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The *TIME FOR COFFEE* Gene Maintains the Amplitude and Timing of Arabidopsis Circadian Clocks[W]

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Plants synchronize developmental and metabolic processes with the earth's 24-h rotation through the integration of circadian rhythms and responses to light. We characterize the *time for coffee* (*tic*) mutant that disrupts circadian gating, photoperiodism, and multiple circadian rhythms, with differential effects among rhythms. *TIC* is distinct in physiological functions and genetic map position from other rhythm mutants and their homologous loci. Detailed rhythm analysis shows that the chlorophyll *a*/*b*-binding protein gene expression rhythm requires *TIC* function in the mid to late subjective night, when human activity may require coffee, in contrast to the function of *EARLY-FLOWERING3* (*ELF3*) in the late day to early night. *tic* mutants misexpress genes that are thought to be critical for circadian timing, consistent with our functional analysis. Thus, we identify *TIC* as a regulator of the clock gene circuit. In contrast to *tic* and *elf3* single mutants, *tic elf3* double mutants are completely arrhythmic. Even the robust circadian clock of plants cannot function with defects at two different phases.

INTRODUCTION

The circadian system includes an oscillator that generates biological rhythms with a period of ~24 h (reviewed by Hayama and Coupland, 2003; Stanewsky, 2003). In higher plants, these circadian rhythms control many processes, including the emission of floral fragrances (Kolosova et al., 2001), elongation growth (Dowson-Day and Millar, 1999; Jouve et al., 1999), photoperiodism (Yanovsky and Kay, 2003), and the expression of ~6% of RNAs in the model plant Arabidopsis (Harmer et al., 2000; Schaffer et al., 2001). The period length under constant environmental conditions has been a useful experimental tool, for example, in identifying mutants that affect the oscillator (Millar et al., 1995), although period also is modulated by light signaling pathways (reviewed by Hayama and Coupland, 2003). The latter normally mediate the entrainment of the whole circadian system to the 24-h period of the environmental day/night cycle, so alterations in the circadian period are not expressed directly in nature. Rather, mutations that would alter the period under constant conditions lead to an altered phase of entrainment (Yanovsky and Kay, 2002). Thus, the entrained circadian system generates appropriate responses throughout the diurnal cycle, coupling a temporal sequence of biological processes to the rhythmic environment.

Mathematical models of gene regulation can give an oscillating solution if they include a negative feedback circuit that operates with a delay (reviewed by Goldbeter, 2002). The first loop proposed within the plant circadian clock depends on the repression of *TIMING OF CAB EXPRESSION1* (*TOC1*) in the early subjective day by the products of *LATE ELONGATED HYPOCOTYL* (*LHY*) and *CIRCADIAN CLOCK-ASSOCIATED 1* (*CCA1*) (Harmer et al., 2000; Alabadi et al., 2001, 2002; Mizoguchi et al., 2002). The CCA1 and LHY proteins are homologous DNA binding proteins that recognize a sequence present in the *TOC1* promoter (Harmer et al., 2000; Alabadi et al., 2001) in the early subjective day (Schaffer et al., 1998; Kim et al., 2003). *TOC1* expression in the late day to early night is proposed to activate the transcription of *CCA1/LHY*, completing the loop (Alabadi et al., 2001). The activation could be indirect, because it takes >8 h from the peak of *TOC1* expression and requires at least three other genes, *EARLY FLOWERING3* (*ELF3*) (Schaffer et al., 1998), *GIGANTEA* (*GI*) (Fowler et al., 1999), and *ELF4* (Doyle et al., 2002). The latter are expressed in the evening and encode plant-specific proteins of unknown biochemical activity. *gi* mutants alter period (Fowler et al., 1999; Park et al., 1999), *elf3* mutants are arrhythmic in light but not in darkness (Hicks et al., 1996), and *elf4* mutants first lose rhythmic accuracy and then become arrhythmic in light and darkness (Doyle et al., 2002).

This diversity of phenotypes indicates that the cognate wild-type genes have different functions in the clock regulatory net-

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work. Mutants in these evening-expressed genes can all have elongated hypocotyls, however, suggesting that their wild-type functions have some overlap. By contrast, the *lhy cca1* double mutant has a short hypocotyl (Alabadi et al., 2002). Arabidopsis *PSEUDO-RESPONSE REGULATOR* genes, with sequence similarity to *TOC1*, also are expressed rhythmically and affect hypocotyl elongation and circadian period and/or amplitude when overexpressed (Matsushika et al., 2002; Sato et al., 2002) or disrupted (Eriksson et al., 2003), although their exact circadian function is unknown.

Here, we describe *time for coffee* (*tic*), a circadian mutant of Arabidopsis that has phenotypes affecting many aspects of biological rhythms and photoperiodism. We located *TIC* function to the mid to late subjective night, a phase at which any human activity often requires coffee, by testing its phase of action within the circadian cycle, its regulation of other clock-related genes, and its interaction with *elf3*. This spectrum of phenotypes indicates that *TIC* is important in the generation of circadian rhythms.

RESULTS

tic Reduces the Amplitude and Accuracy of Circadian Rhythms

tic was identified in an ethyl methanesulfonate–mutagenized population of the *CHLOROPHYLL a/b-BINDING PROTEIN gene promoter*: *LUCIFERASE* (*CAB*:*LUC*) transgenic line by its altered pattern of reporter gene expression under constant light. The observed *tic* phenotype was a very-low-amplitude luminescence rhythm (see Figure 1C of Millar et al., 1995). The *tic* phenotype segregated as a monogenic recessive trait in the F1 and F2 generations of successive backcrosses to the *CAB*:*LUC* parent (data not shown). Because it is fully recessive, the mutation is potentially caused by an absence of *TIC* function.

We tested several circadian rhythms to characterize the mutant and to allow comparisons with other clock mutants. Seedlings were grown under 12-h-light/12-h-dark cycles (12L:12D) at constant temperature to entrain the circadian clock and then transferred to constant conditions to reveal circadian rhythms. Ambient lighting affects all circadian clocks, so we tested rhythms under various light conditions. We confirmed the low amplitude of *CAB*:*LUC* luminescence rhythms in *tic* seedlings under constant bright white light (LL; Figure 1A), dim red plus blue light (R+B; Figure 1C), and dim red light (data not shown). The mean level of *CAB* expression remained within the wild-type range in all conditions, so drastically altered expression levels did not conceal ongoing rhythms. Rather, the rhythms that were detected in *tic* mutants lost amplitude after 2 to 3 days (referred to as "damping"), whereas the wild type remained robustly rhythmic under all conditions (Figures 1A and 1C).

