kay, 2001). Such oscillators are self-sustaining, with a periodicity of ~24 h under constant conditions. However, they are influenced by environmental cues, such as changes in light and temperature conditions (Dunlap, 1999). In higher plants, circadian rhythms control many biological processes, including the floral transition, leaf movement, stomata opening, seed germination, and hypocotyl elongation. It was found that ~10% of all transcripts in *Arabidopsis thaliana* are clock regulated at the steady state level (Harmer et al., 2000; Schaffer et al., 2001; Yanovsky and Kay, 2003). Recent research supports the notion that the plant clock works to increase photosynthesis efficiency, growth survival, and competitive advantage (Dodd et al., 2005) and that allelic variation in the clock is correlated with species migration (Michael et al., 2003). Therefore, deciphering the molecular mechanism of the circadian clock will help us to better understand the dynamics of this oscillator with regard to its role in regulating growth and development.

Circadian rhythms were first described from botanical experiments, but research regarding the molecular mechanism of plant rhythms has lagged behind that of other organisms (Glossop et al., 1999; Shearman et al., 2000; Young and Kay, 2001; Stanewsky, 2003; Houl et al., 2006). During the past decade, with improved experimental techniques and methods, including the use of the firefly luciferase reporter gene (*LUC*) to monitor clock gene expression in vivo, much progress has been made in the plant circadian research field. In *Arabidopsis*, the putative central oscillator genes *CIRCADIAN CLOCK ASSOCIATED1* (*CCA1*) (Wang and Tobin, 1998), *LATE ELONGATED HYPOCOTYL* (*LHY*) (Schaffer et al., 1998), and *TIMING OF CAB2 EXPRESSION1* (*TOC1*) (Strayer et al., 2000; Makino et al., 2002) have been cloned, and an autoregulatory positive–negative feedback loop model was proposed (Young and Kay, 2001; Hayama and Coupland, 2003; Yanovsky and Kay, 2003; Salome and McClung, 2004). *CCA1* and *LHY* are both circadian clock regulated, with a peak in expression soon after dawn. They encode closely related MYB-like transcription factors that can bind in vitro a sequence present in the *TOC1* promoter. This binding activity correlates with the genetic inhibition of *TOC1* expression early in the early subjective day (Strayer et al., 2000). *TOC1* is a member of the *Arabidopsis* pseudoresponse regulator (*PRR*) family, composed of the rhythmically expressed genes *PRR9*, *PRR7*, *PRR5*, *PRR3*, and *TOC1*/*PRR1* (Matsushika et al., 2000). Upon turnover of CCA1 and LHY, by an as yet unidentified mechanism, *TOC1* repression is alleviated and *TOC1* accumulates to peak levels near dusk. This correlates with the evening peak of the *TOC1* transcript during the circadian cycle. The accumulation of TOC1 protein is proposed to activate *CCA1* and *LHY* expression later into the night or in the earlier morning, which closes this loop (Schaffer et al., 1998; Wang and Tobin, 1998; Harmer et al., 2000; Alabadi et al., 2002; Kim et al., 2003a). It is clear that this model is not sufficient to explain current experimental data, as both the *cca lhy* double mutant and the *toc1* single mutant still retain rhythms (Alabadi et al., 2002; Mizoguchi

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et al., 2002; Locke et al., 2005). Mathematical simulations incorporating current experimental data have led to the proposal of a model comprising two interlocking feedback loops, with *GIGANTEA* (*GI*) and *TOC1* as candidates for components of a second loop (Locke et al., 2005). However, this newly proposed transcriptional feedback loop is only a framework onto which other factors must be incorporated. For example, it is clear that *TIME FOR COFFEE* (*TIC*) (Hall et al., 2003) needs to be integrated into the current framework of the molecular oscillator to explain morning-acting features of the clock. This would aid a more correct understanding of the molecular mechanism(s) of the plant circadian system.

We previously executed a screen for rhythm mutants in *Arabidopsis*, from which the *tic-1* mutant allele was reported (Hall et al., 2003). *TIC* was shown to be a regulator of normal clock function. The *tic* mutation affected a range of clock phenotypes, including free-running circadian rhythms under light or in darkness, and this mutant was also altered in rhythmic gating of light-activated gene expression (Hall et al., 2003). To further investigate the molecular role of *TIC*, we characterized the genetic network of *TIC* action and isolated this gene. We suggest that *TIC* encodes a nuclear clock regulator that works close to the central oscillator of *Arabidopsis*.

RESULTS

Effect of the Loss of *TIC* Function on Evening Clock Gene Mutants

To investigate the genetic relationship between *TIC* and evening clock genes, we crossed *tic-1* harboring the *CAB:LUC* reporter to a panel of mutants each defective in evening function and tested clock responses in the resultant double mutants. Established homozygous lines were *elf3-4 tic-1*, *elf4-1 tic-1*, *toc1-21 tic-1*, and *gi-11 tic-1*. The *elf3-4*, *elf4-1*, *toc1-21*, and *gi-11* single mutants harboring the *CAB:LUC* reporter were selected as the appropriate controls. *CAB* expression became arrhythmic immediately after being transferred into constant light conditions in *elf3 tic* and *elf4 tic* (Figures 1A and 1B). The control single mutants of *tic* and *elf4* maintained circadian rhythms of *CAB* expression (albeit with circadian defects) (Figure 1B). The control *elf3* single mutant became arrhythmic after one cycle of expression under constant light conditions, confirming previous reports (Doyle et al., 2002; Hall et al., 2003). Under constant light conditions, *CAB* became strikingly and rapidly arrhythmic in *toc1 tic* and *gi tic* double mutants after one cycle of expression, even though the single mutants each exhibited rhythmic expression of this marker (Figures 1C and 1D). Through fast Fourier transform nonlinear least squares (FFT-NLLS) analysis, a very weak rhythmic expression of *CAB* could be detected in these double mutants, but all mutants exhibited a much higher relative amplitude of error (RAE), and the period was more variable, compared with wild-type plants (see Supplemental Figure 1 online).

To further classify the genetic relationship of *tic* to evening genes, the above panel of double mutants was tested under diurnal conditions. Both the *elf3 tic* and *elf4 tic* double mutants completely lost dawn and dusk anticipation of *CAB* expression under a long-day photoperiod (16 h of light/8 h of dark), exhibiting a square wave form of light-regulated expression (Figures 1E and 1F). In *toc1 tic* and *gi tic* double mutant seedlings, *CAB* expression exhibited only a weak, subtle anticipation under this long-day photoperiod (Figures 1G and 1H). Collectively, all examined double mutants displayed a marked phenotype far more dramatic than a simple compound of the single mutant effects, perhaps indicating that *TIC* and the evening clock genes, such as *TOC1*, *GI*, *ELF3*, and *ELF4*, have partly independent functions in the *Arabidopsis* circadian system. This would be consistent with these genes acting at different circadian times.

Defects in the Regulation of Circadian Clock Genes with Expression Rhythmic Peak in the Evening in *tic*

The current *Arabidopsis* circadian model proposes that CCA1 and LHY repress the expression of evening-acting genes, such as *TOC1*. Since both *CCA1* and *LHY* expression were changed in the *tic* mutant (Hall et al., 2003), the expression of evening clock genes might be indirectly affected through a feedback oscillation. Therefore, we investigated in *tic* the expression profile and waveform of the evening clock genes *TOC1*, *GI*, *ELF3*, and *ELF4*. To this end, *TOC1:LUC*, *GI:LUC*, *ELF3:LUC*, and *ELF4:LUC* fusion genes were introduced into the *tic-1* mutant through crosses, and clock responses of transcription were monitored in these lines. *ELF3* expression in *tic* was highly dampened both under constant light conditions and in constant darkness (Figures 2A and 2B). *TOC1* expression was similarly severely impaired in *tic*. Here, the expression became essentially arrhythmic after only two cycles (Figures 2C and 2D). Through FFT analysis, only ~70% of plants had detectable rhythmic expression of *TOC1* and *ELF3* in *tic* compared with the wild type (see Supplemental Figures 2A to 2D online). By contrast, both *GI* and *ELF4* expression maintained a low level of rhythmicity in *tic*, although both genes displayed a reduced amplitude compared with the wild type (Figures 2E to 2H). As well, these two genes displayed a short-period response. For example, *ELF4* expression almost had a reversed phase position during the second day after transfer into constant conditions of light or dark (Figures 2G and 2H). In contrast with the above FFT results for *TOC1* and *ELF3*, nearly 100% of *tic* plants exhibited rhythmic expression of *GI* and *ELF4* (see Supplemental Figures 2C to 2F online). Collectively, we found that *tic* mutant plants had a much higher RAE for the rhythms of all of the above evening genes, and the periods were more variable, compared with the wild type (see Supplemental Figure 2 online).

