TIME FOR COFFEE Encodes a Nuclear Regulator in the Arabidopsis thaliana Circadian Clock

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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 HYOCOTYL (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 HYOCOTYL (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 (P RR) 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 et al., 1999; Shearman et al., 2000; Young and Kay, 2001; Stanewsky, 2003; Houl et al., 2006).
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 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 $\text{CAB}:\text{LUC}$ luminescence.

(A) $\text{CAB}:\text{LUC}$ bioluminescence rhythms in Ws, tic-1, elf3-4, and elf3-4 tic-1 mutant plants under LL.

(B) $\text{CAB}:\text{LUC}$ 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).
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.
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 seedons. 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 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.
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, 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.
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 \textit{PRR9} were not altered in tic under light/dark cycles (Figure 3F). It can be suggested that under such diurnal conditions, \textit{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 \textit{CCA1} and \textit{PRR9} from \textit{LHY}, with \textit{LHY} expression as a dominant genetic target of TIC action. Furthermore, we also found that both \textit{CCA1} and \textit{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 \textit{CAB:LUC} population mutagenized with ethyl methanesulfonate (Hall et al., 2003). To allow for further

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

Open diamonds and triangles or closed diamonds and triangles represent \textit{CCA1:LUC} and \textit{LHY:LUC} in tic-1 orWs, respectively. tic-1 and Ws harboring the \textit{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) \textit{CCA1:LUC} and \textit{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 \textit{CCA1:LUC} and \textit{LHY:LUC} in Ws and tic-1 mutant plants under LL (B) or in DD (D).

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

(F) \textit{CCA1:LUC} and \textit{LHY:LUC} bioluminescence rhythms in Ws and tic-1 mutant plants under 16L/8D cycles.
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 \( \sim 32 \) centimorgan (Hall et al., 2003). We used recombinant 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 \( tic \)-7 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 possess high sequence similarities to \( TIC \) 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 TickKle 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 support 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 repressible 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. Replica 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.
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

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**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.
ENCODING 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.

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.

Figure 7. TIC Localizes to the Nucleus and Is Constantly Present in 3SS:TIC-GFP Transgenic Plants.

tic1-1 plants harboring with 3SS: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. 3SS: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.
**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).
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-Bogdan, 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-ACCCAGCTATCTC- TAATCAG-3' and 5'-attB2-ACCGAAACTAAACCCCAC-3' and recombined in the pGWLuc vector. The ELF3 promoter represents an ~2.5-kb fragment isolated by PCR with 5'-ATTACCCGGGTGTTGGCTAAAC- TCTACAATATCG-3' and 5'-CATGGATCCCTTTTTCACACTTGGCAAATT- CTG-3'. This fragment was subcloned into the pPCV-LUC:: binary vector at XbaI/blunt BamHI 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 cca1-1, lhy-1, toc1-11, 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; Than 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 (p35SGFP-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**

Seeds 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 

\[
\Delta C_T = \Delta C_{T,\text{reference}}
\]

where \(\Delta C_{T,\text{sample}}\) was the \(C_T\) value for the assay sample normalized to tubulin and \(\Delta C_{T,\text{reference}}\) 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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