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Dual Role of TOC1 in the Control of Circadian and Photomorphogenic Responses in Arabidopsis [W]

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To examine the role of the *TOC1* (*TIMING OF CAB EXPRESSION1*) gene in the Arabidopsis circadian system, we generated a series of transgenic plants expressing a gradation in *TOC1* levels. Silencing of the *TOC1* gene causes arrhythmia in constant darkness and in various intensities of red light, whereas in blue light, the clock runs faster in silenced plants than in wild-type plants. Increments in *TOC1* gene dosage delayed the pace of the clock, whereas *TOC1* overexpression abolished rhythmicity in all light conditions tested. Our results show that *TOC1* RNA interference and *toc1-2* mutant plants displayed an important reduction in sensitivity to red and far-red light in the control of hypocotyl elongation, whereas increments in *TOC1* gene dosage clearly enhanced light sensitivity. Furthermore, the red light–mediated induction of *CCA1*/*LHY* expression was decreased in *TOC1* RNA interference and *toc1-2* mutant plants, indicating a role for TOC1 in the phytochrome regulation of circadian gene expression. We conclude that TOC1 is an important component of the circadian clock in Arabidopsis with a crucial function in the integration of light signals to control circadian and morphogenic responses.

INTRODUCTION

Many physiological and biochemical processes in some prokaryotes and most eukaryotes exhibit a cyclic pattern of activity with a period that approximately matches that of the earth's rotation. These 24-h rhythms are regulated by an internal timing mechanism, the circadian clock, that enables organisms to anticipate rhythmic changes in the environment and to synchronize their physiological states accordingly (Dunlap, 1999; Harmer et al., 2001). Classically, the circadian system has been divided into three conceptual components: the input elements, which perceive and transmit the environmental information to the central oscillator/s, which in turn generate rhythms through multiple output pathways.

In the past, circadian research has been focused primarily on the identification of clock components and signal transduction pathways that generate and maintain rhythmicity. Almost ubiquitously, from vertebrates to bacteria, the general mechanism of circadian clock function seems to be based on delayed negative feedback loops at the core of the oscillator (Dunlap, 1999; Harmer et al., 2001). In broad outline, this common theme involves negative molecular components that feed back to repress their own expression by inhibiting the positively acting elements (reviewed by Dunlap, 1999; Harmer et al., 2001; Young and Kay, 2001). Multiple levels of post-transcriptional controls and the existence of interlocked feedback loops also contribute to the stability and robustness of the cycling activity and provide a mechanism by which the clock can be reset by environmental cues (Glossop et al., 1999; Lee et al., 2000; Shearman et al., 2000). Among these cues, light is a very important factor in the entrainment of the clock, enabling organisms to maintain a stable phase relationship with the external photoperiod (Roenneberg and Foster, 1997). Genetic and biochemical evidence obtained in animal, fungal, and bacterial model systems have suggested that light resets the clock through changes in the phase of negative components that ultimately define the phase of the oscillator so that clock-controlled processes are appropriately phased (Devlin and Kay, 2001).

Despite the increasing progress in our understanding of the plant circadian clock (Barak et al., 2000; Roden and Carré, 2001), the study of its molecular components and intracellular pathways lags behind that of corresponding animal systems. Analysis of mutant phenotypes has provided the main clues regarding clock function in plants. *toc1-1* (*timing of cab expression1-1*), the first circadian mutant characterized in Arabidopsis (Millar et al., 1995a), exhibited

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a shortened period phenotype in multiple rhythms (Somers et al., 1998b; Strayer et al., 2000). Cloning of *TOC1* revealed that it encodes an atypical response regulator with a distinctive C-terminal motif that is conserved within the *CONSTANS* family of plant transcription factors (Strayer et al., 2000). *TOC1* mRNA rhythmically cycles and participates in a negative feedback loop mechanism to control its own expression (Strayer et al., 2000). Together, these results place TOC1 very close to the core of the oscillator. Two single MYB-domain DNA binding proteins, LHY (LATE ELONGATED HYPOCOTYL) and CCA1 (CIRCADIAN CLOCK ASSOCIATED1), were shown independently to be associated with the Arabidopsis clock. Constitutive overexpression of either gene repressed its own and the other's transcription and caused general arrhythmia, whereas the loss-of-function mutation of *CCA1*, *cca1-1*, retained clock function, albeit with a shortened period (Schaffer et al., 1998; Wang and Tobin, 1998; Green and Tobin, 1999). Alabadí et al. (2002) and Mizoguchi et al. (2002) reported recently the absence of circadian rhythms in plants lacking both CCA1 and LHY activities, showing that these transcription factors are critical components of the clock. Furthermore, the reciprocal regulation between *CCA1*, *LHY*, and *TOC1* provided a feedback loop mechanism proposed to be essential for circadian rhythmicity in Arabidopsis, with *TOC1* promoting the transcription of *CCA1* and *LHY*, the negative elements in the loop (Alabadí et al., 2001).

In plants, light is particularly important in the control of many morphogenic and circadian responses (Kendric and Kronenberg, 1994). Studies with photoreceptor-deficient mutants have provided evidence that the light-driven entrainment of the Arabidopsis clock is mediated by two classes of photoreceptors, phytochromes and cryptochromes (Devlin and Kay, 2001). Four of the five phytochromes identified in Arabidopsis (PhyA, PhyB, PhyD, and PhyE) act additively in the red light input to the clock, whereas both Arabidopsis cryptochromes, CRY1 and CRY2, are involved in the blue light signaling pathway (Somers et al., 1998a; Devlin and Kay, 2000). These photoreceptors also have overlapping functions, with CRY1 being required for PhyA signaling to the clock in both red and blue light and PhyB being necessary for CRY2 function in white light (Somers et al., 1998a; Devlin and Kay, 2000; Más et al., 2000).

Here, we have examined circadian and photomorphogenic phenotypes in a series of transgenic plants in which *TOC1* gene expression was either increased, in *TOC1* minigene (TMG) and TOC1-overexpressing (TOC1-ox) plants, or silenced by double-stranded RNA interference (RNAi) methods. In contrast to our expectations based on a previously characterized *TOC1* mutant allele, *toc1-1* (Somers et al., 1998b; Strayer et al., 2000), our results revealed unpredicted and essential roles for TOC1 in sustaining circadian rhythmicity in the dark and in specific monochromatic light conditions. We also report a role for TOC1 in the light-dependent responsiveness and phytochrome-mediated control of circadian gene expression. Together, our findings suggest that TOC1 functions as a molecular link between environmental information and clock outputs.