The above circadian defects prompted us to test in *tic* the evening expression of circadian markers under diurnal conditions. Under long-day photoperiods (16 h of light/8 h of dark), the phases of *ELF3*, *ELF4*, *TOC1*, and *GI* expression were significantly advanced in *tic*. The peaks of all of these evening genes occurred up to 3 h earlier in *tic* than in wild type (see Supplemental Figures 3A to 3C online). A similar effect for *ELF3* and *ELF4* expression was found under a short-day photoperiod (8 h of light/16 h of dark), as both of these peaked at ~3 h earlier in *tic* (see Supplemental Figure 3D online). To examine the steady state transcript levels of several evening genes in *tic*, we performed

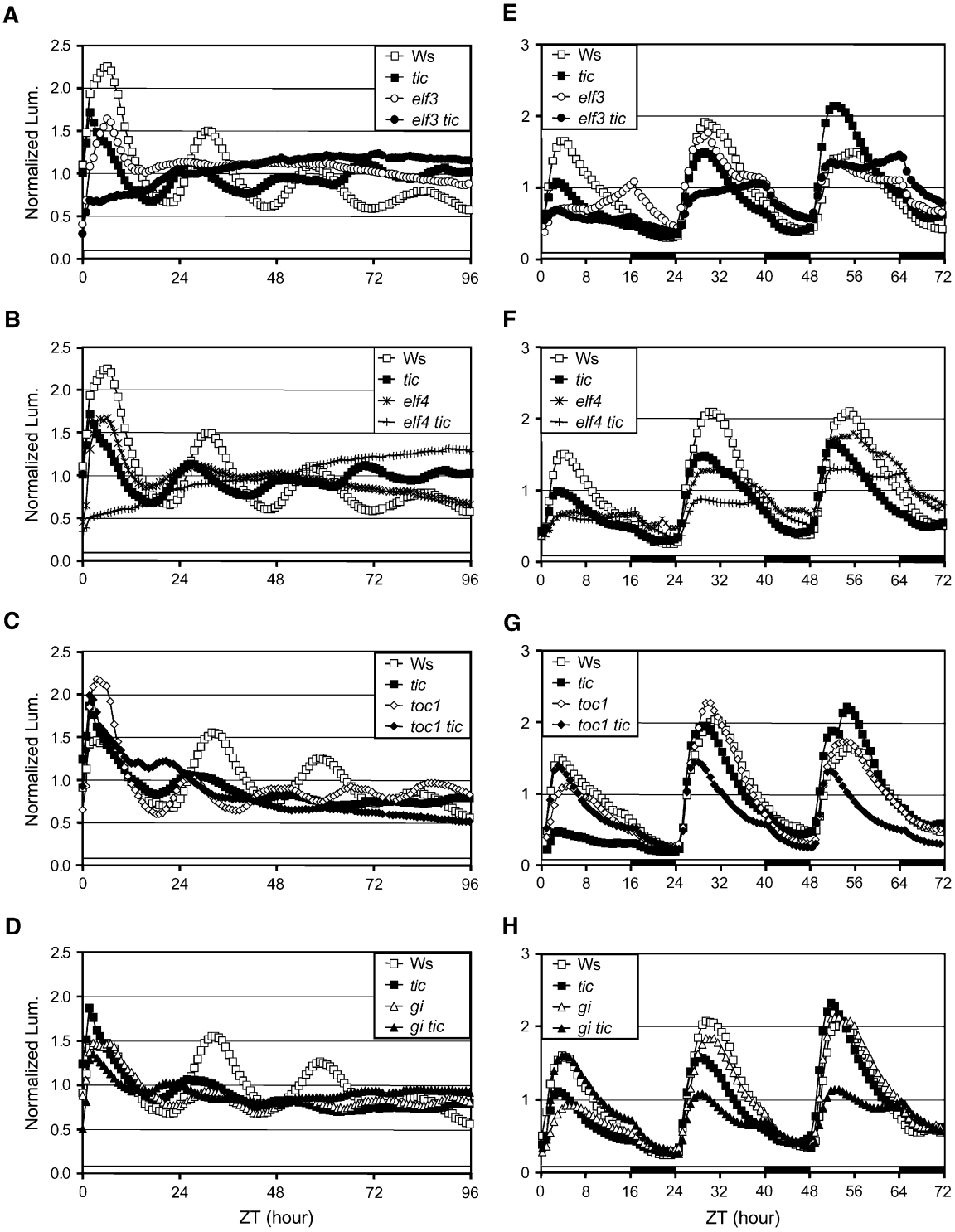

Figure 1. Effects of the Loss of *TIC* Function on Evening-Clock Gene Mutants.

Seedlings of the Wassilewskija (Ws) wild type, *tic-1*, *toc1-21*, *toc1-21 tic-1*, *gi-11*, *gi-11 tic-1*, *elf4-1*, *elf4-1 tic-1*, *elf3-4*, and *elf3-4 tic-1* were grown under standard 12L/12D (12-h-light/12-h-dark) conditions or under 16L/8D conditions for 7 d, then transferred to LL (constant light) or to 16L/8D conditions, to monitor *CAB*:*LUC* luminescence.

(A) *CAB:LUC* bioluminescence rhythms in Ws, *tic-1*, *elf3-4*, and *elf3-4 tic-1* mutant plants under LL.

(B) *CAB:LUC* bioluminescence rhythms in Ws, *tic-1*, *elf4-1*, and *elf4-1 tic-1* mutant plants under LL.

real-time PCR on RNA extracted from replicate time points from *tic-1* seedlings grown under 12-h-light/12-h-dark cycles. Through this assay, we found no major changes in mean expression levels for *GI*, *TOC1*, and *ELF3* in *tic* under our assay conditions (Figures 3A to 3C). (The RNA results reported here for *GI* in *tic* are not identical to those reported previously [Hall et al., 2003]. The reason for this discrepancy is not clear, but our results were repeated multiple times with multiple samples, and the steady state *GI* levels are in agreement with the reporter studies [Figures 2 and 3; see Supplemental Figure 3 online; data not shown]). As with the above reporter studies, here we found that the major evening-gene phenotype in *tic* was an early advancement in peak expression (Figures 3A to 3C). Thus, the RNA and luciferase assays are tightly correlated.

Genetic Interaction between *TIC* and *CCA1*/*LHY*

tic was reported to be a low-amplitude clock mutant defective in late-night to early-morning detection of dawn (Hall et al., 2003), and the data shown above support that assertion. As *CCA1* and *LHY* are both morning-clock genes, we sought to disclose potential genetic relationships between *TIC* and *CCA1* and/or between *TIC* and *LHY*. For this, we separately crossed *tic-1* to *cca1-11*, to *lhy-21*, and to the double *cca1-11 lhy-21*, established all possible combinatorial double and triple mutants, each harboring *CAB:LUC* as a reporter, and tested their clock responses. Under constant light conditions, we found that the circadian phenotypes of both *cca1* and *lhy* could be enhanced by *tic. CAB* expression dampened much earlier in the *tic cca1* and *tic lhy* double mutants than in the wild-type plant or in any single mutant (Figures 4B and 4C). Furthermore, the free-running periods of these double mutants were found to be variable, and this could be confirmed mathematically through FFT-NLLS analysis. Each of these double mutants exhibited a much higher RAE compared with the wild-type plants, thus indicating a lack of proper clock precision (see Supplemental Figure 4 online). Both the *cca1 tic* and *lhy tic* double mutants were found to have a similar free-running period length to *tic* (Figure 4H). We also found that *CAB:LUC* displayed a 2-h earlier phase position in the *lhy* mutant than in the *cca1* mutant (Figure 4A). Strikingly, under constant light conditions, *CAB* was found to lack rhythmic expression in the *cca1 lhy tic* triple mutant (Figure 4D). However, in *cca1 lhy* double mutants, *CAB* expression was maintained for at least three short-period cycles after transfer into constant light conditions (Figure 4D). Unlike the effect of *tic* on evening-gene mutants even under light/dark cycles (Figures 1E to 1H), we could not detect major phase differences between *cca1 tic*, *lhy tic*, and *tic* (Figures 4E to 4G). In the *cca1 lhy tic* triple mutant, there was still essentially no phase difference observed compared with the *tic* single mutant (Figure 4G). Furthermore, the periodicity of the *CAB:LUC* rhythm under constant light in the *cca1 tic* and *lhy tic* double mutants was indistinguishable from that in *tic* (Figure 4H). An evident epistatic relationship is thus apparent between *TIC* and *LHY*/*CCA1* for these responses. Collectively, we conclude that there were genetic interactions of *TIC* with *CCA1* and *LHY*.

TIC Differentially Regulates *CCA1* and *LHY* Expression

The above experiments led us to hypothesize that *TIC* functions close to either *CCA1* and/or *LHY*. To test such a hypothesis, we introduced *CCA1:LUC* and *LHY:LUC* fusion genes into the *tic-1* mutant through genetic crossing and monitored the luminescence rhythms of these lines under different regimes. Under constant-light conditions, *CCA1* maintained strong, rhythmic expression in *tic* for at least two cycles, albeit with an earlier phase and a short period compared with wild-type plants, and mean levels were always found to be nearly as high as the wild-type levels (Figure 5A). By contrast, *LHY* expression in *tic* approached arrhythmicity immediately after being transferred into constant-light conditions. Furthermore, the mean levels of *LHY* were lower than in the wild-type plants (Figure 5A). Note that the maximum of *LHY* in *tic* was essentially at the minimum levels seen in the wild type. In constant darkness, both *CCA1* and *LHY* expression dampened faster in *tic* than in wild-type seedlings. However, *LHY* expression dampened even earlier than *CCA1* expression in *tic* (Figure 5C). Through FFT-NLLS analysis, a very weak rhythm of *LHY* expression in *tic* could be mathematically detected under constant light or in constant darkness, but this rhythm had a much higher variability in period length and RAE compared with that in the wild type (Figures 5B and 5D). As well, *CCA1* expression exhibited a higher variability in period length and RAE in *tic* compared with the wild type under these conditions, but a less severe period phenotype than *LHY*. Specifically, only ~20% of *tic* individuals exhibited rhythmic expression of *LHY*, whereas nearly 100% of *tic* individuals exhibited rhythmic expression of *CCA1* (Figures 5B and 5D).