Mathematical analysis confirmed that rhythms in *tic* seedlings were significantly weaker than those in the wild type: only 1 of 20 and 4 of 46 *tic* seedlings gave rhythms within the wild-type range of robustness in LL (see supplemental data online) and R+B (Figure 1D), respectively. Although the detected rhythms were weaker than those seen in the wild type, they were stronger than those detected in arrhythmic mutants such as *elf3* (McWatters et al., 2000), because at least 70% of

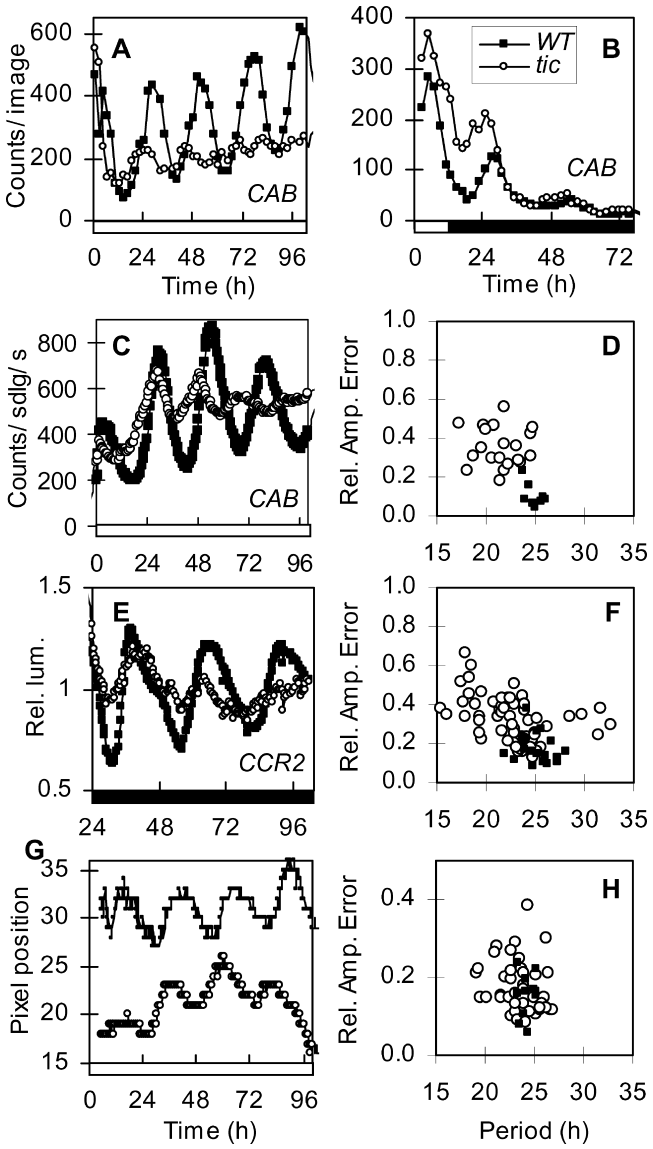

Figure 1. *TIC* Affects the Amplitude, Accuracy, and/or Period Length of Multiple Circadian Rhythms.

Transgenic seedlings carrying the *LUC* reporter genes indicated were entrained under 12L:12D cycles for 7 days, after which luminescence (Counts) was monitored in the wild type (WT; closed squares) and in *tic* (open circles).

(A) *CAB*:*LUC* under constant white light.

(B) *CAB*:*LUC* under one LD cycle followed by DD.

(C) *CAB*:*LUC* under R+B.

(E) *CCR2*:*LUC* under DD.

(G) Leaf movements were monitored under constant white light.

(D), **(F)**, and **(H)** Mathematical analysis of experiments represented in **(C)**, **(E)**, and **(G)**, respectively. Period estimates for individual seedlings **[D]** and **[F]**) and leaves **(H)** are plotted against their relative amplitude errors (Rel. Amp. Error).

Open bars indicate light intervals, and closed bars indicate dark intervals. The data shown represent mean luminescence from representative pools of 10 to 14 seedlings (**[A]** and **[B]**), mean luminescence **(C)** or normalized luminescence (Rel. lum.; **[E]**) from 17 to 45 individual seedling records, or representative traces of vertical leaf positions (Pixel position; **[G]**) from images of 11 wild-type and 41 *tic* leaves.

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tic seedlings produced a period in the circadian range under all conditions. The mean period of the *CAB* expression rhythm in *tic* was shorter than that in the wild type: 21.8 ± 0.7 h (SE) for *tic* in LL versus 23.9 ± 0.2 h for the wild type, and 20.9 ± 0.3 h for *tic* in R+B versus 26.1 ± 0.2 h for the wild type. In each case, the variance of period was significantly greater in *tic* than in the wild type, as demonstrated by the *F* statistic ($P < 0.005$ in each case). The *tic* mutation profoundly affected several circadian characteristics, consistent with a function for *TIC* in the circadian clock that controls *CAB* expression in the light.

When plants were transferred to constant darkness (DD), *tic* seedlings showed a clear peak of rhythmic *CAB*:*LUC* expression at a phase ~5 h earlier than did wild-type seedlings (Figure 1B). The decrease in mean *CAB* expression level was unchanged in *tic* (Figure 1B), so circadian period cannot be assessed with this marker under DD. Expression of *COLD AND CIRCADIAN REGULATED2* (*CCR2*; also known as *AtGRP7*) did not show such damping in wild-type plants under DD. *tic* mutants had a shortened period of *CCR2* expression, with an average period of 21.3 ± 0.41 h compared with 25.0 ± 0.35 h in the wild type (Figures 1E and 1F). *CCR2* expression rhythms also lost amplitude in *tic* mutants during the DD time course, which was reflected in the fact that only 38 of 81 *tic* seedlings had a *CCR2* expression rhythm within the wild-type range of robustness. Therefore, *tic* affects clock function in both light and darkness. However, a greater proportion of *tic* mutants had robust *CCR2* expression in DD than had robust *CAB* expression in the light, and the initial phase of *CCR2* expression in *tic* was not obviously different from that in the wild type (Figure 1E), in contrast to the early phase of *CAB* expression.

We tested rhythms of leaf movement in LL as an independent rhythmic marker. Again, *tic* plants showed a broader range of periods than did wild-type plants (Figures 1G and 1H), but the mean period was longer (25.5 ± 0.3 h) than in the wild type (23.8 ± 0.1 h) and the majority of *tic* leaf movement rhythms (35 of 41) were within the wild-type range of robustness, in contrast to *CAB* expression rhythms. Rhythms of *CCR2* expression in R+B (see supplemental data online) also showed a high percentage of robustly rhythmic *tic* plants (29 of 34) with a slightly longer mean period but greater variability (23.8 ± 0.3 h versus 23.0 ± 0.3 h for the wild type). Thus, the *tic* mutation reduced the robustness and/or period accuracy of circadian timing in all our assays, showing qualitatively that wild-type *TIC* is a component of the circadian system. However, the importance of *TIC* function varies among the many circadian clocks that are present in the plant, because the *tic* mutation altered the period and phase of some but not all circadian rhythms.

tic Affects the Circadian Gating of Light Responses

The complex interaction of light signaling with the plant circadian clock is affected specifically by mutations such as *elf3*. The pattern of *CAB* expression reflects several aspects of this interaction (Millar and Kay, 1996), including the alteration of circadian phase according to the photoperiod of the entraining day/night cycle and the rhythmic antagonism of light responses in the early subjective night (termed circadian gating). To determine whether the *tic* mutant affected this regulation, we monitored *CAB* expression under 8L:16D or 16L:8D cycles followed by a transfer to DD. The light intervals consisted of low-intensity red light to reveal any enhanced responsiveness to light. During 8L:16D cycles, the acute and circadian peaks could not be distinguished within the short photoperiod. During 16L:8D cycles, the acute response was relatively small in the wild type, and the higher circadian peak at approximately midday was followed by a decline in *CAB* expression (Figure 2A). Neither a circadian peak nor a decline before dusk was evident in *tic* seedlings under 16L:8D (Figure 2B), consistent with the low amplitude of circadian rhythms in LL (Figure 1). The peak of *CAB* expression in DD occurred ~4 h earlier after 8L:16D cycles than after 16L:8D in both genotypes, indicating that entrainment in *tic* remains sensitive to photoperiod despite its earlier average phase (Figures 2A and 2B). Both genotypes showed increased *CAB* expression in anticipation of dawn and acute activation of similar magnitude after each light-on signal, indicating that this light response is not enhanced in *tic* mutants.