RESULTS

The *TOC1* RNAi Transgene Shortens the Free-Running Period of Circadian Gene Expression in Continuous White Light

In an effort to examine the role of TOC1 in circadian function, we silenced the *TOC1* gene using RNAi methods. Three different *TOC1* cDNA regions of 400 to 600 bp were cloned in the sense and antisense orientations into the RNAi vector (see Methods) (Table 1). The design of the constructs was based on the fact that the sense and antisense sequences will produce loopless hairpin RNA that may efficiently silence the gene when the intron is spliced out (Smith et al., 2000). The *TOC1* RNAi constructs were used to transform Arabidopsis transgenic plants expressing the *CCR2* (*COLD-CIRCADIAN RHYTHM–RNA BINDING2*) promoter fused to the *luciferase* (*luc*) reporter gene (Strayer et al., 2000). Our results showed that under constant white light, 90% of the *TOC1* RNAi transgenic (T1) plants displayed a 3- to 4-h shorter period length than that observed for wild-type plants (20.10 ± 0.74 h versus 24.12 ± 0.20 h, variance-weighted mean ± variance-weighted SD; Figure 1A). The short-period phenotype was maintained in transgenic T2 lines that were used for the production and subsequent selection of T3 lines. Bioluminescence analysis of different homozygous T3 *TOC1* RNAi lines (Figure 1B) revealed period lengths varying from 20.00 ± 0.30 h (line 65) to 20.98 ± 0.48 h (line 49) (Table 1).

To analyze the circadian expression of a gene that peaks at a different phase than *CCR2*, *TOC1* RNAi constructs were used to transform Arabidopsis plants expressing the morning-phased clock-controlled reporter *CAB2*::*luc* (Millar et al., 1995a). Similar to the effect of *TOC1* RNAi constructs on *CCR2*::*luc* expression, the period length of *CAB2*::*luc* expression was shortened by 3 to 4 h in 95% of the *TOC1* RNAi T1 seedlings (Figure 1D). The short-period phenotype under constant white light was maintained in T2 (data not shown) and T3 homozygous lines (Figure 1E, Table 1).

RNA gel blot analysis of *TOC1* RNAi-transformed *CCR2*::*luc* and *CAB2*::*luc* plants revealed very low levels of *TOC1* mRNA at times when *TOC1* expression was maximal in wild-type plants (cf. zeitgeber time [ZT] 8 in wild-type and *TOC1* RNAi plants in Figures 1C and 1F). The reduced levels of *TOC1* mRNA at ZT 8 are not likely attributable to advances in the phase of *TOC1* expression, because no *TOC1* mRNA was detected at earlier time points (ZT 0 and ZT 4). RNA gel blot and period length analysis of several RNAi lines confirmed that the observed short-period circadian phenotypes were correlated with decreased levels of *TOC1* mRNA (see supplemental data online). Although the *TOC1* regions selected for the RNAi constructs showed a very low

Table 1. Free-Running Period Estimates of *CCR2*::*luc* and *CAB2*::*luc* Expression in Wild-Type and Different Homozygous T3 *TOC1* RNAi Lines in Constant White Light

Line	Construct	Arm (nucleotides)	Reporter	Period (h ± SD)	*n*
T3 line 49	a3s3	415	*CCR2*::*luc*	20.98 ± 0.48	24
T3 line 65	a1s1	659	*CCR2*::*luc*	20.00 ± 0.30	36
T3 line 69	a1s1	659	*CCR2*::*luc*	20.53 ± 0.28	36
T3 line 81	a2s2	619	*CCR2*::*luc*	20.89 ± 0.90	29
Columbia wild type	–	–	*CCR2*::*luc*	24.07 ± 0.28	18
T3 line 10	a1s1	659	*CAB2*::*luc*	20.34 ± 0.32	25
T3 line 24	a2s2	619	*CAB2*::*luc*	19.83 ± 0.22	19
T3 line 57	a3s3	415	*CAB2*::*luc*	20.98 ± 0.85	15
Columbia wild type	–	–	*CAB2*::*luc*	24.27 ± 0.17	15

Plants were entrained for 6 days in 12-h/12-h light/dark cycles before being released and imaged in white light (60 μmol·m^{-2}·s^{-1}). Period estimates (variance-weighted mean ± variance-weighted SD) were obtained by fast Fourier transform nonlinear least-square best-fit algorithm analysis as described (Millar et al., 1995b; Plautz et al., 1997).

degree of identity at the nucleotide level with the rest of the members of the TOC1 family (Matsushika et al., 2000; Strayer et al., 2000), we confirmed by reverse transcriptase–mediated PCR analysis that the expression of the four other genes was similar to that observed in wild-type plants (see supplemental data online). These results confirmed the specificity of the RNAi constructs.

Role of TOC1 in the Red Light–Dependent Control of Circadian Gene Expression

Previous phenotypic analyses of *toc1-1* mutant plants revealed that this semidominant allele did not have light-dependent defects (Somers et al., 1998b; Strayer et al., 2000). To determine whether this also was the case for *TOC1* RNAi plants, bioluminescence rhythms of *CCR2*::*luc* and *CAB2*:: *luc* expression were monitored in wild-type and *TOC1* RNAi seedlings grown under 20 μmol·m^{-2}·s^{-1} continuous red (Figures 2A and 2C) or blue (Figures 2B and 2D) light. Our studies revealed that under constant red light, 93% (*CCR2*::*luc*) and 95% (*CAB2*::*luc*) of *TOC1* RNAi seedlings exhibited arrhythmic expression, with relative amplitude errors of >0.7. By contrast, wild-type plants displayed robust circadian rhythmicity, with amplitude errors of <0.4 in all cases (values > 0.7 represent very altered circadian rhythms [Dowson-Day and Millar, 1999]). Our results showed that there was a good correlation between decreased levels of *TOC1* mRNA and altered circadian expression in continuous red light: the lower the *TOC1* mRNA level, the higher the relative amplitude value of period estimates (see supplemental data online). In blue light, the oscillations in *TOC1* RNAi plants were rhythmic, but with a free-running period 3 to 4 h shorter than in wild-type plants (Figures 2B and 2D).