Our previous report on *tic* supported the idea that neither a circadian peak nor a decline before dusk was evident for *CAB:LUC* expression in *tic* seedlings under a long-day photoperiod, while there was no clear defect for this rhythm in *tic* under short-day conditions (Hall et al., 2003). To further examine the regulatory mechanism of *TIC*, we tested in *tic* the expression of *CCA1* and *LHY* under light/dark cycles. We found that under short-day

Figure 1. (continued).

(C) *CAB:LUC* bioluminescence rhythms in Ws, *tic-1*, *toc1-21*, and *toc1-21 tic-1* mutant plants under LL.
(D) *CAB:LUC* bioluminescence rhythms in Ws, *tic-1*, *gi-11*, and *gi-11 tic-1* mutant plants under LL.
(E) *CAB:LUC* bioluminescence rhythms in Ws, *tic-1*, *elf3-4*, and *elf3-4 tic-1* mutant plants under 16L/8D conditions.
(F) *CAB:LUC* bioluminescence rhythms in Ws, *tic-1*, *elf4-1*, and *elf4-1 tic*-mutant plants under 16L/8D conditions.
(G) *CAB:LUC* bioluminescence rhythms in Ws, *tic-1*, *toc1-21*, and *toc1-21 tic-1* mutant plants under 16L/8D conditions.
(H) *CAB:LUC* bioluminescence rhythms in Ws, *tic-1*, *gi-11*, and *gi-11 tic-1* mutant plants under 16L/8D conditions.
Open bars indicate light intervals, and closed bars indicate dark intervals. The data shown represent normalized luminescence from >20 seedlings.

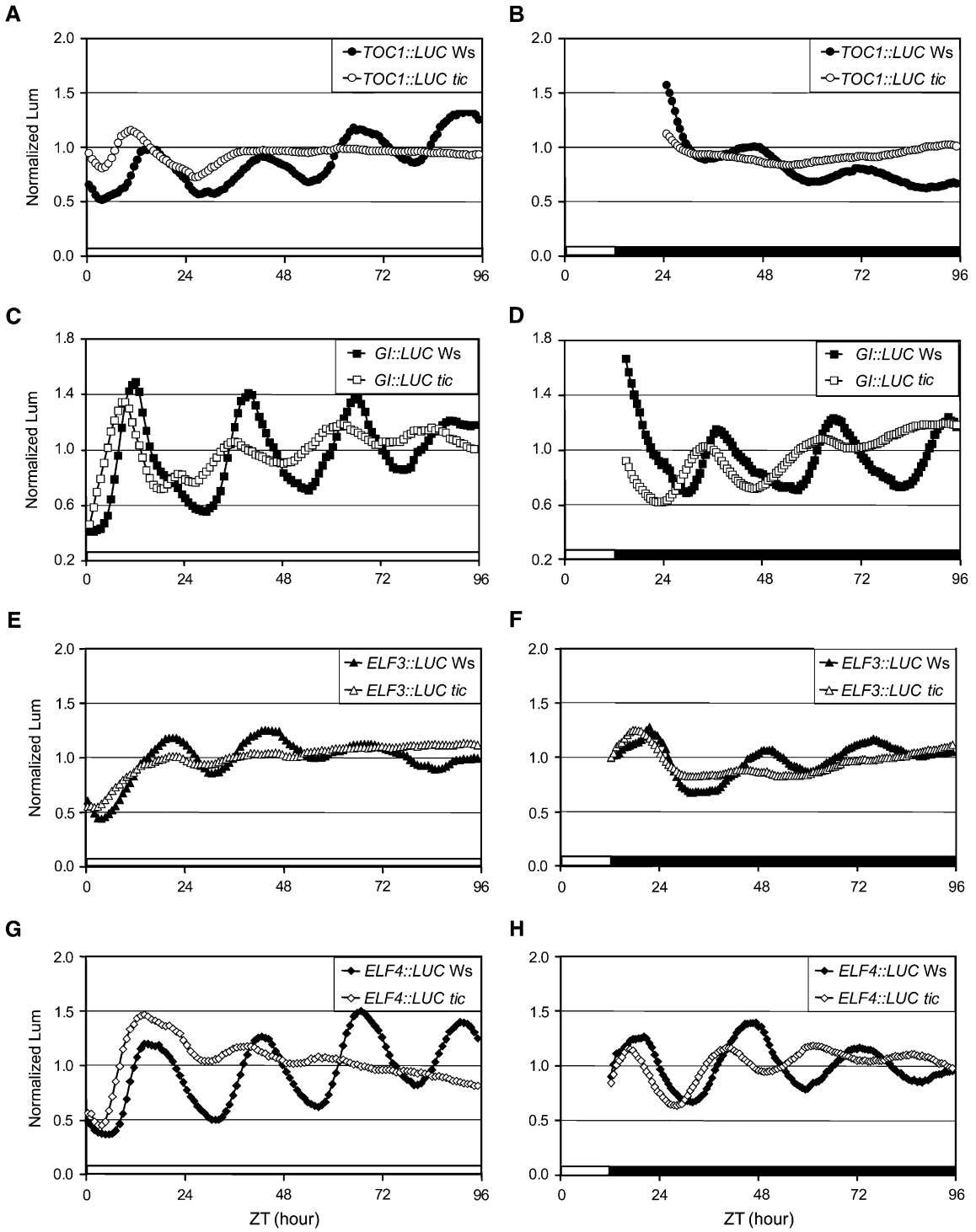

Figure 2. Effects of the Loss of *TIC* Function on Circadian Genes with Expression Rhythmic Peak in the Evening.

(A) and **(B)** *ELF3:LUC* bioluminescence rhythms in Ws and *tic-1* mutant plants under LL **(A)** or in DD (constant darkness) **(B)** conditions.
(C) and **(D)** *TOC1:LUC* bioluminescence rhythms in Ws and *tic-1* mutant plants under LL **(C)** or in DD **(D)**.
(E) and **(F)** *GI:LUC* bioluminescence rhythms in Ws and *tic-1* mutant plants under LL **(E)** or in DD **(F)**.
(G) and **(H)** *ELF4:LUC* bioluminescence rhythms in Ws and *tic-1* mutant plants under LL **(G)** or in DD **(H)**.
Closed circles, squares, triangles, and diamonds represent in Ws wild type *TOC1:LUC*, *GI:LUC*, *ELF3:LUC*, and *ELF4:LUC*, respectively. Open circles, squares, triangles, and diamonds represent in *tic-1 TOC1:LUC*, *GI:LUC*, *ELF3:LUC*, and *ELF4:LUC*, respectively. The seedlings in Ws and *tic-1* harboring the *LUC* reporter genes indicated were entrained under 12L/12D cycles before monitoring under LL or in DD. Open bars indicate light intervals, and closed bars indicate dark intervals. The data shown represent normalized luminescence from >20 seedlings.

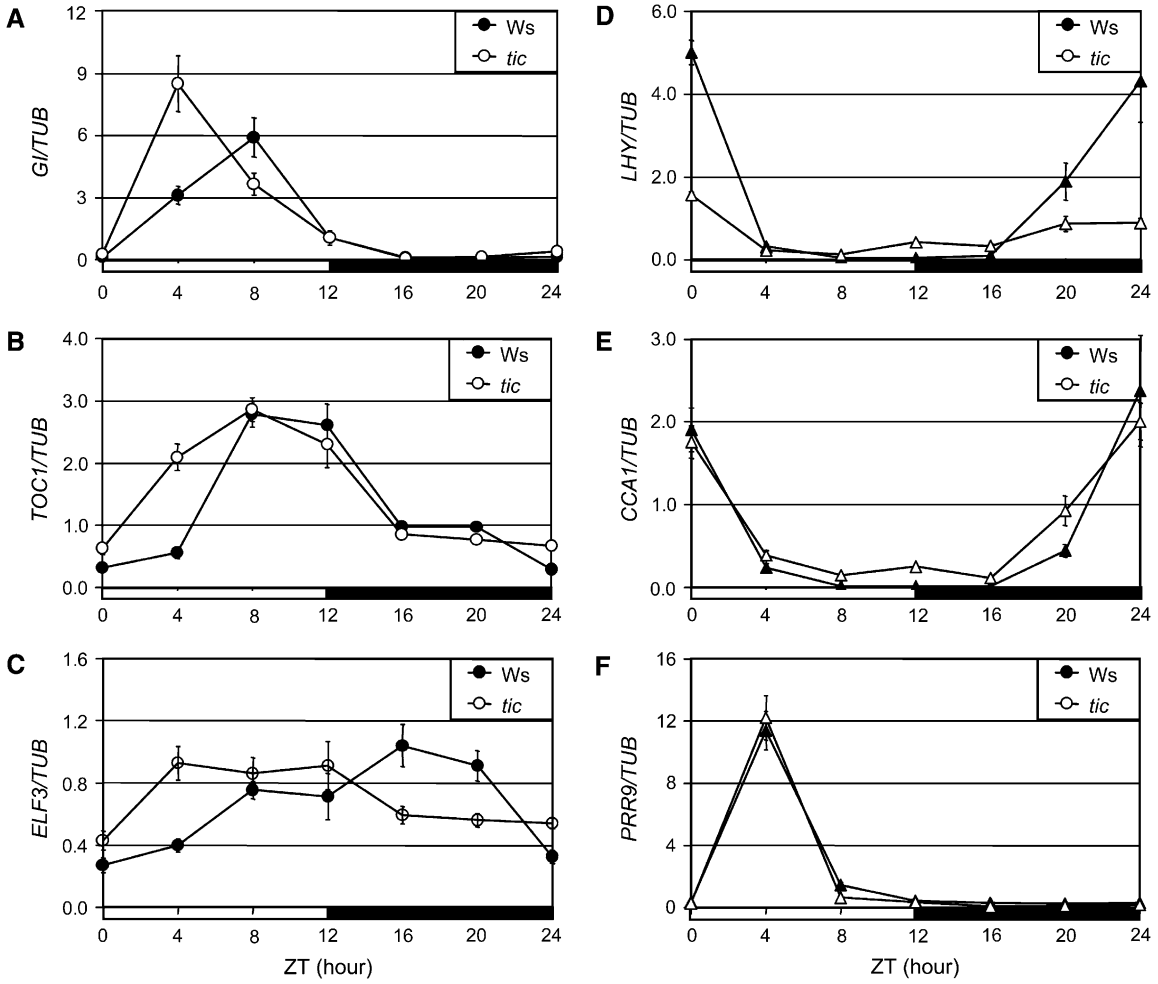

Figure 3. *TIC* Regulates the Normal Expression of Clock Genes.