We tested the circadian gating of the acute response to light by transferring plants grown in 12L:12D to DD and monitoring the induction of *CAB* expression after treatment at various times with a white light pulse. Wild-type seedlings showed clear circadian gating of *CAB* induction (Figure 2C). The size of the acute response to a light pulse varied rhythmically, with maximal responses at 28 h (coinciding with peak *CAB* expression in DD) and minimal responses during the subjective nights at 14 h and 38 to 40 h (Figure 2C) (Millar and Kay, 1996; McWatters et al., 2000). Light pulses at all phases activated *CAB* expression in *tic* seedlings (Figure 2C). The maximal acute response occurred at an earlier phase (24 h) but reached a level similar to that of the wild type (Figure 2C). The smallest acute response in *tic* (at 14 h) was fourfold larger than the wild-type minima; for comparison, the acute response in *elf3* at 14 h was 15-fold greater than that in the wild type (McWatters et al., 2000). Therefore, *tic* showed a partial defect in circadian gating; the altered waveform of *CAB* expression at the end of a long photoperiod (Figure 2B) is consistent with the mutant's failure to suppress light responses fully at this phase.

Genetic Mapping

The *tic* mutation was mapped to chromosome III by scoring the early-phase phenotype of *CAB*:*LUC* in DD in the F2 generation after a cross of *tic* to the Columbia accession (see Methods). *tic* is not allelic with *elf3*, which maps to chromosome II, but it is linked to an *ELF3*-like sequence named *ESSENCE OF ELF3 CONSENSUS* (*EEC*) (Liu et al., 2001). Given the phenotypic similarities between *tic* and *elf3*, *EEC* was a candidate gene for *TIC*. First, we created a cleaved amplified polymorphic sequence marker that segregated in our mapping population based on genomic sequence in the upstream region of *EEC*. Three recombination events were present between *EEC* and *tic* in a population of 46 *tic* mutants. Second, rhythms of leaf movement and gene expression were very similar to those seen in the wild type (data not shown), unlike the *tic* phenotype, in the transgenic line *eec-1* that carried a homozygous T-DNA insertion within the *EEC* coding region (see supplemental data online). Third, the F1 progeny

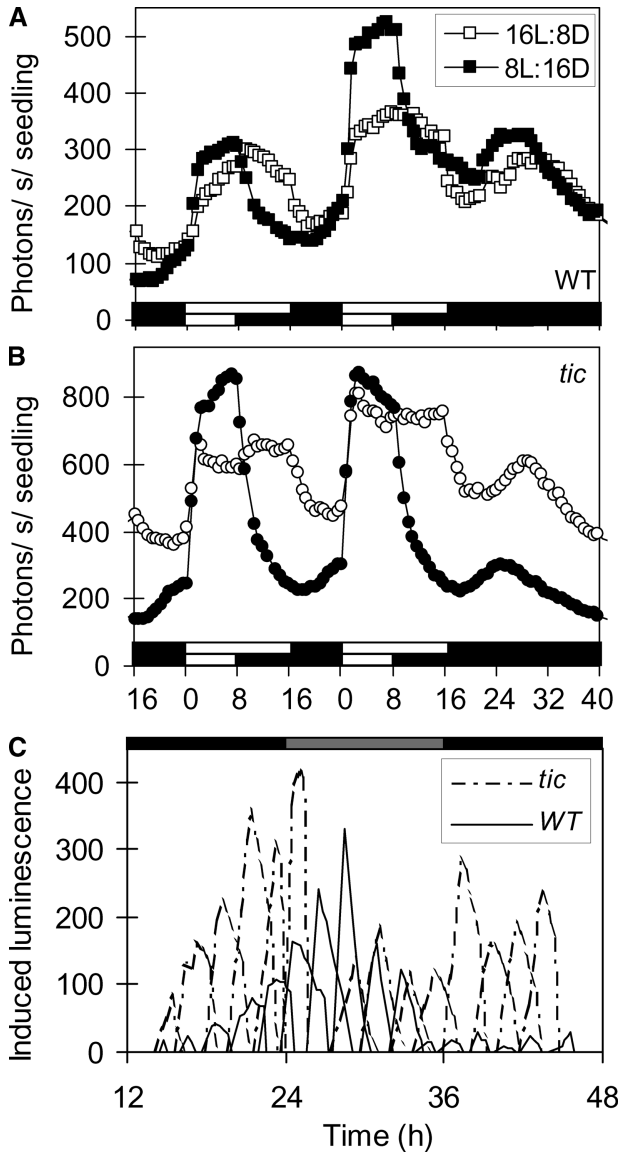

Figure 2. *TIC* Is Required for the Diurnal and Gated Regulation of *CAB* Expression.

(A) and **(B)** Wild-type (WT) **(A)** and *tic* **(B)** seedlings were entrained to either 16L:8D (open symbols) or 8L:16D (closed symbols). *CAB*:*LUC* luminescence was monitored under red light/dark cycles with the same photoperiod for 3 days before transfer to DD. Open bars indicate light intervals, and closed bars indicate dark intervals. Data shown represent means of luminescence from 16 to 24 individual seedling records.

(C) Wild-type (solid lines) or *tic* (dashed lines) seedlings were entrained to standard 12L:12D cycles and transferred to DD at 12 h after lights on (time 12 h). Replicate samples of 16 to 24 seedlings were exposed to 20 min of white light every 2 h. *CAB*:*LUC* luminescence was monitored for 2 h before and 4 h after the light pulse. The mean acute response (Induced luminescence) was calculated by subtracting of each seedling's prepulse luminescence. The black bar indicates subjective night, and the gray bar indicates subjective day, both in constant darkness.

of a cross between *tic* and *eec-1* showed rhythms of *CCR2* gene expression that were indistinguishable from those of wild-type controls (data not shown). Thus, *tic* is not an *EEC* allele.

tic Mutants Are Early Flowering and Have Altered Morphology

Arabidopsis flowers seasonally, partly in response to daylength. The photoperiodic mechanism depends on the circadian gating of *CONSTANS* (*CO*) activation by light (reviewed by Yanovsky and Kay, 2003). Because *tic* affected the gated light induction of *CAB*, we tested flowering time in *tic* plants under long and short photoperiods to determine if the *tic* mutation altered this photoperiodic response. Most wild-type Arabidopsis lines are facultative long-day plants (Figure 3C) that flower with fewer leaves in long days than in short days. *tic* was early flowering and insensitive to photoperiod, because it flowered with fewer leaves than its parent line in long and short days (Figure 3C). The C24 genetic background of the *tic* mutant delayed its flowering compared with the Columbia accession; otherwise, the photoperiodic defect in *tic* was broadly comparable to that of aphotoperiodic mutants such as *elf3* (Figure 3C).

Although *elf3* plants share some circadian phenotypes with *tic* mutants, the mutants' morphology is strikingly different. *elf3* seedlings have elongated hypocotyls, particularly when grown in short photoperiods of white light, whereas *tic* hypocotyls are similar to wild-type hypocotyls or slightly shorter (Figures 3A and 3D). *elf3* plants have long petioles with small leaf blades (Zagotta et al., 1992), whereas the *tic* rosette is close to that of the wild type in architecture but slightly smaller (Figure 3B). Both mutants are paler green than the wild type (Figure 3B), and *tic* plants form new leaves more slowly than do wild-type plants, especially in short days (data not shown); the latter phenotype might be masked in *elf3*, because it produces so few leaves. Such alterations in color and gross morphology have been noted in other clock-related mutants and attributed to altered circadian regulation and/or interactions with phytochrome B signaling (Green et al., 2002). In particular, *lhy-11 cca1-1* and *lhy RNAi cca1-1* are small, paler plants with short hypocotyls and normal leaf shape, similar to *tic* (Alabadi et al., 2002; Mizoguchi et al., 2002). The short-period, damping circadian rhythms of the double mutants in LL and DD also are similar to but more severe than the *tic* phenotypes.