The specificity of the RNAi-induced phenotypes was confirmed by the analysis of *CAB2*::*luc* bioluminescence rhythms in *toc1-2* mutant plants. *toc1-2* is a recessive mutant allele that expresses an incorrectly spliced transcript that results in a truncated protein of only 59 residues (Strayer et al., 2000). Our studies revealed that under constant red light, *CAB2*::*luc* was expressed arrhythmically in 85% of the *toc1-2* seedlings (Figure 2E), displaying relative amplitude errors of >0.7. In constant blue light, *CAB2*::*luc* period length was shortened by 3 to 4 h (20.17 ± 0.17 h) compared with the wild-type period (23.39 ± 0.10 h) (Figure 2F). In both *TOC1* RNAi and *toc1-2* plants, the arrhythmic circadian expression in red light (see supplemental data online) and the short-period phenotypes in blue light (data not shown) also were observed at different light intensities (1, 10, and 40 μmol·m^{-2}·s^{-1}).

Collectively, these results reveal an unexpected and important role for TOC1 in the integration of red light signals to maintain the circadian expression of differently phased genes (*CCR2* and *CAB2*). The effect on period length under blue light suggests that TOC1 is important in controlling the pace of the clock under these conditions, although other clock components are able to compensate partially for the loss of TOC1 in blue light.

Increased *TOC1* Gene Dosage Lengthens the Free-Running Period, whereas TOC1 Overexpression Abolishes Circadian Rhythmicity

Circadian expression also was examined in TMG transgenic lines expressing additional copies of the *TOC1* locus (see Methods). Bioluminescence analysis of T3 homozygous transgenic plants transformed with the TMG construct showed a lengthening of the free-running period of *CAB2*::*luc* expression in plants maintained under constant white (Figure 3A), red (Figure 3B), and blue (Figure 3C) light. RNA gel blot analyses revealed that *TOC1* transcript levels were higher in TMG than in wild-type plants (Figure 3D). TMG lines with lower *TOC1* levels (TMG13) displayed as much of a phase shift as

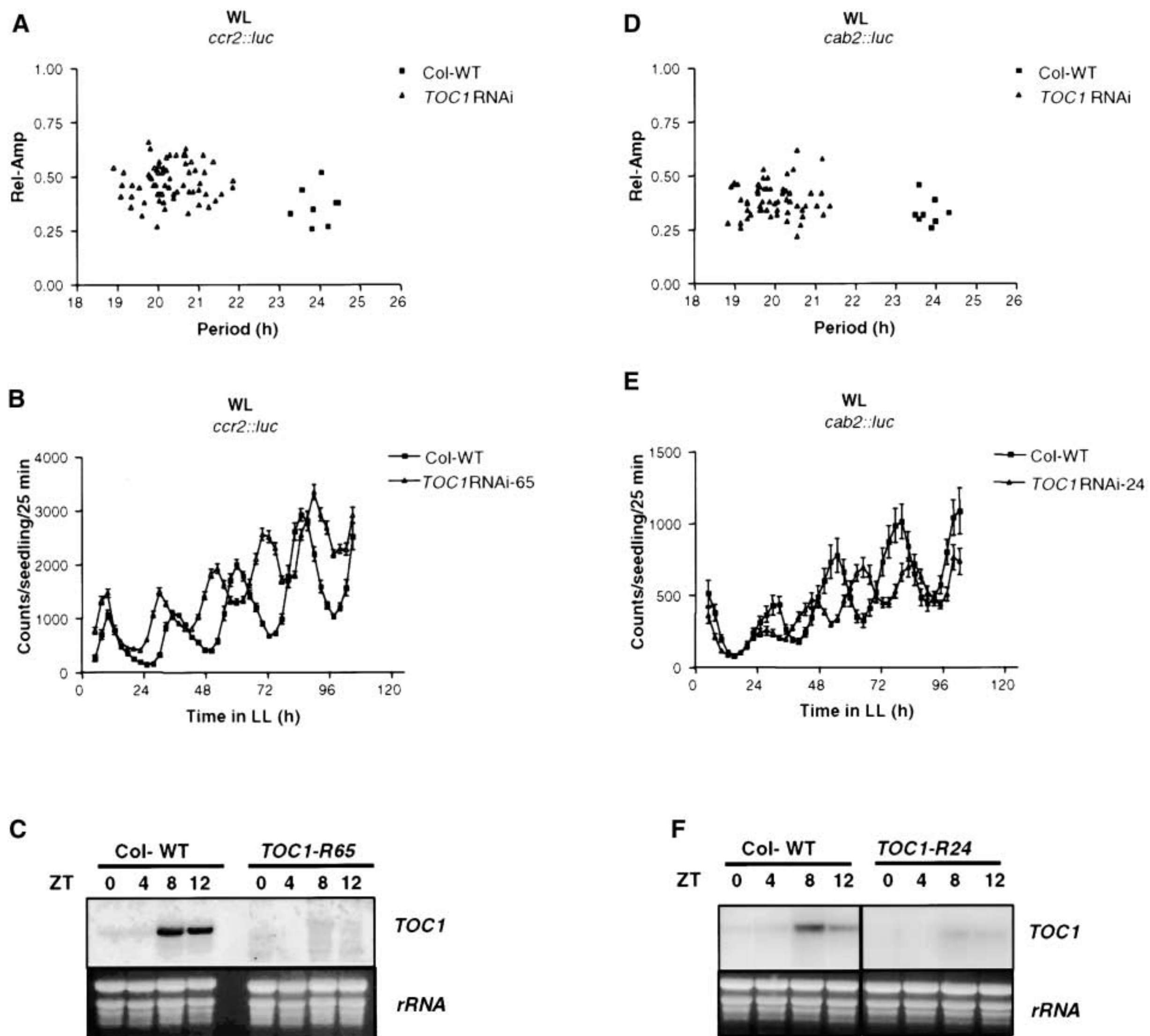

Figure 1. *TOC1* RNAi Transgene Decreases *TOC1* mRNA Levels and Shortens the Period Length of Gene Expression in Constant White Light.