Ws and *tic-1* mutant seedlings were grown for 7 d in standard 12L/12D conditions, then seedlings were harvested every 4 h. Total RNA was assayed from these samples by real-time PCR, and the accumulation of *GI* **(A)**, *TOC1* **(B)**, *ELF3* **(C)**, *LHY* **(D)**, *CCA1* **(E)**, and *PRR9* **(F)** was measured relative to an internal tubulin (TUB) control. *GI*, *TOC1*, and *ELF3* are represented with closed and open circles in Ws and *tic*, respectively. *CCA1*, *LHY*, and *PRR9* are represented with closed and open triangles in Ws and *tic*, respectively. The error bars indicate SE. Each point represents an average of three replicates within each sample. Open bars indicate light intervals, and closed bars indicate dark intervals. This experiment was repeated two times with similar results.

photoperiods, there was little distinguishable difference in *CCA1* expression between *tic* and the wild type, with the small exception of a minor peak in *tic* just before lights on (Figure 5E). This result leads us to believe that in the *tic* mutant, although circadian-regulated *CCA1* expression is no longer synchronized with light-induced expression at dawn, diurnal control can restore most of this expression defect. Compared with *CCA1* expression under a short-day photoperiod, the *LHY* expression profile was more severely altered. Besides a modest peak of expression just before/at lights on, a quite low amplitude of *LHY* expression was observed in *tic* compared with the wild type (Figure 5E). Under long-day photoperiod conditions, both *CCA1* and *LHY* expression in *tic* were affected, and for *LHY*, this was extremely dramatic. *CCA1* expression had an earlier phase in *tic* than in wild-type seedlings. Besides the peak expression at dawn, there was also another clear peak of expression just preceding lights off at dusk, perhaps indicative of the coming circadian peak suppressed by the dark transition (Figure 5F). *LHY* expression was found to be nearly arrhythmic in *tic* under long-day photoperiod conditions. In addition, the expression level of *LHY* was greatly reduced (Figure 5F). To confirm the expression level with regard to endogenous *LHY* and *CCA1* mRNA in *tic*, we performed real-time PCR on RNA extracted from replicate time points from *tic-1* seedlings grown under 12-h-light/12-h-dark cycles and examined *LHY* transcript abundance. With this assay, we found in *tic* low transcript levels of *LHY*, whereas *CCA1* transcript levels were nearly at wild-type levels (Figures 3D and 3E). We also examined the expression profile of *PRR9* (Ito et al., 2003) as

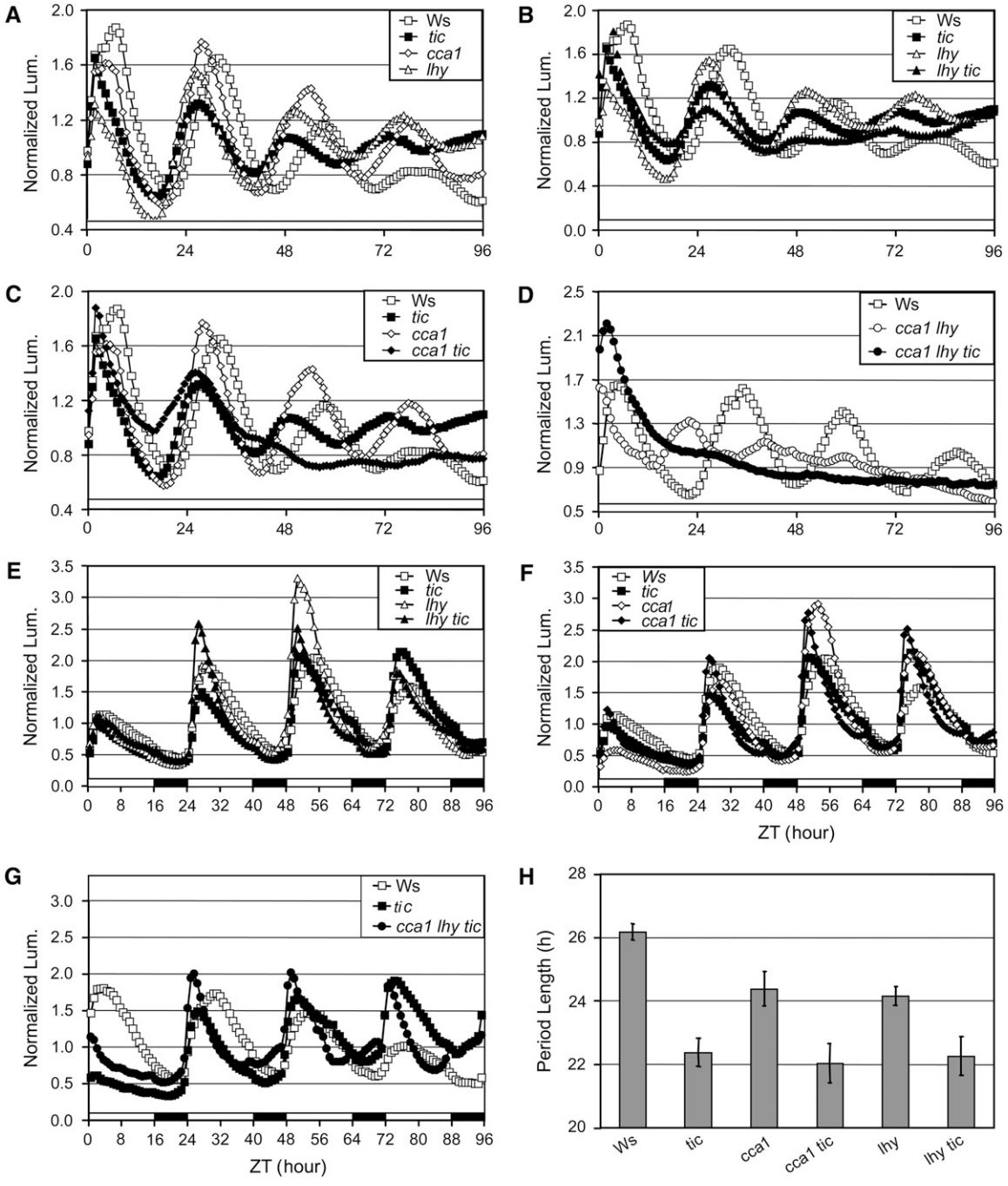

Figure 4. Genetic Interaction between *TIC* and *CCA1* or *LHY*.

Seedlings of the Ws wild type, *tic-1*, *cca1-11*, *lhy-21*, *cca1-11 lhy-21*, *cca1-11 tic-1*, *lhy-21 tic-1*, and *cca1-11 lhy-21 tic-1* were grown under standard 12L/12D conditions. *CAB:LUC* luminescence was monitored under LL. Ws, *tic-1*, *cca1-11*, *cca1-101 tic-1*, *lhy-101*, *lhy-21 tic-1*, *cca1-11 lhy-21*, and *cca1-11 lhy-21 tic-1* are represented with open squares, closed squares, open diamonds, closed diamonds, open triangles, closed triangles, open circles, and closed circles, respectively. Error bars indicate SE. Open bars indicate light intervals. The data shown represent normalized luminescence from >20 seedlings.

(A) *CAB:LUC* bioluminescence rhythms in Ws, *cca1-11*, *tic-1*, and *lhy-21* mutant plants under LL.

(B) and **(E)** *CAB:LUC* bioluminescence rhythms in Ws, *tic-1*, *cca1-11*, and *cca1-11 tic-1* mutant plants under LL **(B)** or under 16L/8D **(E)** conditions.

(C) and **(F)** *CAB:LUC* bioluminescence rhythms in Ws, *tic-1*, *lhy-21*, and *lhy-21 tic-1* mutant plants under LL **(C)** or under 16L/8D **(F)** conditions.

(D) *CAB:LUC* bioluminescence rhythms in Ws, *cca1-11 lhy-21*, and *cca1-11 lhy-21 tic-1* mutant plants under LL.

(G) *CAB:LUC* bioluminescence rhythms in Ws, *tic-1*, and *cca1-11 lhy-21 tic-1* mutant plants under 16L/8D conditions.

(H) Period analysis of *CAB:LUC* bioluminescence rhythms shown in Figures 3A to 3C under LL.