Therefore, we tested hypocotyl elongation in more detail, comparing *tic* with transgenic lines that carry homozygous T-DNA insertions that disrupt the *LHY* and/or *CCA1* genes in a homogeneous genetic background (Wassilewskija). *tic* and the single *lhy* mutant had slightly reduced hypocotyl elongation in all fluence rates of red light (Figures 3E and 3F); *cca1* behaved identically to *lhy* (data not shown), whereas *elf3* has a long-hypocotyl phenotype in red light (Zagotta et al., 1996; Reed et al., 2000). *lhy cca1* showed a striking short-hypocotyl phenotype that was fluence rate dependent, indicating that *LHY* and *CCA1* negatively regulate high-fluence-rate red light signaling in the hypocotyl (Figure 3) (Alabadi et al., 2002). Therefore, the morphology of *tic* mutants is closer to that of *lhy* and *cca1*, which are defective in genes that are expressed approximately at dawn (Schaffer et al., 1998; Wang and Tobin, 1998; Alabadi

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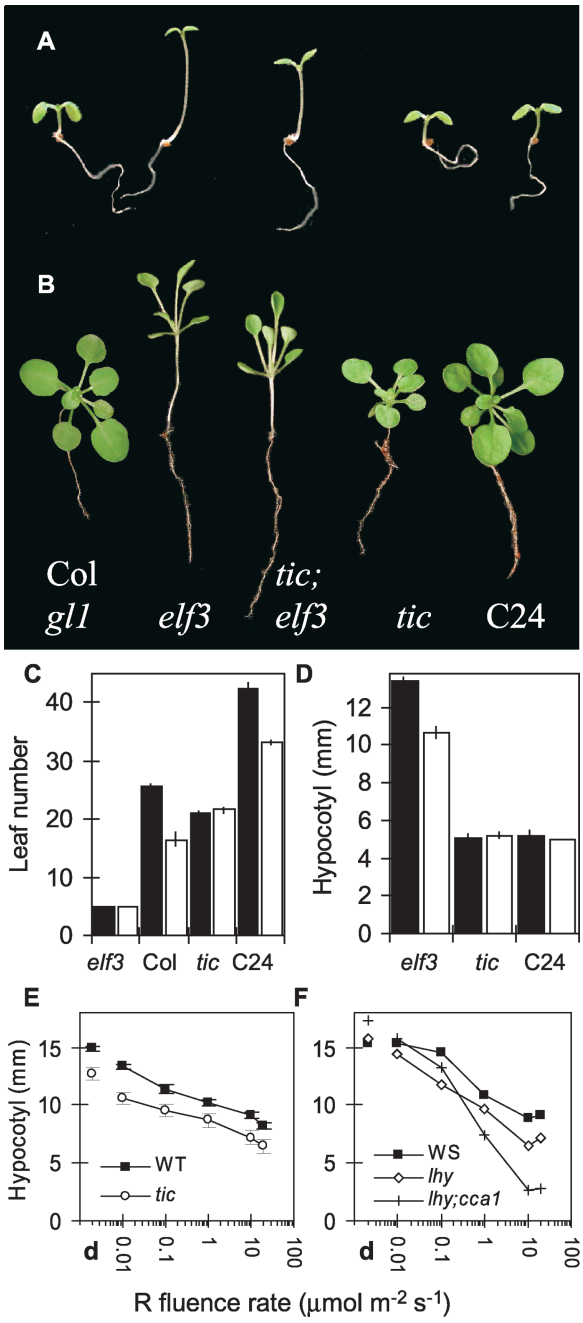

Figure 3. *TIC* Affects Light- and Photoperiod-Regulated Development.

(A) and **(B)** Morphology of the *elf3-1* and *tic* mutants, their cognate parents, and a double mutant (labeled at bottom) after 7 days **(A)** or 21 days **(B)** of growth in 8L:16D. Col, Columbia wild type.

(C) and **(D)** Flowering time **(C)** and hypocotyl elongation **(D)** of *elf3-1* and *tic* under 16L:8D (open bars) and 8L:16D (closed bars). Total fluence was equal under both photoperiods. Flowering time is scored as the number of rosette leaves when inflorescence reached 1 cm (mean ± SE, n = 16 to 29). Hypocotyl lengths are means ± SE (n = 30 to 55) after 4 days of growth.

(E) and **(F)** Fluence rate response curves for hypocotyl elongation after 4 days of growth under constant red light (R) at the fluence rates indicated or in darkness (d).

(E) *tic* mutants (open circles) compared with the C24 parent (closed squares).

(F) T-DNA insertion mutants *lhy* (open diamonds) and *lhy cca1* (crosses) compared with the Wassilewskija parent (WS; closed squares).

Data are means ± SE (n = 30 to 35); SE bars are smaller than the symbols in **(F)**. WT, wild type.

et al., 2001), than it is to that of *elf3*, which lacks a dusk-expressed gating function (McWatters et al., 2000; Covington et al., 2001; Liu et al., 2001).

tic Affects the *CAB* Clock in the Mid to Late Night

We previously used a "release" protocol to show that the circadian oscillator in *elf3* was arrested by light ~10 h after lights on (McWatters et al., 2000), which coincides with the peak of *ELF3* expression (Hicks et al., 2001; Liu et al., 2001). We repeated the release assay, comparing *tic* with its wild-type parent (Figure 4). Briefly, seedlings were entrained to 12L:12D before being transferred to LL at predicated dawn (0 h). Replicate samples were transferred to darkness at 2-h intervals, so a sample receiving 12 h of light would be equivalent to that shown in Figure 1B. The average time of the first peak of *CAB* expression in DD was plotted against the duration of the preceding light interval (Figure 4A). The peak phase in the wild type was affected only marginally by the single light-dark transition, because the peaks of *CAB* expression occurred close to the phases predicted from the discontinued light/dark (LD) cycle, at 28 to 30 h or 52 to 54 h after the last dark-light transition at 0 h (Figure 4A). *CAB* expression in *tic* seedlings peaked ~6 h earlier than that in the wild type (as in Figure 1B) in samples that received up to 15 to 17 h of light. With 19 h of light or more, the time of the peak was set by the final light-dark transitions, not by the preceding LD cycle (Figure 4A), indicating that the oscillator was arrested in the light and restarted in darkness. The apparent arrest in *tic* plants occurred after 9 h longer light exposure than in *elf3-1* plants (McWatters et al., 2000). Thus, *TIC* function affects the circadian clock in the mid to late night phase and not at the dusk phase when *ELF3* functions.