(A) and **(D)** Period estimates of *CCR2*::*luc* **(A)** and *CAB2*::*luc* **(D)** expression from individual traces examined by fast Fourier transform nonlinear least-squares best-fit algorithm analysis as described (Millar et al., 1995b; Plautz et al., 1997). Wild-type (WT) and *TOC1* RNAi (T1) seedlings were entrained for 6 days in 12-h/12-h light/dark cycles before being released and imaged in constant white light (WL; 60 $\mu mol \cdot m^{-2} \cdot s^{-1}$). More than 60 T1 transgenic seedlings were analyzed in each case.

(B) and **(E)** Bioluminescence analysis of *CCR2*::*luc* **(B)** and *CAB2*::*luc* **(E)** expression in wild-type and *TOC1* RNAi (T3) lines 65 and 24. Plants were grown in 12-h/12-h light/dark cycles for 6 days before being transferred to constant white light (LL; 60 $\mu mol \cdot m^{-2} \cdot s^{-1}$). Traces represent averages of 30 to 40 seedlings. The experiments were repeated three times with similar results in all cases.

(C) and **(F)** *TOC1* mRNA levels of expression in wild-type and *TOC1* RNAi lines 65 (*CCR2*::*luc*) and 24 (*CAB2*::*luc*). Plants were maintained in 12-h/12-h light/dark cycles for 12 days before samples were harvested at zeitgeber times (ZT) 0, 4, 8, and 12; ZT 0 represents lights on. Total RNA was extracted, blotted, and hybridized as described (Somers et al., 2000; Alabadí et al., 2001). rRNA levels served as a loading control.

the lines expressing higher levels (TMG14) (Figure 4). Interestingly, *TOC1* expression was rhythmic in TMG plants (Figures 3D and 4), with lines with higher levels of *TOC1* mRNA displaying more disrupted circadian expression under constant red light (note the higher relative amplitude values in Figure 4).

We also investigated circadian function in the presence of constitutive levels of TOC1 using transgenic plants expressing the *TOC1* coding region controlled by the strong 35S promoter of *Cauliflower mosaic virus* (see Methods). In all of the T3 homozygous TOC1-ox seedlings maintained under constant white light, *CAB2*::*luc* transcription was arrhythmic (Figure 3E), with relative amplitude errors of >0.7 in all cases. Similar arrhythmic phenotypes were observed in

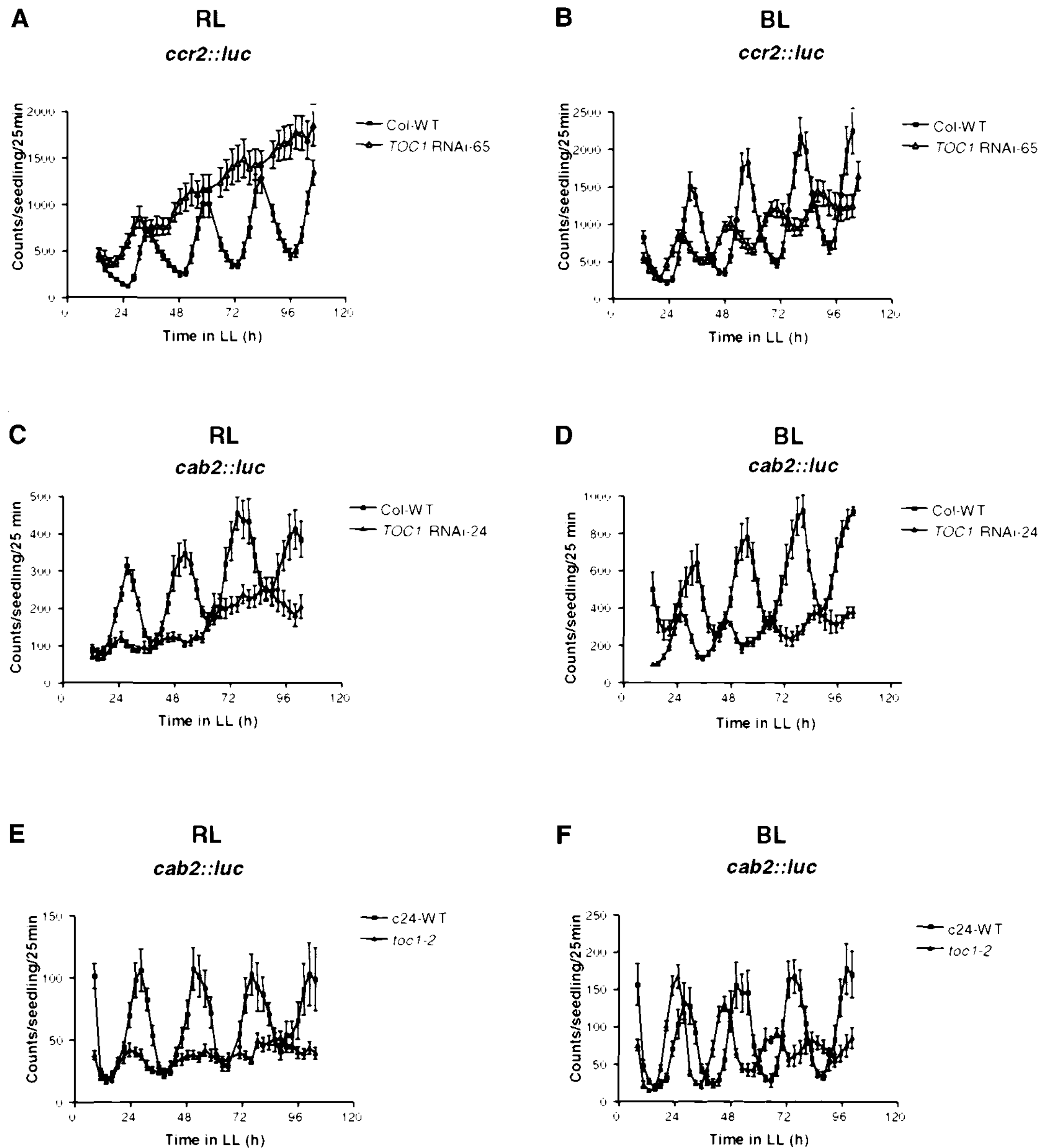

Figure 2. Effects of Monochromatic Red and Blue Light on Circadian Gene Expression in Wild-Type, *TOC1* RNAi, and *toc1-2* Plants.