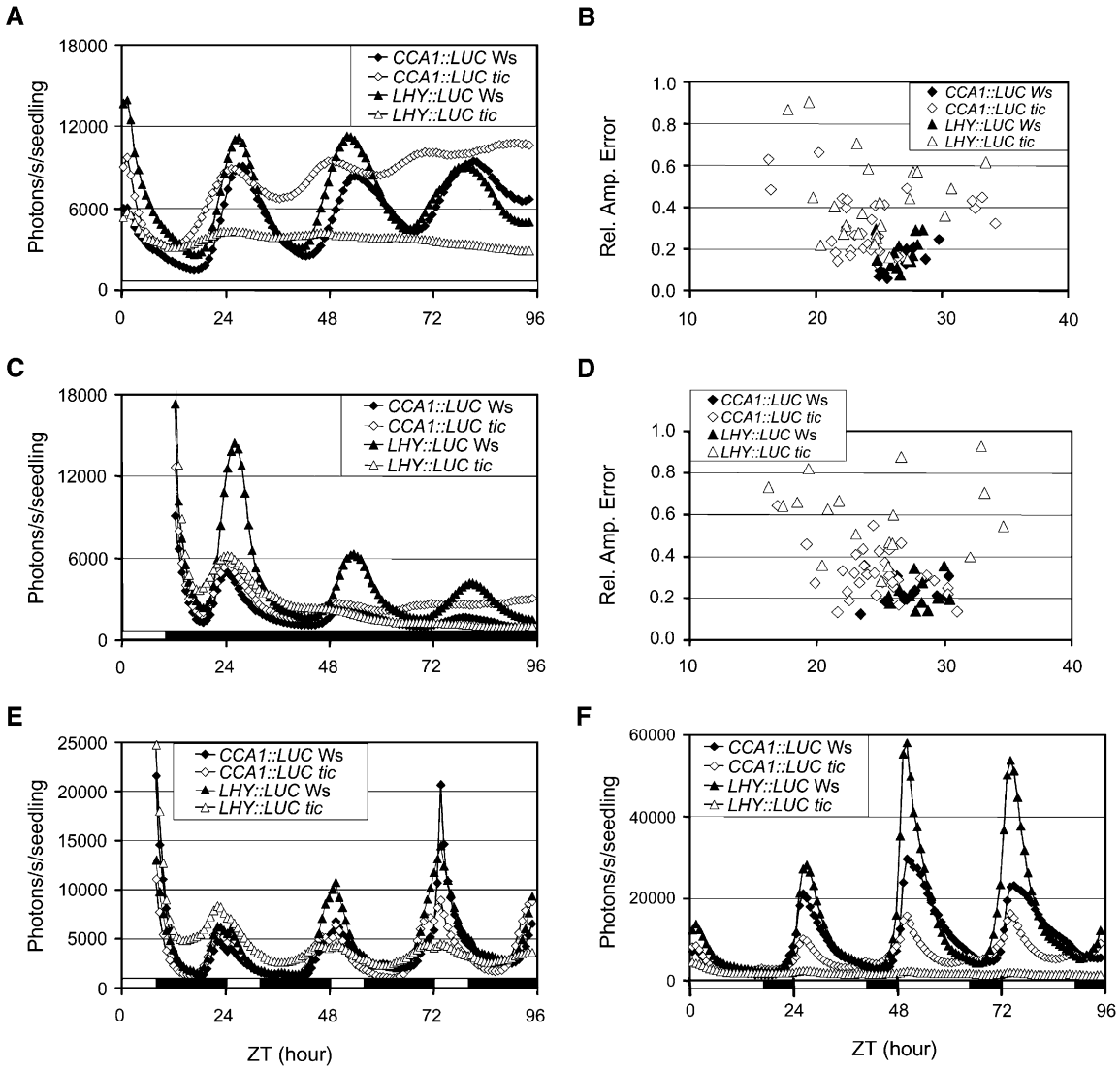

Figure 5. *LHY* Expression Is Dramatically Attenuated in *tic*.

Open diamonds and triangles or closed diamonds and triangles represent *CCA1:LUC* and *LHY:LUC* in *tic-1* or Ws, respectively. *tic-1* and Ws harboring the *LUC* reporters indicated were entrained under 12L/12D cycles before monitoring under LL or in DD conditions. Open bars indicate light intervals, and closed bars indicate dark intervals. Data shown represent means of luminescence from at least 20 seedlings.

(A) and **(C)** *CCA1:LUC* and *LHY:LUC* bioluminescence rhythms in Ws and *tic-1* mutant plants under LL **(A)** or in DD **(C)**.

(B) and **(D)** Period analysis plotted against the respective RAE for *CCA1:LUC* and *LHY:LUC* in Ws and *tic-1* mutant plants under LL **(B)** or in DD **(D)**.

(E) *CCA1:LUC* and *LHY:LUC* bioluminescence rhythms in Ws and *tic-1* mutant plants under 8L/16D cycles.

(F) *CCA1:LUC* and *LHY:LUC* bioluminescence rhythms in Ws and *tic-1* mutant plants under 16L/8D cycles.

another morning-acting factor involved in circadian function under the above assay conditions. We found that both the expression level and the phase position of *PRR9* were not altered in *tic* under light/dark cycles (Figure 3F). It can be suggested that under such diurnal conditions, *PRR9* transcript accumulation was independent of TIC regulation. Thus, we conclude that only a subset of clock-regulated transcripts are underexpressed in *tic*. Taken together, we found that TIC differentially regulates *CCA1* and *PRR9* from *LHY*, with *LHY* expression as a dominant genetic target of *TIC* action. Furthermore, we also found that both *CCA1* and *LHY* expression were more dramatically affected under long-day photoperiods than under short-day photoperiods.

Positional Isolation of the *TIC* Locus

The *tic-1* allele was reported as a mutant isolated in a direct circadian screen of a *CAB:LUC* population mutagenized with ethyl methanesulfonate (Hall et al., 2003). To allow for further

characterization of *TIC* and the original *tic* allele, we isolated the *TIC* gene via a position-cloning strategy. Previous work mapped *TIC* to chromosome 3, between molecular markers EEC and g4711, with a genetic position at ~32 centimorgan (Hall et al., 2003). We used recombination mapping and newly developed markers to delimit *TIC* to a 28-kb region that contained six genes as candidates (see Supplemental Figure 5A online). Sequencing of all of these genes in *tic-1* and in the wild type (both in the C24 background) led us to identify a sequence polymorphism in *At3g22380*. The *tic-1* allele was found to have a C-to-T transition in this gene (see Supplemental Figure 5B online), as expected from an ethyl methanesulfonate–induced event. This mutation conceptually results in a premature stop codon in the *TIC* open reading frame. Thus, *tic-1* is a putative null allele. A full-length *TIC* cDNA clone was isolated by RT-PCR with primers designed according to *At3g22380*. Our sequencing analysis indicated that *TIC* has a coding region of 4650 bp that when translated conceptually encodes for a 1550–amino acid polypeptide (data not shown). Using BLAST (Altschul et al., 1997), we found expressed sequence tags of cDNAs that posses high sequence similarities with *TIC* in a number of plant species, but none from animals or fungi (data not shown), indicating that *TIC* is restricted to plants. Using the conceptual protein sequence from *TIC* and a related sequence from *Arabidopsis* (termed here *TKL*, an acronym of TicKLe derived from TIC-like) and identified sequences from *Medicago truncatula* and rice (*Oryza sativa*), we generated a tree. From this tree, we could clearly see a sequence relationship among these conceptual protein sequences (see Supplemental Figure 6 online). Furthermore, these sequences were found to contain sequence relationships over their entire lengths (data not shown). This could suggest the conservation of biochemical function between *TIC*-related proteins.

To demonstrate that the *tic* mutant phenotype was caused by *At3g22380* loss of function, we complemented *tic-1* by transforming it with the coding region of *At3g22380* cDNA driven by the cauliflower mosaic virus 35S promoter. In these transgenic plants, the *tic* phenotype restored circadian responses under light/dark cycles or under constant light conditions (see Supplemental Figures 7B and 7C online). Furthermore, these *35S:TIC* transgenic plants displayed a slightly longer period phenotype compared with wild-type plants under constant light conditions. This mild overexpression phenotype could be an indication that *TIC* has a repressive role in period-length control of the clock (see Supplemental Figures 7C and 7D online). An increased *TIC* transcript level from *35S:TIC* transgenic plants was consistent with the longer period phenotype and an overexpression phenotype from complementation (data not shown). As well, suites of growth phenotypes were also rescued by the introduction of *35S:TIC* into the *tic* mutant (data not shown). We further confirmed that *At3g22380* encodes *TIC* by phenotypic analysis of a T-DNA insertion allele (SAIL_753_E03), termed here *tic-2*. The mutational insert in *tic-2* was found to be located in the second exon of *TIC*. In *tic-2* mutant plants, both *CAB:LUC* and *CCA1:LUC* dampened rapidly with earlier phases, as occurs in *tic-1* (see Supplemental Figure 7A online). *tic-2* also exhibited previously reported growth phenotypes, including altered leaf movement rhythms (data not shown). We conclude from these collective experiments that *At3g22380* encodes *TIC*.