We then tested *tic* mutants in a release assay after entrainment to warm 24°C/cold 18°C (WC) cycles in constant light instead of LD cycles at a constant 22°C. Previously, this treatment had clearly restored free-running circadian oscillations to plants carrying the weak allele *elf3-7*, although their *CAB* expression was completely arrhythmic in LL (McWatters et al., 2000; Reed et al., 2000). The phase of *CAB* expression under DD in the wild type was set largely by the entraining WC cycle, with little modification by the final light-dark transition (Figure 4B), similar to the result after LD entrainment (Figure 4A). Plants that were held for 4 h or 30 to 32 h in constant conditions had an intermediate phase of *CAB* expression that was not observed after LD entrainment (Figure 4B). The fact that the light-dark transition had reset the circadian clock of these plants indicates that WC entrainment left the circadian clock more sensitive to the light-dark transition than did LD entrainment. *tic* plants that were entrained to WC cycles and held for up to 4 h in constant condi-

tions peaked at the same intermediate phase in DD as the wild type. Thus, WC cycles corrected the early phase of *CAB* expression observed in *tic* plants under LD cycles (Figure 4A). *CAB* expression reverted to an early phase in *tic* plants kept for up to 16 h in constant conditions (Figure 4B). After 18 h or more under constant conditions, phase was set again by the final light-dark transition, consistent with the result after LD entrainment (Figure 4B). Thus, the *tic* mutation severely affected the circadian clock that controls *CAB* expression in a manner that could not be rescued by a light-independent entraining signal.

TIC Affects Distinct Circadian Components from *ELF3*

The release assays (Figure 4) showed that *TIC* functioned in the mid to late night. The circadian gating assay (Figure 2C), by contrast, revealed a gating defect in *tic* that was manifest approximately at dusk, similar to the *elf3* defect, raising the possibility that *TIC* and *ELF3* functioned together at the dusk phase. To resolve these two possibilities, we constructed and characterized *tic elf3-1*. Double mutant plants have the elongated hypocotyl and rosette architecture of *elf3* but at the reduced size of *tic* (Figures 3A and 3B). The absence of epistasis indicated that *TIC* and *ELF3* have at least partly independent functions. In LL, *CAB* expression of double mutant seedlings was not distinguishable from the complete arrhythmia of the *elf3* parent (Figure 5A).

Both single mutants produced a peak of *CAB* expression at an early phase in DD (Figure 5B) and showed clear anticipation of dawn in all LD cycles tested (Figures 5C and 5D). In DD, populations of the double mutant appeared completely arrhythmic after entrainment under all conditions tested, including R+B (Figure 5B), low red light (see supplemental data online), and bright white light (see supplemental data online). Individual plants exhibited a variety of fluctuations with few or no circadian characteristics (see supplemental data online). Under LD cycles, *CAB* expression in the double mutant formed a square wave without anticipation of light-dark transitions (Figure 5C), in contrast to the wild type and single mutants. Circadian regulation is required for anticipation, whereas the square wave observed is consistent with a response to light that lacks any circadian component. The double mutant phenotypes clearly were more severe than those of either parent, again indicating that *TIC* and *ELF3* affect rhythmic regulation at least in part by different mechanisms. We tested *CAB* expression in the double mutant during and after temperature entrainment but found no consistent rhythms or anticipation of temperature transitions (data not shown). The double mutant abolished circadian function as completely as any mutant genotype yet described, comparable to plants that overexpress *CCA1* and *LHY* (Schaffer et al., 1998; Wang and Tobin, 1998).

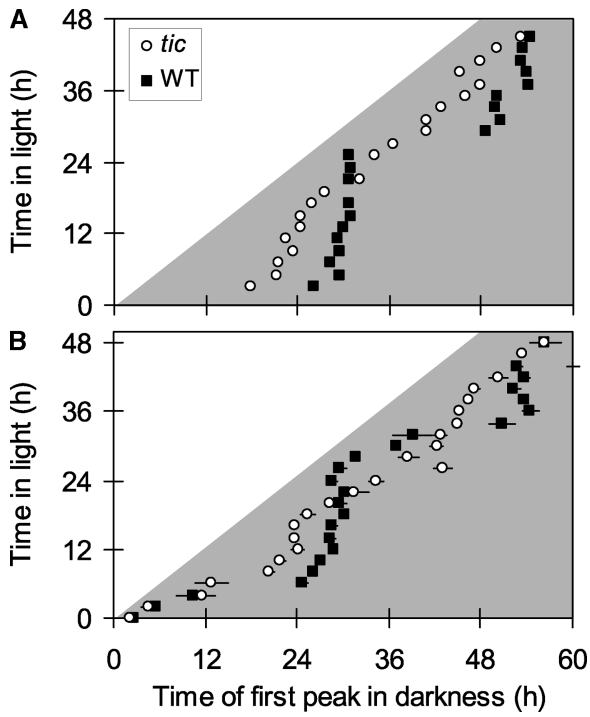

Figure 4. Release Assays Show That the Clock in *tic* Arrests in the Subjective Morning.

Wild-type (WT; closed squares) and *tic* (open circles) seedlings were entrained for 7 days to 12L:12D cycles at constant 22°C **(A)** or for 12 h at 24°C and 12 h at 18°C in LL **(B)**. At 0 h (subjective dawn), all seedlings were transferred to LL and 22°C (open area). Replicate samples of 10 to 16 seedlings were transferred to DD and 22°C (shaded area) at 2-h intervals, and *CAB*:*LUC* luminescence was monitored. Data shown represent mean phases of peak luminescence ± SE (most error bars are smaller than the symbols).

TIC Affects the Expression of Candidate Clock Components

We tested the accumulation of transcripts that encode candidate clock components in the single *tic* and *elf3* mutants and in *tic elf3* plants transferred from LD cycles to constant light. Figure 6 shows that *CCA1* and *LHY* RNA levels peaked at approximately the time of actual and predicted lights on in the wild type, whereas *TOC1* and *GI* RNA levels peaked at the end of the day (10 h after lights on), as described previously (Schaffer et al., 1998; Wang and Tobin, 1998; Fowler et al., 1999; Strayer et al., 2000). *CCA1* expression in *tic* reached slightly lower peak levels than that in the wild type before and after lights on (Figure 6A) and showed a normal pattern of diurnal regulation. The *TOC1* RNA level in *tic* plants was normal before dawn, increased slightly after dawn, and reached almost its peak level at 6 h after dawn, when the wild type hardly expressed *TOC1* (Figure 6B). *elf3* plants accumulated very little *CCA1* RNA at any phase, reaching ~10% of the wild-type peak at 2 h after dawn; this pattern is similar to the weak, light-induced expression of *CCA1* in *elf4* mutants (Doyle et al., 2002).

The pattern of *TOC1* RNA abundance in *elf3* mutants was very similar to that in *tic* plants, so the effect of the *tic* mutation may be similar to the severe reduction of *CCA1* expression in the *elf3* mutant. The maximal *TOC1* RNA level in *tic* and *elf3* mutants was approximately half that in the wild type, resulting in a 5- to 10-fold lower amplitude of rhythmic *TOC1* expression overall. Peak levels of *LHY* transcript were reduced approximately twofold in *tic* mutants, although the peak time remained

