The TIME FOR COFFEE Gene Maintains the Amplitude and Timing of Arabidopsis Circadian Clocks

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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 H11601 genes (Dowson-Day and Millar, 1999; Jouve et al., 1999), photoperiodism, and multiple circadian rhythms, with differential effects among rhythms. 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 H11601 genes (Dowson-Day and Millar, 1999; Jouve et al., 1999), photoperiodism, and multiple circadian rhythms, with differential effects among rhythms. The first loop proposed within the plant circadian clock depends on the repression of TIMING OF CAB EXPRESSION1 (TOC1) in the early subjective day (Schaffer et al., 1998; Kim et al., 2003). The latter normally mediate the entrainment of the whole circadian system to the 24-h period of the environmental day/night 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 (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). 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-
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 *Ily 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.](image)
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 than 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...
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 day-length. 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 alterations in the hypocotyl (Figure 3) (Alabadi et al., 2002). 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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TIC in the Clock (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 circa- dian 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 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 entrain- ment 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 4A), 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-
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).

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...
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.

**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 BL: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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**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 mean ± 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.
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.

**METHODS**

**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 overly 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.

**TIC in the Clock**

**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 (Dowson 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).

**Rhythm Analysis**

Luminescence levels were measured and analyzed either by ultra-low-light video imaging (Thain et al., 2000) or with an automated luminoimeter (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 mean periods and standard errors were variance-weighted
Table 1. Primers Used in This Study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Gene Number</th>
<th>Direction</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACT2</td>
<td>At5g09810</td>
<td>Forward</td>
<td>CAGTGTCTGATCGAGGAT</td>
</tr>
<tr>
<td>ACT2</td>
<td>At5g09810</td>
<td>Reverse</td>
<td>TGAACATCGATGGACCTGA</td>
</tr>
<tr>
<td>CCA1</td>
<td>At2g4830</td>
<td>Forward</td>
<td>GATGTGTTGAGCCGATG</td>
</tr>
<tr>
<td>CCA1</td>
<td>At2g4830</td>
<td>Reverse</td>
<td>TGTGTTAATGGATGGAGA</td>
</tr>
<tr>
<td>LHY</td>
<td>At1g01060</td>
<td>Forward</td>
<td>CCTGCTGTATCGGGCTCC</td>
</tr>
<tr>
<td>LHY</td>
<td>At1g01060</td>
<td>Reverse</td>
<td>CGGAGATTGGAAGCCAGA</td>
</tr>
<tr>
<td>TOC1</td>
<td>At5g61380</td>
<td>Forward</td>
<td>TCACCAATGACCCATTGAA</td>
</tr>
<tr>
<td>TOC1</td>
<td>At5g61380</td>
<td>Reverse</td>
<td>TTGAAACTTCTCAGACCAAC</td>
</tr>
<tr>
<td>GI</td>
<td>At1g22770</td>
<td>Forward</td>
<td>GTGTCGACGTTTATCCAACTA</td>
</tr>
<tr>
<td>GI</td>
<td>At1g22770</td>
<td>Reverse</td>
<td>CGGACTATTCGCGTCCTTC</td>
</tr>
<tr>
<td>ELF3</td>
<td>At2g25930</td>
<td>Forward</td>
<td>AGCTTCTGGAGAGCAAGGA</td>
</tr>
<tr>
<td>ELF3</td>
<td>At2g25930</td>
<td>Reverse</td>
<td>GAGACAGAGACTCCCGTGAT</td>
</tr>
</tbody>
</table>

(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 std (cf. 1 std 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 (Bio-gene, Cambridge, UK). PCR conditions for each primer set were optimized using a glass capillary MgCl2 optimization kit (Biogene; 4 mM gene, Cambridge, UK). PCR conditions for each primer set were optimized using a glass capillary MgCl2 optimization kit (Biogene; 4 mM gene, Cambridge, UK). PCR conditions for each primer set were optimized using a glass capillary MgCl2 optimization kit (Biogene; 4 mM gene, Cambridge, UK). PCR conditions for each primer set were optimized using a glass capillary MgCl2 optimization kit (Biogene; 4 mM gene, Cambridge, UK).

Acknowledgments

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