Bioluminescence analysis of *CCR2*::*luc* (**[A]** and **[B]**) and *CAB2*::*luc* (**[C]** to **[F]**) expression at 20 μmol·m^{-2}·s^{-1} constant red light (RL; **[A]**, **[C]**, and **[E]**) and constant blue light (BL; **[B]**, **[D]**, and **[F]**). Seedlings were grown in 12-h/12-h light/dark cycles for 6 days before being transferred to continuous light (LL). Traces represent averages of 10 to 15 seedlings per line. The experiment was repeated three times with similar results in all cases. WT, wild-type.

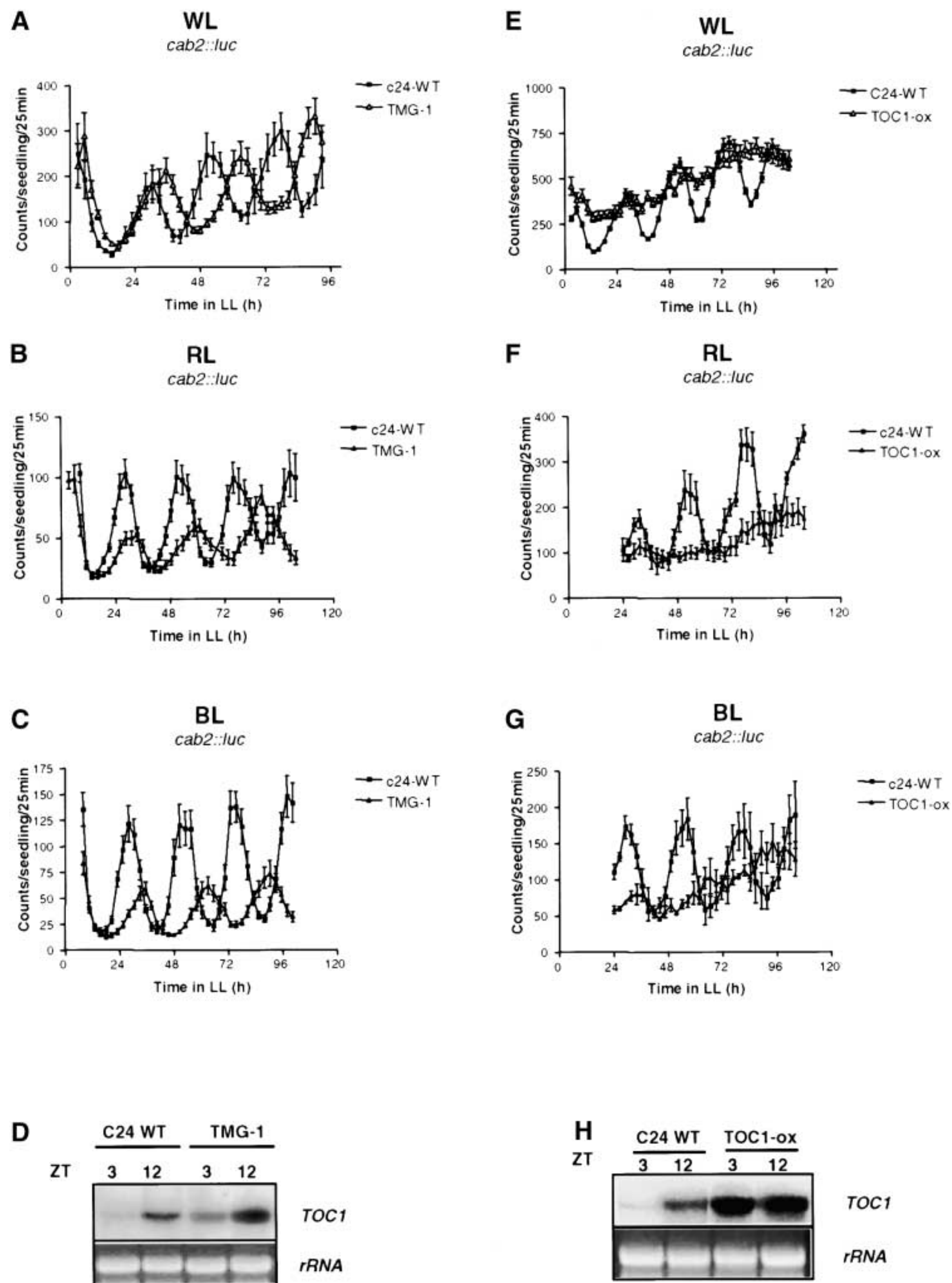

Figure 3. Effects of Increased TMG and TOC1-ox on *CAB2*::*luc* Circadian Expression.

(A) to **(G)** Bioluminescence analysis of *CAB2*::*luc* expression in TMG plants (**[A]** to **[C]**) and TOC1-ox plants (**[E]** to **[G]**) under constant white light (WL; 40 μmol·m^{-2}·s^{-1}) (**[A]** and **[E]**), constant red light (RL; 40 μmol·m^{-2}·s^{-1}) (**[B]** and **[F]**), and constant blue light (BL; 40 μmol·m^{-2}·s^{-1}) (**[C]** and **[G]**). Seedlings were grown in 12-h/12-h light/dark cycles for 6 days before being transferred to constant light (LL). Traces represent averages of 15 to 20 seedlings. The experiment was repeated at least twice with similar results. WT, wild type.

(D) and **(H)** *TOC1* mRNA levels of expression in wild-type, TMG, and TOC1-ox plants. Plants were maintained in 12-h/12-h light/dark cycles for 6 days before samples were harvested at the zeitgeber times (ZT) indicated. Total RNA was extracted, blotted, and hybridized as described (Somers et al., 2000; Alabadí et al., 2001). rRNA was used as a loading control.

TOC1-ox plants maintained under constant red (Figure 3F) or blue (Figure 3G) light. The phenotypes were most likely caused by high and constant *TOC1* mRNA levels compared with those observed in wild-type plants (Figure 3H).