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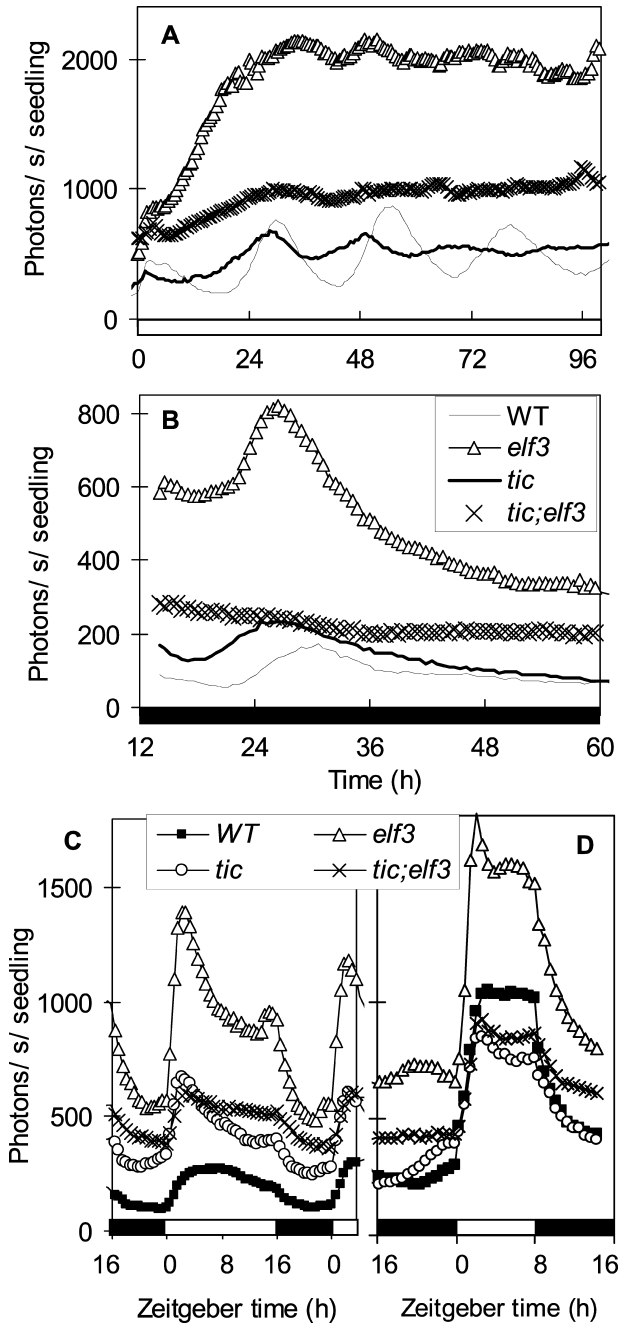

Figure 5. *CAB2*:*LUC* Luminescence Is Arrhythmic in *tic elf3-1*.

(A) and **(B)** Seedlings of the C24 parent (fine line), *tic* (heavy line), *elf3-1* (open triangles), and *tic elf3-1* (crosses) were grown in standard 12L: 12D conditions. *CAB*:*LUC* luminescence was monitored under constant R+B (as in Figure 1C) **(A)** or DD (as in Figure 1B) **(B)**. Data presented are means of 22 to 25 individual seedling traces. SE values (not shown) are smaller than the symbols representing the double mutant in all cases. WT, wild type.

(C) and **(D)** Seedlings of the C24 parent (closed squares), *tic* (open circles), *elf3-1* (open triangles), and *tic elf3-1* (crosses) were entrained to and *CAB*:*LUC* luminescence was monitored under 16L:8D **(C)** or 8L: 16D **(D)**. Open bars indicate light intervals, and closed bars indicate dark intervals. Data shown represent means of 12 individual seedling traces.

just after dawn, as in the wild type (Figure 6C). *GI* RNA levels in *tic* mutants peaked well before those in the wild type, at a level fourfold lower than in the wild type. Thus, rhythms of *LHY* and *GI* transcript accumulation also had lower amplitudes in the *tic* mutants.

tic elf3 showed very-low-amplitude rhythms for both *CCA1* and *TOC1* transcripts (Figures 6D and 6E). The mean levels varied between experiments, possibly indicating that the clock gene network in the double mutant can be driven by environmental or developmental signals, which the wild-type network is buffered against. *CCA1* RNA levels peaked during the day, as in *elf3*, rather than at dawn, as in the wild type. The peak level was intermediate between the low level in *elf3* and the nearly normal peak in *tic*. *TOC1* RNA accumulation in the double mutant was similar to that in the single mutants.

DISCUSSION

We have identified *TIC* as a component of the Arabidopsis circadian system. The *tic* mutant affected a range of phenotypes, including free-running circadian rhythms in light and darkness (Figure 1), rhythmic gating of light-activated *CAB* expression (Figure 2), hypocotyl elongation, and early, aphotoperiodic flowering (Figure 3). However, the morphological phenotype of *tic* was distinct from that of *elf3*, a previously described aphotoperiodic and gating mutant. *tic* mutants are morphologically more similar to *lhy* and *cca1* (Figure 3). A release assay for circadian clock function (Figure 4) indicated that *TIC* affects *CAB* rhythms in the mid to late night, in contrast to *ELF3*, which both functions and is expressed approximately at dusk (McWatters et al., 2000; Covington et al., 2001; Liu et al., 2001). The RNA accumulation patterns of candidate clock components were reduced to low amplitudes in *tic* (Figure 6). *tic elf3* showed additive morphological, rhythmic, and gene expression phenotypes (Figures 3, 5, and 6), consistent with the *ELF3* and *TIC* functions affecting different components of the circadian system.

tic Affects Rhythmic Markers Differentially

The *tic* mutation affects *CAB* expression rhythms more than the rhythms of leaf movement (Figures 1G and 1H) and *CCR2* expression (see supplemental data online) in the light. The latter have increased variability of period but retain a mean period close to or slightly longer than the wild type, in contrast to the short period of *CAB* expression in *tic*. This effect is unlikely to reflect a trivial difference in the light intensity or sample preparation for leaf-movement assays, because the period of *CAB* expression was affected in both dim and bright light (Figures 1A and 1C) and because the *CCR2* expression assays were conducted in exactly the same conditions as the *CAB* assays that showed a short period (Figure 1C). Rather, the *tic* phenotype emphasizes the heterogeneity of circadian rhythms in wild-type plants.

Plants and animals contain many copies of the circadian clock mechanism, probably one per cell; the clocks in plants are not tightly coupled to each other or to a central pacemaker (Thain et al., 2000). Wild-type plants maintain circadian rhythms

with different periods (Sai and Johnson, 1999; Hall et al., 2002; Michael et al., 2003), indicating a subtle difference in the underlying circadian oscillators. For example, *CCR2* expression can have a different period than *CAB* and *CCA1* expression (Eriksson et al., 2003). In several such cases, the rhythms are known to be expressed in different cell types (Hennessey and Field, 1992; Thain et al., 2002). Mutations in candidate clock components typically affect all of the rhythms tested, indicating that a qualitatively similar biochemical mechanism underlies the clocks of all cells. The common mechanism is likely to be quantitatively modulated in a cell-specific manner, but the nature of this modulation is unclear, not least because the spatial expression patterns of clock-associated genes are poorly described. In general, the circadian rhythms assayed in whole plants reflect a sample of the circadian clocks. Results derived from different rhythms must be compared with caution, because the sample of clocks will differ among rhythms. Specifically, *CCR2*:*LUC* is expressed in a wide range of cells, many of which are outside of the mesophyll layers that express *CAB*:*LUC* (our unpublished results). The differential effects we observed in *tic* could be attributable to an allele-specific effect, such as a local expression defect in the mesophyll. More likely, *TIC* function in the wild type may be most important for controlling the circadian period in *CAB*-expressing cells, although it is required for accurate and/or robust rhythms in all cells.