TIC Transcription Is Constantly Present over Diurnal Time and Is Independent of the Regulation of Clock Genes

A release-assay experiment using *CAB:LUC* as a marker indicated that *tic* affected the circadian clock somewhere between the late-night phase and the early-morning phase (Hall et al., 2003). We thus hypothesized that *TIC* mRNA accumulation might be circadian clock regulated, with a peak just before dawn. *TIC* mRNA expression was measured in both wild-type plants and in *tic-1*. Seedlings were entrained for 7 d under light/dark cycles before transfer to constant-light conditions. Replicate RNA samples were extracted at the given times. Transcript abundance was assayed by real-time PCR. Contrary to our expectations, we found that *TIC* mRNA expression was largely independent of circadian regulation. In addition, *TIC* mRNA expression was not impaired in *tic*, indicating that this gene is not autoregulated (Figure 6A). To further support this result, bioluminescence was monitored from wild-type transgenic plants harboring the *TIC* promoter fused to the luciferase reporter gene. A *CCR2:LUC* line was used as a robustly rhythmic control; it peaks around dusk under a wide range of assay conditions (Doyle et al., 2002; Hall et al., 2003; McWatters et al., 2007). *TIC:LUC* plants exhibited only a modest light regulation in LUC activity under diurnal conditions, with a higher activity in the light period and a lower activity during the dark period (Figure 6B). The marginal diurnal expression of *TIC* observed in the *TIC:LUC* transgenic seedlings had a particularly low amplitude, which could result from a number of factors, including light regulation of LUC activity. Under free-running conditions, no luminescence rhythm was detected (data not shown). We conclude that, as was seen for *ZTL* and *LKP2* transcripts (Somers et al., 2000; Schultz et al., 2001), *TIC* regulation at the RNA level is independent of the circadian oscillator. Interestingly, although *ZTL* mRNA expression is not clock regulated, its protein level oscillates in a rhythmic manner (Somers et al., 2000; Kim et al., 2003b). Thus, clock control of a gene product could be manifest at many conceptual steps. Therefore, we tested whether TIC protein abundance is clock regulated. Analysis of TIC:green fluorescent protein (GFP) accumulation by protein gel blot revealed that this protein was present at similar levels over circadian time (Figure 7E), and no abundance changes were found at the dark-to-light transition (data not shown).

Previous reports defined *TOC1*, *GI*, *ELF3*, and *ELF4* as potential positive regulators of the morning-clock genes *CCA1* and *LHY* (Fowler et al., 1999; Alabadi et al., 2001; Doyle et al., 2002). To establish whether such a relationship between morning-acting *TIC* and the above evening genes exists, *TIC* mRNA expression was examined in *toc1-21*, *gi-11*, *elf3-4*, and *elf4-1*. Seedlings were grown under 12-h-light/12-h-dark conditions for 7 d before replicate samples were harvested at different *Zeitgeber* (ZT) times. Transcript abundance of *TIC* was assayed by real-time PCR. Compared with wild-type plants, the transcript abundance and expression pattern of *TIC* was not altered in *toc1*, *gi*, *elf3*, or *elf4* (Figures 6C and 6D). Given the relationship of *TIC* with *CCA1* and *LHY*, an indirect effect of *CCA1* or *LHY* on *TIC* is conceptually possible. We tested *TIC* expression in *cca1-11* and *lhy-21* by real-time PCR under the above assay conditions. There was no detectable difference in *TIC* mRNA abundance

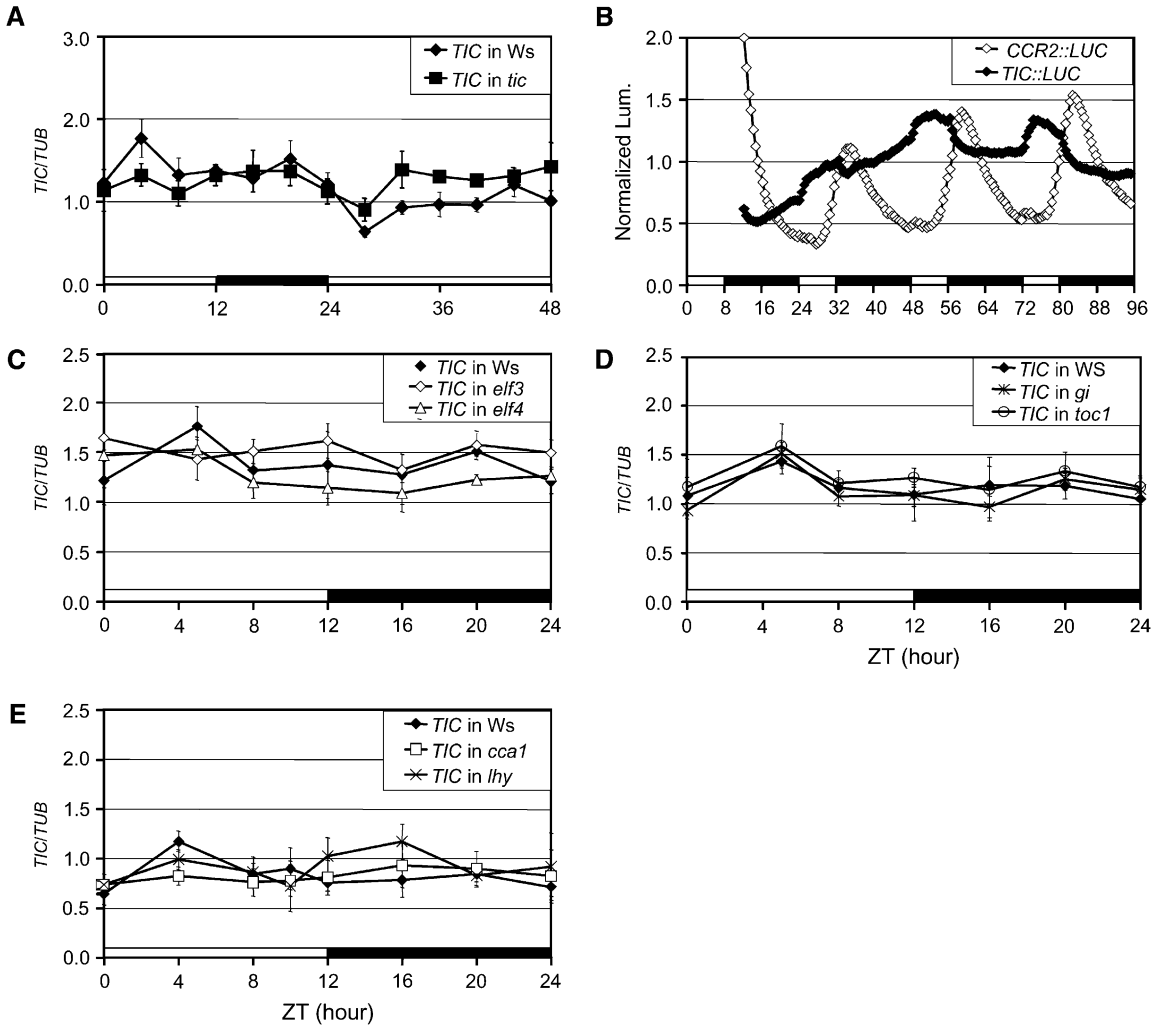

Figure 6. *TIC* mRNA Expression Is Not Clock Regulated.

(A) Real-time PCR analysis of *TIC* mRNA expression in wild-type and *tic-1* mutant plants. One-week-old seedlings in Ws and *tic* were grown under 12L/12D cycles before harvesting. Closed squares and diamonds represent *TIC* in *tic-1* and Ws, respectively.

(B) Bioluminescence was monitored from transgenic T2 seedlings harboring *TIC:LUC* and *CCR2:LUC* constructs, as indicated. Seedlings were germinated and entrained for 7 d under light/dark cycles (12L/12D) and then released into constant light. Traces represent averages from ~20 seedlings. Closed and open diamonds represent *TIC:LUC* and *CCR2:LUC*, respectively.

(C) to **(E)** Ws with *elf3-4* and *elf4-1* **(C),** *gi-11* and *toc1-21* **(D)**, and *cca1-11* and *lhy-21* **(E)** mutant seedlings were grown for 7 d in standard 12L/12D conditions, then seedlings were harvested every 4 h. Total RNA was assayed by real-time PCR, and the accumulation of *TIC* was measured relative to an internal tubulin control.

Error bars indicate SE. Each point represents an average of three repeats with each sample. Open bars indicate light intervals, and closed bars indicate dark intervals. This experiment was repeated two times with similar results.

in *cca1* or *lhy* compared with the wild-type plants (Figure 6E). Our data leads us to strongly suggest that clock genes do not genetically regulate *TIC* mRNA abundance and, furthermore, that neither its mRNA abundance nor its protein accumulation is physiologically regulated by the clock.

TIC Is Localized to the Nucleus

We found a predicted nuclear localization signal at amino acid positions 67 to 78 of the TIC protein through sequence analysis with the PROSITE database (Sigrist et al., 2005). This prediction was conserved in the analysis of TIC-related sequences in public databases (data not shown). To assess directly the intracellular localization of TIC in vivo, we generated a construct that would synthesize in plants a GFP fusion protein to TIC under the transcriptional control of the cauliflower mosaic virus 35S promoter. Transgenic *Arabidopsis* plants expressing *TIC:GFP* complemented the *tic-1* mutant phenotype, indicating to us that TIC:GFP protein is biochemically functional in vivo (see Supplemental Figures 7B and 7C online). Fluorescent examination of *TIC:GFP*