Together, these results demonstrate that increased *TOC1* expression that retains rhythmic oscillation (TMG plants) delays the pace of the clock, whereas *TOC1* constitutive overexpression completely abolishes rhythmicity.

TOC1 Is Involved in the Light-Mediated Control of Hypocotyl Elongation during Seedling Deetiolation

We next examined the possible involvement of TOC1 in the light-dependent control of hypocotyl growth. Hypocotyl length was measured in *TOC1* RNAi, *toc1-1*, *toc1-2*, and TMG plants exposed to various intensities of red, far-red, and blue light. Our results show that the *toc1-2* and *TOC1* RNAi lines displayed a clear reduction in sensitivity to red light and a significant but less pronounced effect in far-red light (Figures 5C to 5F). By contrast, TMG lines were significantly more sensitive to red light and far-red light over the entire fluence-rate range (Figures 5C to 5F). Hypocotyl length in *TOC1* RNAi, *toc1-2*, and TMG plants was nearly identical to that in the wild type at all blue light intensities tested (Figures 5A and 5B) as well as in the dark (Figures 5G and 5H). Interestingly, TOC1-ox seedlings exhibited an increased sensitivity to light, with plants displaying extremely short hypocotyls in all light conditions (white, blue, and red) tested (data not shown).

The decreased sensitivity to red light and far-red light observed in *toc1-2* and in *TOC1* RNAi lines clearly contrasted with the absence of hypocotyl phenotypes in *toc1-1* mutant plants. These results suggest that altered levels of *TOC1* are responsible for the perturbations in photoresponsiveness and assign a novel and unexpected role for TOC1 in the red and far-red light control of hypocotyl elongation during seedling deetiolation.

Previous reports have shown that the daylength-dependent circadian control of flowering time is affected severely

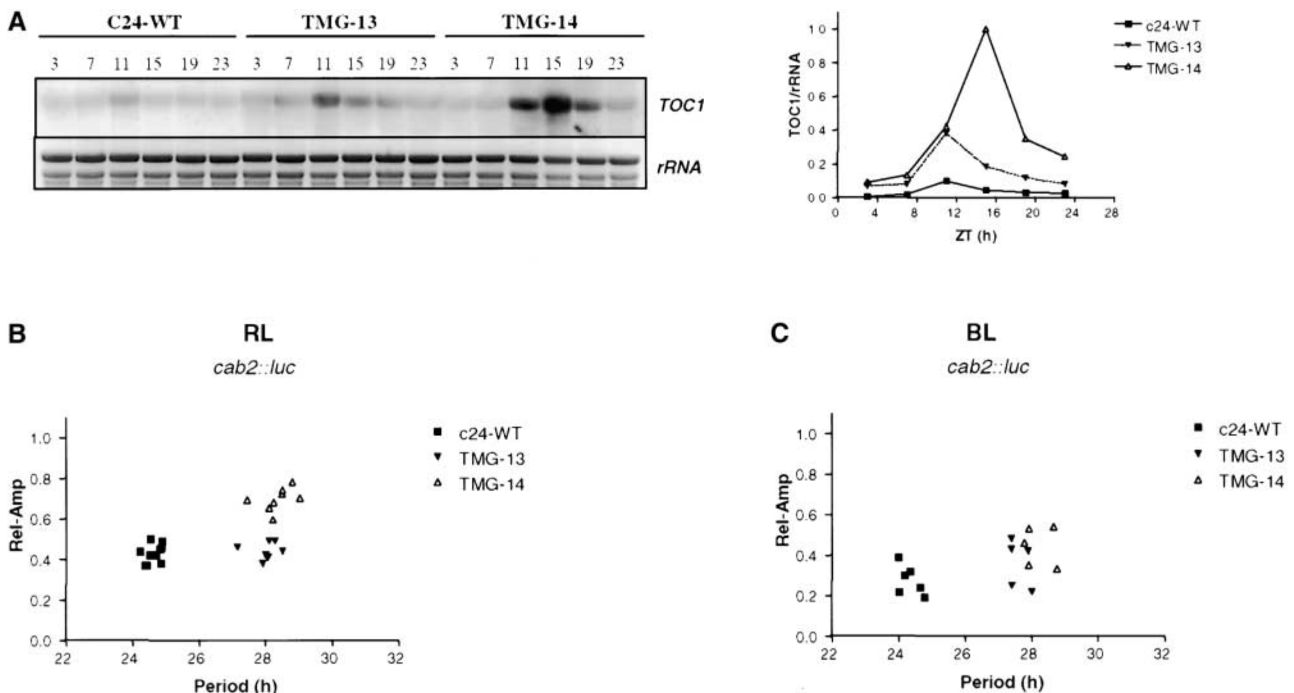

Figure 4. *TOC1* mRNA Levels of Expression in Wild-Type and TMG Transgenic Lines.

(A) Plants were maintained in 12-h/12-h light/dark cycles for 12 days before the samples were collected every 4 h during one light/dark cycle. Total RNA was extracted, blotted, and hybridized as described (Somers et al., 2000; Alabadí et al., 2001). rRNA levels served as a loading control. WT, wild type; ZT, zeitgeber time.

(B) and **(C)** Period estimates of individual wild-type and TMG bioluminescence traces of *CAB2*::*luc* expression at 20 μmol·m^{-2}·s^{-1} red light (RL; **[B]**) and blue light (BL; **[C]**). Period estimates were analyzed using the fast Fourier transform nonlinear least-squares best-fit algorithm as described (Millar et al., 1995b; Plautz et al., 1997). Relative amplitude error values of $>$0.7 represent very altered circadian rhythms (Dowson-Day and Millar, 1999).