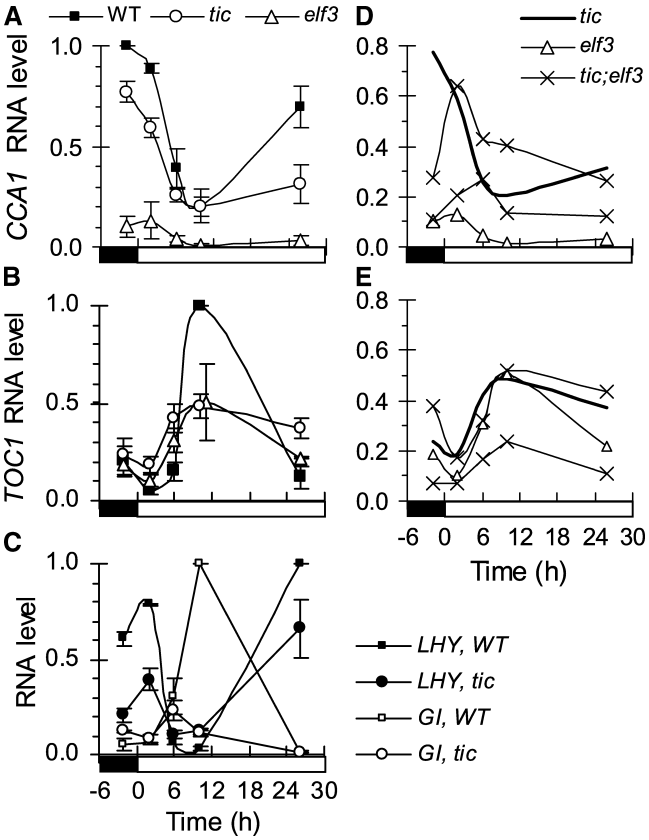

Figure 6. *TIC* Is Required for the Normal Regulation of Clock-Associated RNAs.

Seedlings were grown in standard 12L:12D conditions followed by constant light and harvested at the times indicated. Total RNA was assayed by real-time reverse transcriptase–mediated PCR for the accumulation of *CCA1* RNA (**[A]** and **[D]**), *TOC1* RNA (**[B]** and **[E]**), or *GI* and *LHY* RNA **(C)** relative to an internal *ACTIN* control. In **(A)** and **(B)**, symbols represent means ± SE of the C24 parent (closed squares), *tic* (open circles), and *elf3-1* (open triangles). In **(C)**, symbols represent RNA of *LHY* (closed symbols) and *GI* (open symbols) in *tic* (large symbols) and the C24 parent (small symbols). In **(D)** and **(E)**, RNA levels in the double mutant had consistently low amplitude but were variable in mean levels, so the results of two independent experiments are shown: *tic elf3-1* (crosses) compared with the single mutants *tic* (solid line) and *elf3-1* (open triangles). The maximum level in the wild type (WT) was set to 1 for each experiment.

TIC Functions in the Circadian Clock

Our results suggest that *TIC* contributes to the amplitude of circadian clocks. All of the transcripts of candidate clock components showed low-amplitude rhythms in *tic* (Figure 6). If these components directly regulate output genes, then low-amplitude oscillations in these components will cause low-amplitude output rhythms. Consistent with this notion, LHY and CCA1 are thought to bind to the *CAB* and *CCR2* promoters, from which we observed low-amplitude expression rhythms (Wang and Tobin, 1998; Green and Tobin, 1999; Harmer et al., 2000; Michael and McClung, 2002). The amplitude of output rhythms need not directly reflect the oscillator's amplitude if a more complex output pathway is involved, as may be the case for leaf movement. Variation in the period of all rhythms will be increased, as we observed, because the noise inherent in biological systems will have a greater impact on a low-amplitude regulator than on a higher amplitude regulator, all else being equal.

The release assay (Figure 4) provides a more discriminating, phase-specific test for clock function by using the rhythm of *CAB* expression. A defect in circadian timing appeared late in the subjective day in *elf3* mutants under these conditions (McWatters et al., 2000) but in the mid to late night in *tic* mutants (Figure 4). Thus, wild-type *TIC* functions ~9 h after *ELF3* in the circadian clock that controls *CAB* expression. The loss of amplitude in other rhythms presumably is the result of a smaller effect at the same, mid to late night phase, although *TIC* could have additional functions at other phases. The *CAB* gating defect in *tic* (Figure 2C) is obvious 5 h earlier than the *CAB* rhythm arrest, raising the possibility that the effect on gating is secondary. The early flowering of *tic* in 8L:16D is likely to result from an early phase of the photoperiodic response rhythm, such as that caused by the early expression of *CO* RNA (Figure 3) (Yanovsky and Kay, 2002).

Two factors complicate the inference of a mechanism of *TIC* function. First, the phase of oscillator arrest in *tic* mutants, 19 h after lights on, might best be compared with a phase of ~24 h in the wild type, because the phase of *CAB* expression in *tic* is ~5 h early. Second, our whole-seedling RNA samples include transcripts from many cells that do not express *CAB*, so the RNA data do not necessarily reflect the mid to late night arrest. Nonetheless, the early peak of *TOC1* RNA is consistent with reduced repression by CCA1 and LHY (Alabadi et al., 2001). It is

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unclear whether the minor reductions in *LHY* and *CCA1* RNA accumulation in *tic* are sufficient to cause the observed derepression of *TOC1*, so post-transcriptional effects and/or effects on other, similar repressors are possible. The *tic* mutation also must affect unknown activators of *TOC1* and *GI*, because neither transcript reaches its wild-type peak level in *tic* mutants.

TIC is unlikely to function specifically in light signaling. This is because *tic* mutants can show similar circadian defects under all lighting conditions (Figure 1). Depending on the rhythm tested (see above), the phase of *CAB* expression had a normal dependence on the entraining photoperiod (Figure 2), the acute light activation of *CAB* expression reached levels close to wild-type levels (Figures 2 and 5), and the hypocotyl-elongation phenotype in *tic* had little dependence on fluence rate (Figure 3). Only the restoration of a wild-type phase of *CAB* expression by temperature entrainment in LL (Figure 4B) linked *TIC* function to a clock component(s) that is more important for light than for temperature input.

Arrhythmia in *tic elf3*

A function for *TIC* in the mid to late night also explains the phenotypes of *tic elf3*. We constructed the double mutants because the overtly similar phenotypes of *elf3* and *tic* (defective gating and early phase of *CAB* expression in DD) suggested that the two mutations might show an epistatic interaction if *TIC*, like *ELF3*, affected the circadian clock via the gating mechanism. Almost all phenotypes in the double mutant were additive or intermediate between the single mutant parents (intermediate morphology [Figure 3]; complete arrhythmia [Figure 5]; intermediate *CCA1* expression levels [Figure 6]), consistent with the two genes affecting the clock at different phases through different molecular components. The pattern of *TOC1* expression, by contrast, was similar in *tic* and *elf3* single mutants and in their double mutants (Figure 6), indicating that both genes regulate *TOC1* by the same, or overlapping, mechanisms. This is the result expected if the strong *elf3-1* mutation reduced *CCA1* transcript levels to such an extent (Figure 6) that *CCA1* was no longer an effective repressor of *TOC1*. Any effect of *tic* in altering the repressive function of CCA1 would have little or no effect on *TOC1* expression in the *elf3* background. *tic elf3* was as fully arrhythmic as any Arabidopsis mutant described to date (Figures 5 and 6), presumably because the circadian cycle cannot function with defects in two distinct phases.