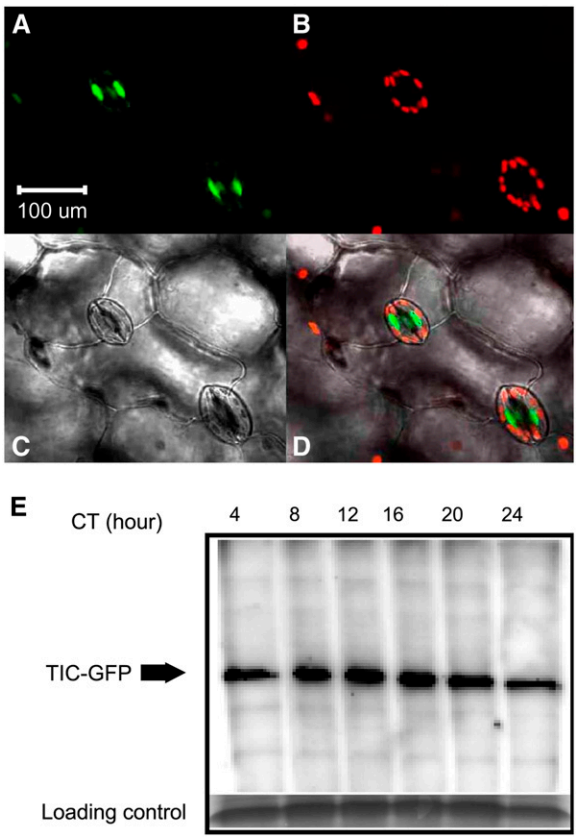

Figure 7. TIC Localizes to the Nucleus and Is Constantly Present in *35S:TIC-GFP* Transgenic Plants.

tic1-1 plants harboring with *35S:TIC-GFP* were imaged with confocal microscopy. **(A)** shows imaging in stomata of a C-terminal fusion of GFP to TIC. The signal from the red channel **(B)**, the GFP signal **(A)**, and the background are overlaid in the composite image **(D)**. **(C)** represents the same image from bright-field microscopy. *35S:TIC-GFP* transgenic seedlings were grown for 7 d under 12L/12D conditions, then replicate samples were harvested every 4 h after transfer to LL conditions. Protein expression from resultant extracts was assayed by protein gel blot hybridization **(E)**. This experiment was repeated twice with similar results.

transgenic plants was performed via confocal microscopy. Confocal image panels are presented in Figures 7A to 7D. Fluorescence signal from GFP was exclusive to the nucleus. This was confirmed with spectral analysis of the fluorescent images and with bleaching experiments (data not shown). Examining the fluorescence accumulation from the *TIC:GFP* lines under diurnal or circadian regimes, we did not find alterations in cellular distribution or in fluorescence intensity (data not shown). Thus, *TIC* encodes a protein that is strictly localized to the nucleus.

DISCUSSION

tic was a reported circadian mutant (Hall et al., 2003). Here, we further characterized the spectrum of phenotypic alterations in this mutant to more fully understand the roles of TIC in the circadian clock. One interesting feature of the *tic* phenotype is the prevalent low-amplitude response. This is reminiscent of analogous mutant studies, such as those of the *FRQ* locus in fungus and *CLOCK* studies in the mouse (Merrow et al., 1999, 2006; Debruyne et al., 2006). Collectively, these analogous studies provide insight into amplitude per se as a regulator of circadian processes. Beyond this, to assess *TIC*'s placement within the plant clock, we found that the presence of the *tic* mutation exacerbated evening-clock gene mutants. This is consistent with TIC action outside of evening times. Real-time bioluminescence and quantitative RNA analysis were used to indicate that the morning clock gene *TIC* had a closer relationship with *LHY* than it did with *CCA1* or *PRR9*. After positional isolation of the *TIC* locus, we found that both the *TIC* mRNA and its protein were not circadian clock regulated and that GFP-tagged TIC was strictly localized to the nucleus independent of circadian control. Although TIC had a strong effect on the rhythmic expression of all clock genes tested, we found that the expression profile and level of *TIC* were neither rhythmic nor affected by the mutation of clock genes. Our studies lead us to suggest that TIC has a key role in the clock, where it works close to the central oscillator of *Arabidopsis*, and that *LHY* transcriptional induction is a main target gene of TIC action.

TIC Encodes a Protein Predicted to Have P-Loop Motifs

Although we could find sequences related to *TIC* in other plants through BLAST analysis, to our knowledge, none of these genes has been characterized, nor do any of their primary amino acid sequences provide strong clues to putative biochemical activity. Through PROSCAN analysis, we found that the TIC protein is predicted to have ATP/GTP binding site A motifs (P-loop) (data not shown). The conserved amino acids [AG]-x(4)-G-K-[ST] have been found in several protein classes, including kinases, transporters, and structural proteins, and are implicated in nucleotide binding (Bork and Koonin, 1994). The significance of these protein motifs would be an important area to explore in the future, considering the potentially important consequences of nucleotide binding on a range of protein activities. For example, ATP and/or GTP binding could lead to changes in the kinetic properties of enzymatic activity or to changes in structural characteristics, such as surface charge and conformation (Leipe et al., 2003). Interestingly, in preliminary experiments, we found that a TIC fragment containing this P-loop could bind to, and be phosphorylated by, a protein kinase (our unpublished data). The bioinformatic finding of a P-loop–related motif and preliminary findings that TIC can associate with a kinase might relate TIC activity to elements of a kinase cascade. In mammals and flies, protein phosphorylation plays a critical role in circadian regulation. It regulates both protein stability and the nuclear localization of clock proteins. For example, in the *Drosophila* circadian feedback loop that includes the negative-acting factor PER and the positive-acting factor CLK, both PER and CLK activities are regulated by their phosphorylation (Yu et al., 2006). Therefore, it is possible that the TIC protein mediates circadian rhythms dependent on its in vivo phosphorylation state. This is under current study.

TIC Functions Close to the Central Oscillator

Our previous report on *tic* stated that both *CAB* and *CCR2* expression were impaired in this mutant (Hall et al., 2003). To better understand the roles of *TIC* in the *Arabidopsis* circadian clock, we further investigated the molecular–genetic network regarding where TIC operates in generating normal rhythmic responses. Since *CCA1* and *LHY* are key components of the clock model in *Arabidopsis* (Schaffer et al., 1998; Wang and Tobin, 1998), we analyzed in *tic* the expression profiles of *CCA1* and *LHY* by monitoring the luminescence of *CCA1:LUC* and *LHY:LUC* under different ambient conditions. It was found in these studies that *LHY* expression is especially dampened either under constant light or in constant darkness. Only modest effects on *CCA1* expression were noticed (Figure 5). In addition, we found that the expression waveforms of *ELF3*, *ELF4*, *GI*, and *TOC1* were all dramatically altered. Especially for *ELF3* and *TOC1*, we found that their rhythmic expression cannot be maintained in *tic* (Figures 2A to 2D). Although *ELF4* and *GI* still displayed a low-amplitude waveform of expression, both dampened earlier in *tic* than in wild-type plants, and the phase position became reversed in the wild-type plants after day 2 under constant light or in constant darkness (Figures 2E to 2H). Taken together, all evening genes tested in the *tic* background exhibited a shorter period, dampened more rapidly, and had a reduced amplitude over the oscillation (Figure 2). Moreover, although we found that the expression profiles of *PHYA*, *PHYB*, *PHYC*, *PHYD*, *CRY1*, and *CRY2* were all changed in *tic*, in which a low amplitude and a short period rhythm were detected, the mean levels of expression were not altered (data not shown). Thus, photoreceptor accumulation is likely to persist normally in *tic*. Collectively, we found that all clock-regulated genes tested were in one way or another impaired in *tic*, suggesting that *TIC* has a role close to the central oscillator of *Arabidopsis*.

tic Affects Compound Evening-Gene Mutations

Our epistatic studies revealed that all tested double mutants between *tic* and evening-gene mutants had an enhanced circadian phenotype. For example, *toc1 tic* and *gi tic* retained only one rhythm cycle of *CAB* expression under constant-light conditions, and the acute responses under light/dark cycles were greatly reduced (Figures 1C, 1D, 1G, and 1H). The displayed response effect was clearly more pronounced than that seen in each of these single mutants. Furthermore, an immediate lack of persistent rhythms in *elf3 tic* and *elf4 tic* was detected under constant light conditions (Figures 1A and 1B). We also found that *TIC* mRNA expression was not impaired in *toc1*, *gi*, *elf3*, and *elf4* (Figures 6C and 6D). In addition, although evening-clock genes had phase-shifted phenotypes under light/dark cycles in *tic*, the expression level was not altered (Figures 3A to 3C; see Supplemental Figure 3 online). These data are collectively consistent with our suggestion of a combinatorial effect of *tic* on evening mutants, thus suggesting that *TIC* and evening genes function at different arms of the circadian loop.

LHY Expression Requires *TIC* Function

From the collective work we report here, the *LHY* expression pattern was found to be dramatically altered in *tic*. Either under constant light or in constant darkness, *LHY* rapidly approached arrhythmic expression (Figures 5A and 5C). The absolute level of *LHY* transcript was also greatly reduced, especially under long-day photoperiods (Figure 5F). By contrast, although *CCA1* expression was also impaired in *tic*, its expression level was near that of the wild type under most physiological conditions (Figures 5A, 5C, 5E, and 5F). The low expression level of *LHY* in *tic* under light/dark cycles was also confirmed by real-time PCR (Figure 3D). The earlier phase shifting of all evening clock genes in *tic* was also consistent with that in *lhy* or *cca1*, which exhibited a phase-advanced phenotype for clock genes with a rhythmic peak in the evening (Alabadi et al., 2002; Mizoguchi et al., 2002). Moreover, we also found that the mean transcript levels of evening genes, such as *GI*, *TOC1*, and *ELF3*, were not altered in *tic* mutant plants, although the rhythmic peak expression of these genes was advanced under light/dark cycles, which might result from the low expression of *LHY* (Figures 3A to 3C). In addition, our genetic studies indicated that, in *cca1 tic* and *lhy tic* compared with *tic*, the phase position of *CAB* under a photoperiod and the circadian period length under free-running conditions are similar (Figures 4E to 4H). Under a long-day photoperiod, a similar waveform was detected for both *cca1 tic* and *lhy tic* double mutants (Figures 4E and 4F). The above data support our hypothesis that *LHY* is genetically downstream of TIC action. That other genetic target genes exist is plausible, but we have yet to identify them. In support, we note that *tic* enhanced the circadian dampening defect phenotype of *cca1* and *lhy*, especially in the *cca1 lhy tic* triple mutant. Here, rhythms were completely disrupted under constant light, in contrast with the extremely short-period phenotype of the *cca1 lhy* double mutant (Figures 4B to 4D). This finding lends support to the notion that TIC is predicted to have additional target genes besides *LHY*.