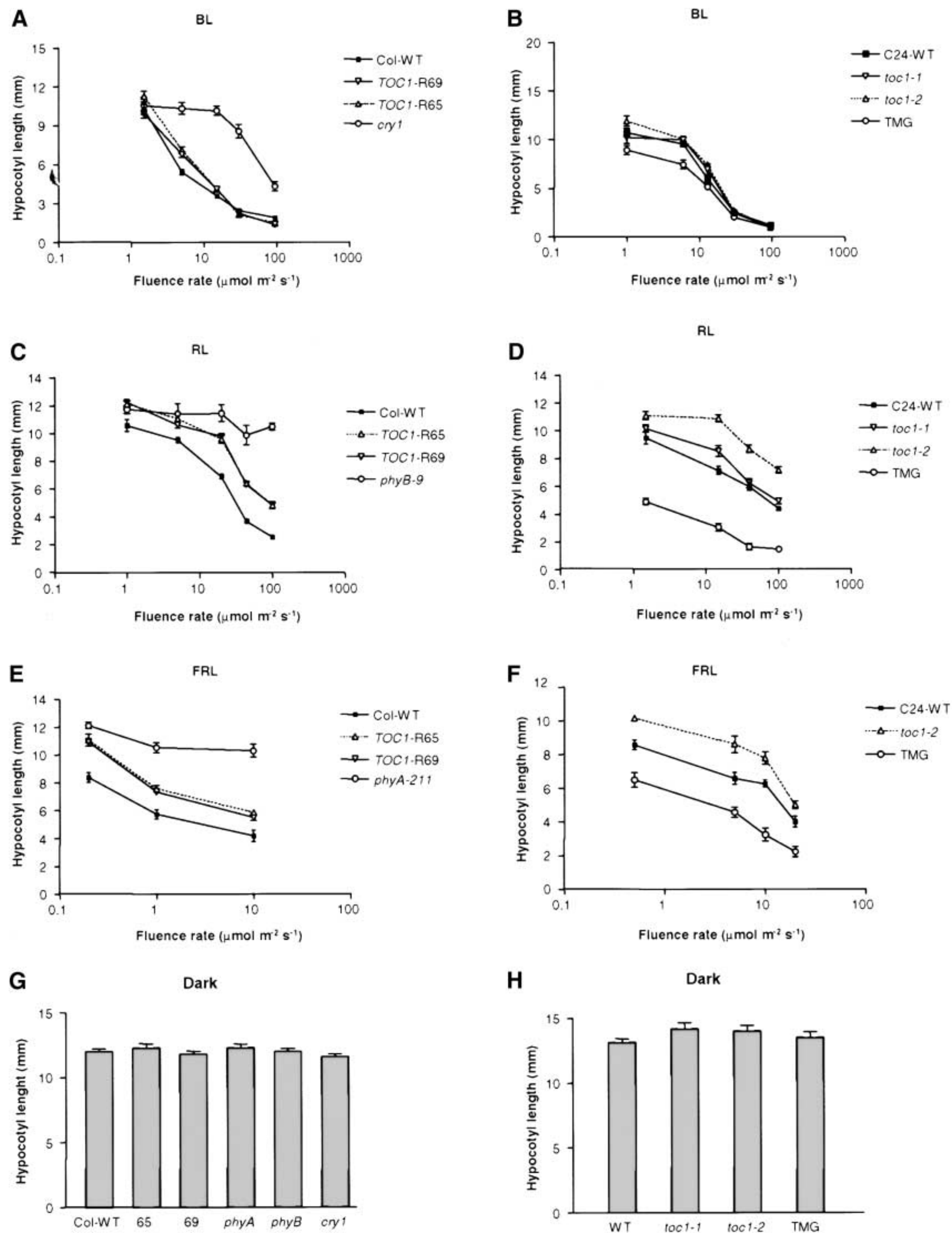

Figure 5. Involvement of TOC1 in the Control of Hypocotyl Elongation during Seedling Deetiolation.

(A) to **(F)** Inhibition of hypocotyl extension at the indicated intensities of blue light (BL; **[A]** and **[B]**), red light (RL; **[C]** and **[D]**), and far-red light (FRL; **[E]** and **[F]**). Seedlings were stratified in the dark at 4°C for 4 days, held in white light (50 μmol·m^{-2}·s^{-1}) for 6 h, and maintained in the dark for 18 h before exposure under the appropriate light quality and fluence rate for 6 days. WT, wild type.

(G) and **(H)** As a control, seedlings were maintained in constant darkness without light treatment. Each experiment was performed at least twice with similar results.

in *toc1-1* mutant plants (Somers et al., 1998b). In our studies, *TOC1* RNAi plants showed almost no difference in flowering time under long days (16 h of light and 8 h of dark) and short days (8 h of light and 16 h of dark), as measured by leaf production or by days to flowering (data not shown). These results indicate that the photoperiodic regulation of floral induction is altered in *TOC1* RNAi plants.

Red Light Induction of *CCA1* and *LHY* Expression Is Reduced in *TOC1* RNAi and *toc1-2* Plants

Previous reports have described that *CCA1* gene expression is induced by light in etiolated seedlings (Wang and Tobin, 1998). Furthermore, TOC1 has been shown to act positively on *CCA1/LHY* expression (Alabadí et al., 2001). Here, we examined the effect of a 1-h red light pulse on *CCA1* transcript levels in wild-type, *TOC1* RNAi, and *toc1-2* etiolated seedlings. As shown by RNA gel blot analysis and after normalization to the 18S rRNA (Figure 6), *CCA1* mRNA levels in wild-type seedlings were enhanced strongly after 1 h in red light. By contrast, a clear reduction in the extent of *CCA1* induction was observed in *TOC1* RNAi plants (Figure 6A) and in *toc1-2* mutant plants (Figure 6B). Similarly, *LHY* induction was decreased significantly in *TOC1* RNAi and *toc1-2* plants compared with wild-type plants (data not shown). These results indicate that TOC1 is involved in the red light–mediated, phytochrome-dependent regulation of *CCA1/LHY* expression.