METHODS

Plant Materials

tic was identified as a low-amplitude ethyl methanesulfonate mutant of the *CHLOROPHYLL a/b-BINDING PROTEIN gene promoter*: *LUCIFERASE* (*CAB*:*LUC*) transgenic line in the C24 ecotype of *Arabidopsis thaliana*, as described (Millar et al., 1995). *tic* was backcrossed at least three times to the *CAB*:*LUC* parent before the physiological tests described. The *CCR2*:*LUC* construct (Doyle et al., 2002) was transformed into *tic* and its C24 parent line; several independent transformants gave essentially identical results. Transgenic *CAB*:*LUC* lines in the *elf3-1* background have been described (Hicks et al., 1996) and were crossed to *tic* mutants to produce *tic elf3*. Putative double mutants were selected by their flowering time and morphology. Their genotypes were confirmed using a cleaved amplified polymorphic sequence marker for the *elf3-1* mutant allele (Hicks et al., 2001) and markers closely linked to *tic* (see below). Wassilewskija transgenic lines carrying insertions in the *EEC*, *LHY*, and *CCA1* genes (referred to as *eec*, *lhy*, and *cca1*) were identified in the Arabidopsis Functional Genomics Consortium population (Krysan et al., 1999). Allele numbers *eec-1*, *lhy-21*, and *cca1-11* distinguish them from previously described alleles. The T-DNA locations can be viewed in the supplemental data online: *cca1-11*, 80 bp upstream from the translation start codon; *lhy-21*, 100 bp after the start of the seventh exon; and *eec-1*, 100 bp after the start of the first intron. No cognate RNA transcripts were detectable by reverse transcriptase–mediated PCR in homozygous mutants (data not shown).

Growth Conditions

Seedlings for luminescence or leaf-movement analysis were grown under 12-h-light/12-h-dark cycles (12L:12D) as described (Dowson-Day and Millar, 1999; Thain et al., 2000). Light sources were as described (McWatters et al., 2000; Doyle et al., 2002) except that dim red light (Figures 2A and 2B) was provided by red light–emitting diodes ($\sim$2 $\mu mol \cdot m^{-2} \cdot s^{-1}$). Photoperiod effects on hypocotyl growth and flowering time (Figure 3) were assessed in conditions that provided equal fluences: 8L:16D at 80 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and 16L:8D at 40 $\mu mol \cdot m^{-2} \cdot s^{-1}$ cool-white fluorescent light. Fluence response curves for hypocotyl elongation (Figure 3) were performed on standard medium without sucrose, as described (Halliday et al., 1999). Plants for RNA analysis (Figure 6) were grown in the same conditions used for luminescence assays (Figure 1A).

Genetic Mapping

The *tic* mutation was mapped to chromosome III by scoring the early-phase phenotype in DD in the F2 generation of a cross to the Columbia wild type. The phenotypic analysis was confirmed in later generations by scoring the photoperiodic defects of *CAB*:*LUC* expression under 16L:8D photoperiods. DNA from 46 mutant F2 plants was analyzed using the cleaved amplified polymorphic sequence markers g4711, EEC, and AP600. g4711 (38 centimorgan [cM] on chromosome 3) was assayed as described (http://www.arabidopsis.org); after DdeI cleavage, the C24 allele has the same banding pattern as Landsberg *erecta*. EEC is at positions 84,000 to 85,104 bp of clone MXL8 (28 cM), amplified with PCR primers 5′-ACCAGTGACCGTGTGAGACTGTG-3′ and 5′-AGAGGATCTAAAACTTTTTTTTCGTT-3′ and cleaved with AluI. AP600 is located at 439 to 1439 bp of clone MAG2 (20 cM on chromosome 3), amplified with PCR primers 5′-GTTGAAGATCATTATACTGCAGGAA-3′ and 5′-TGGATGTGTAGAAGTTAACAACACA-3′ and cleaved with AluI. The AP600 amplicon spans the Cereon polymorphisms CER464273 and CER464279, one of which gave an AluI-cleavable polymorphism (Jander et al., 2002). Of 92 *tic* chromosomes tested, 12 recombinations were observed with AP600, 5 with g4711, and 3 with EEC, giving a map position of $\sim$32 cM on the Arabidopsis genetic map.

Rhythm Analysis

Luminescence levels were measured and analyzed either by ultra-low-light video imaging (Thain et al., 2000) or with an automated luminometer (McWatters et al., 2000). Leaf movement rhythms were measured by time-lapse imaging (Dowson-Day and Millar, 1999). In each case, rhythmic traces were scored by fast Fourier transform-non-linear least squares analysis (Plautz et al., 1997) as having a circadian period if the strongest period detected was in the 15- to 35-h range (Dowson-Day and Millar, 1999). Mean periods and standard errors were variance-weighted

Table 1. Primers Used in This Study

Primer	Gene Number	Direction	Sequence (5′ to 3′)
ACT2	At5g09810	Forward	CAGTGTCTGGATCGGAGGAT
ACT2	At5g09810	Reverse	TGAACAATCGATGGACCTGA
CCA1	At2g46830	Forward	GATGATGTTGAGGCGGATG
CCA1	At2g46830	Reverse	TGGTGTTAACTGAGCTGTGAAG
LHY	At1g01060	Forward	CTTCTTCCTGTATGCGCTCC
LHY	At1g01060	Reverse	CTGGAGATTTCGAAGCCAAG
TOC1	At5g61380	Forward	TCACCATGAGCCAATGAAAA
TOC1	At5g61380	Reverse	TTGAAACTTCTCCGCCAAAC
GI	At1g22770	Forward	GGTCGACGGTTTATCCAATCTA
GI	At1g22770	Reverse	CGGACTATTCATTCCGTTCTTC
ELF3	At2g25930	Forward	ACGTTTCTGGAGAGCAAGGA
ELF3	At2g25930	Reverse	GAGCAAGAGATCCGGTGATG

(Millar et al., 1995). The variability of periods was compared using the *F* statistic based on the unweighted (arithmetic) standard deviation of period estimates. Rhythmic robustness was assessed using the relative amplitude error (RAE): a rhythm is described as being within the wild-type range of robustness if its RAE value is less than the wild-type mean RAE plus 2 SD (cf. 1 SD in Hicks et al., 1996). Release assays (Figure 4) were conducted as described (McWatters et al., 2000). All data are representative of two or three independent experiments.

Quantitative PCR Analysis

Seven-day-old seedlings that had been entrained in 12L:12D at 22°C were harvested intact into liquid nitrogen. Total RNA was isolated and treated with DNase using a Qiagen RNeasy kit (Qiagen, Crawley, UK) according to the manufacturer's instructions. One microgram of total RNA was reverse-transcribed according to the manufacturer's instructions (BD Bioscience, Cowley, UK). A total of 2.5 μL of each cDNA preparation was assayed by quantitative PCR in a LightCycler (Roche Diagnostics, Mannheim, Germany) using premixed buffer and CyberGold dye (Biogene, Cambridge, UK). PCR conditions for each primer set were optimized using a glass capillary $MgCl_2$ optimization kit (Biogene; 4 mM $MgCl_2$ for ACT2 and 3 mM for other primers). The primers used are listed in Table 1. The efficiency of amplification was assessed relative to an actin standard. Each RNA sample was assayed in triplicate. RNAs were assayed from two to three independent biological replicates. Expression levels were calculated relative to ACT2 using a comparative threshold cycle method (Applied Biosystems, 1997); levels were normalized to the maximum level of each RNA in the wild type, which was set to 1.

Upon request, materials integral to the findings presented in this publication and not available in public stock centers will be made available in a timely manner to all investigators on similar terms for noncommercial research purposes. To obtain materials, please contact Andrew J. Millar, andrew.millar@warwick.ac.uk.

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