Uncoupling of *CCA1* and *LHY* Function

CCA1 and *LHY* genetic activities are reported as largely redundant. Mutations in these genes singly were found to have closely related phenotypic circadian responses. The double mutant was dramatically enhanced for these phenotypic responses. Such an enhancement was hypothesized to be due to each protein partially compensating for the loss of the other (Alabadi et al., 2002; Mizoguchi et al., 2002). Differences in CCA1 and LHY activity are just beginning to emerge. Gould et al. (2006) recently reported that *CCA1* and *LHY* contribute differentially to temperature compensation. At cooler temperatures, *CCA1* plays a greater role than *LHY* in temperature compensation and the maintenance of rhythm robustness, while *LHY* is the main regulator at warmer temperatures. Here, we also found several different characteristics between *CCA1* and *LHY*. For example, using *CAB:LUC* as a circadian marker, we noted a 2-h earlier phase position in *lhy* than in *cca1* (Figure 4A). We also found *LHY* expression to have an earlier phase compared with *CCA1* expression in wild-type plants under constant light (Figure 5A). These data led to the implication that the activities of CCA1 and LHY might be partly separated at different ZT phases. In addition, the *LHY* expression profile was affected dramatically, compared with that of *CCA1*, in the *tic* mutant (Figures 3A, 3B, and 5). Our

data, together with the findings of Gould et al. (2006), imply that *CCA1* and *LHY* functions can be uncoupled in the *Arabidopsis* circadian system. TIC partially mediates this difference.

METHODS

Plant Material and Growth Condition

The *tic-1* mutant of *Arabidopsis thaliana* was introgressed into the Ws ecotype through a minimum of four outcrosses. To generate *LUC* constructs containing promoter fusions of *CCA1*, *LHY*, *TOC1*, *GI*, *ELF3*, and *ELF4* in the *tic-1* mutant, we systematically crossed mutant plants with Ws wild-type plants harboring the *LUC* marker of interest and selected homozygous lines for all combinations. *CCA1:LUC*, *LHY:LUC*, *TOC1:LUC*, and *ELF4:LUC* have been described (Doyle et al., 2002; Hall et al., 2003; McWatters et al., 2007). The *LUC* constructs for *GI:LUC* and *ELF3:LUC* were obtained from G. Coupland and L. Kozma-Bognar, respectively. Both constructs will be more fully described by the respective investigators. Briefly, the *GI* promoter represents an ~2.5-kb fragment generated by PCR with the primers 5′-attB1-ACCAGCATATCTCTAATCAG-3′ and 5′-attB2-ACCGAAACTAAACCCCAAC-3′ and recombined in the pGWLuc vector. The *ELF3* promoter represents an ~2.5-kb fragment isolated by PCR with 5′-ATTACCCGGGGTTTGGCTTAAAATCTACAATATCG-3′ and 5′-CATGGATCCCCTTTTTCACTTGCAAACTTCTC-3′. This fragment was subcloned into the pPCV-LUC+ binary vector at *Xba*I/blunt *Bam*HI sites. Both of these vectors were used separately to transform the Ws wild type, and this was used in crosses for mutant studies. To construct the *TIC* promoter *LUC* vector, the 5′ upstream DNA sequence encompassing the *TIC* promoter (2690 bp) was amplified by PCR with TICF1 and TICR1 primers (see Supplemental Table 1 online). The *TIC:LUC* construct was generated by introducing this fragment into the binary vector pPZP211-LUC. We isolated several independent transgenic plants with this construct for analysis, as described above. For double mutant analyses of clock mutations to *tic*, *tic-1* mutant plants with *CAB:LUC* (6A) introgressed into the Ws ecotype were used in crosses with *cca1-11*, *lhy-21*, *gi-11*, *toc1-21*, *elf3-4*, and *elf4-1* mutants (Doyle et al., 2002; Hall et al., 2003; Gould et al., 2006; Ding and Davis, 2007). Seedlings for luminescence were grown as described (Dowson-Day and Millar, 1999; Thain et al., 2000; Ding and Davis, 2007). Growth conditions before and during assay were as described by Hall et al. (2003). Period length and RAE were estimated using the FFT-NLLS program (Plautz et al., 1997). The sustainability of rhythmicity was derived from measurements of the RAE.

TIC Gene Isolation

TIC was initially mapped on chromosome 3 between cleaved-amplified polymorphic sequence (CAPS) markers AP600 and g4711, giving a position of ~32 centimorgan on the *Arabidopsis* genetic map (Hall et al., 2003). We used the same parental lines to develop a new mapping population for fine mapping. We developed additional dCAPS markers according to the polymorphism between Columbia and Landsberg *erecta* in The Arabidopsis Information Resource database (http://www.arabidopsis.org/). Fine mapping delimited *TIC* to a 28-kb interval between dCAPS markers MCB17D and MCB17.5 (see Supplemental Table 1 online), which delineated six candidate genes. Genomic DNA from C24 and *tic-1* mutant plants was extracted, and the six genes were amplified separately by PCR with appropriate primers. These products were sequenced directly and compared to determine the mutation site.

Transgenic Complementation of *tic-1*

The coding region of *TIC* was generated by PCR with cDNA as template and ligated into the Gateway donor vector pDONOR207 through a BP reaction (Invitrogen). The primers D9F and D9R used for PCR are listed in Supplemental Table 1 online. Through an LR reaction, the *TIC* coding region was shuttled to the plant expression vector p35SGFP, and the *TIC* open reading frame was thus fused to a GFP coding region. The resulting construct (*p35S:GFP-TIC*) was introduced into *Agrobacterium tumefaciens* strain *GV3101*, which was then introduced into the *tic* mutant plants via the floral dip method (Clough and Bent, 1998). T2 plants from multiple independent transformants were used to confirm circadian complementation via testing for the rescue of proper *CAB:LUC* expression under 16-h-light/8-h-dark photoperiods. From such confirmed lines, the fluorescence signal was assayed with confocal laser scanning microscopy from 3-week-old leaves. Confocal images were captured on either a LSM410 or a LSM510 microscope (Zeiss) using 488-nm laser excitation for GFP. Photobleaching and spectral analyses were performed on the LSM510 microscope according to the manufacturer's instructions. Images were processed using Zeiss LSM510 software and processed with Adobe Photoshop.

Expression Analysis by Real-Time PCR

Seedlings were grown for 1 week in 12-h-light/12-h-dark cycles before replicate samples were harvested at the indicated ZT times. RNA was extracted with the Qiagen RNeasy plant mini kit. First-strand cDNA was synthesized using 2 μg of total RNA as template with the Invitrogen SuperScript II first-strand synthesis system for RT-PCR. The cDNA was diluted six times with water, and 2 μL was used for PCR amplification using a Bio-Rad real-time detection system, according to the manufacturer's suggestions, with primers for detecting *TIC*, *CCA1*, *LHY*, *PRR9*, *GI*, *TOC1*, *ELF3*, and *TUB*. These primer pairs are described in Supplemental Table 1 online. The efficiency of amplification was assessed relative to a tubulin standard. Each RNA sample was assayed in triplicate. Each experiment was repeated at least two times with independent biological materials. Expression levels were calculated relative to tubulin using a comparative threshold cycle method (CT method) with $\Delta\Delta C_t = \Delta C_{t,sample} - \Delta C_{t,reference,}$ where $\Delta C_{t,sample}$ was the C_t value for the assay sample normalized to tubulin and $\Delta C_{t,reference}$ is the C_t value for calibration, also normalized to tubulin, or expression levels were calculated according to the standard curve method, as appropriate. Levels of samples that were calculated according to the CT method were normalized to the maximum level of each RNA in the wild type for the CT method, which was set to 1.

Protein Gel Blot Analysis

Protein extraction and protein gel blot hybridization were performed as described (Kim et al., 2003b).

Accession Numbers

GenBank/EMBL accession numbers and Arabidopsis Genome Initiative locus identifiers for the genes mentioned in this article are as follows: proteins with sequence similarity to TIC were *Arabidopsis TIC* cDNA (AF367257) At *TIC*; *Arabidopsis TICKLE* (CAB86419) At *TKL*; rice cDNA clone (XP_478776) Os *TIC*; and cDNA manually annotated from genomic sequences (AC137838) according to the EST sequences in *Medicago truncatula* Mt *TIC*.

Supplemental Data

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

Supplemental Figure 1. Period Analysis Plotted against the Respective RAE of Figure 1.

Supplemental Figure 2. Period Analysis Plotted against the Respective RAE of Figure 2.

Supplemental Figure 3. Evening-Clock Gene Expression in *tic* under the Different Photoperiods.

Supplemental Figure 4. Period Analysis Plotted against the Respective RAE of Figure 4.

Supplemental Figure 5. Map-Based Cloning of *TIC*.

Supplemental Figure 6. Sequence Relationship between Various TIC-Related Proteins.

Supplemental Figure 7. Confirmation of *TIC* Cloning.

Supplemental Table 1. Primer Sequences.

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