Arrhythmic *CCR2* Expression in *TOC1* RNAi and *toc1-2* Plants under Constant Darkness

Our results show a role for TOC1 in the light-dependent control of circadian gene expression (Figure 2). To determine whether the circadian clock is affected in *TOC1* RNAi plants in the absence of light input, bioluminescence rhythms were assayed in *TOC1* RNAi and wild-type seedlings that were entrained for 6 days under 12-h/12-h light/dark cycles before being transferred to constant darkness. As shown in Figures 7A and 7B, wild-type plants displayed robust circadian rhythms of *CCR2*::*luc* expression, with a period length of 25.91 $\pm$ 0.80 h. By contrast, after the first circadian peak, the rhythmic expression of *CCR2*::*luc* clearly was disrupted in *TOC1* RNAi plants (Figure 7A). Most of the seedlings examined by fast Fourier transform nonlinear least-squares analysis gave period values scattered over the entire circadian range and with relative amplitude errors of $>$0.7 (Figure 7B), indicating that *CCR2* circadian transcription was altered in constant darkness. Analysis of several RNAi lines (see supplemental data online) demonstrated a good correlation between the altered expression of *CCR2*::*luc* and decreased levels of *TOC1* mRNA. The role of TOC1 in controlling *CCR2* expression in constant darkness was confirmed by RNA gel blot analyses of wild-type and *toc1-2* plants. The seedlings were entrained for 6 days in light/dark cycles and maintained for another 2 days in constant darkness before samples were harvested every 4 h during the next 24 h. In accordance with the results obtained with *TOC1* RNAi plants, *CCR2* circadian expression clearly was altered in *toc1-2* plants compared with wild-type plants (Figures 7C and 7D).

In contrast to the shortened circadian phenotype displayed by *toc1-1* mutant plants in constant darkness (Strayer et al., 2000), in *TOC1* RNAi lines and *toc1-2* plants, circadian rhythmicity clearly was affected, demonstrating the crucial role of *TOC1* in sustaining *CCR2* circadian expression in constant darkness.

DISCUSSION

The data presented here provide evidence that silencing of the *TOC1* gene, increments in *TOC1* gene dosage, and *TOC1* overexpression affect oscillator function. In constant darkness and in continuous red light, we found no functional redundancy of *TOC1* with other clock components. *TOC1* is involved in the phytochrome-dependent control of hypocotyl elongation and in the red light induction of *CCA1/LHY*. We propose that TOC1 integrates light signaling from phytochromes to clock outputs, controlling circadian gene expression and other light-dependent developmental processes in the plant.

Dual Role of TOC1 as an Essential Component of the Plant Clock

TOC1 was identified initially in a screen for mutants with aberrant cycling patterns of *CAB2*::*luc* expression in constant white light (Millar et al., 1995a). The circadian defect of the identified mutant, *toc1-1*, was shown to be independent of light quantity and quality, suggesting a role for TOC1 outside of the light input to the clock (Somers et al., 1998b; Strayer et al., 2000). Our results showed that the circadian rhythmic expression of two differently phased genes, *CAB2* and *CCR2*, was abolished in *TOC1* RNAi and *toc1-2* plants maintained under different intensities of red light. These results are consistent with an essential role for TOC1 in the red light control of clock function. Plants lacking CCA1 activity show a short-period phenotype under red light, indicating the presence of a functional clock (Alabadí et al., 2002). However, to maintain proper circadian expression under continuous red light, plants specifically require the *TOC1* gene (Figure 2).

The Arabidopsis *early flowering3* (*elf3*) mutation causes arrhythmic outputs in continuous light, but there is evidence for clock function in darkness (Hicks et al., 1996; Covington et al., 2001). In the case of TOC1, our results show that *TOC1* RNAi and *toc1-2* plants exhibited altered circadian expression in constant darkness, indicating a severe impairment in oscillator function and/or regulation. Together, these

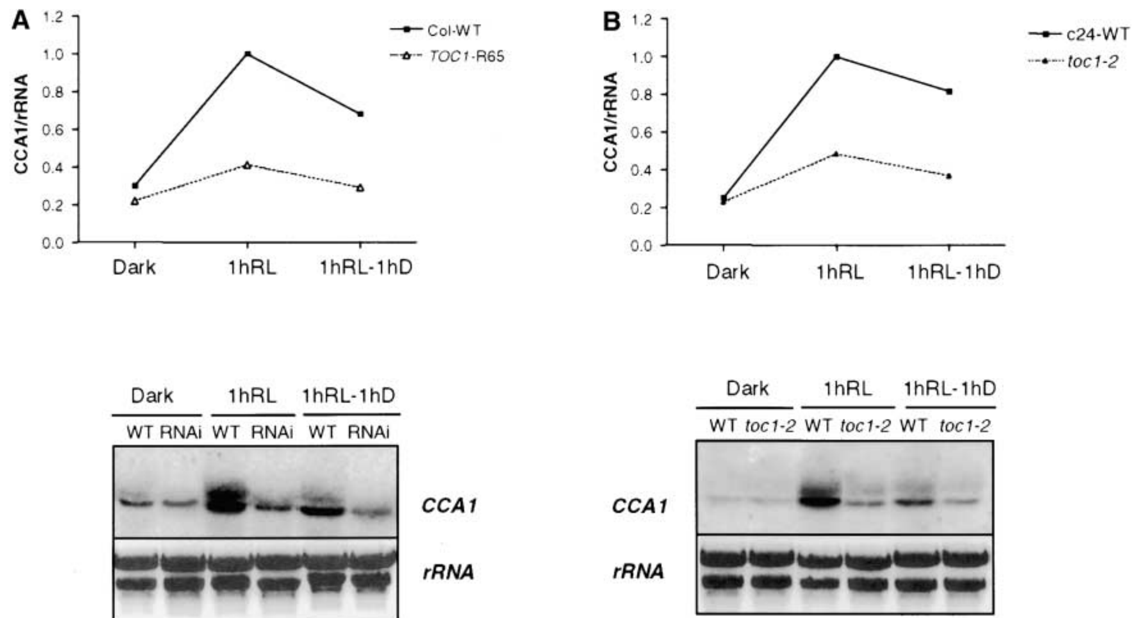

Figure 6. The Light-Mediated Induction of *CCA1* Is Altered in *TOC1* RNAi and *toc1-2* Plants.

CCA1 levels of expression in wild-type and *TOC1* RNAi **(A)** and *toc1-2* **(B)** plants. Seedlings maintained for 5 days in the dark were treated for 1 h with red light (RL; 40 μmol·m^{-2}·s^{-1}) followed by 1 h in the dark (D). RNA was extracted, blotted, and hybridized as described (Somers et al., 2000; Alabadí et al., 2001). As a control, RNA was extracted from samples kept in the dark with no light treatment (Dark). rRNA was used as a loading control. Each experiment was performed at least twice with similar results. WT, wild type.

results suggest that there is no functional redundancy in the circadian control of *CCR2* expression and that the presence of *TOC1* is required to sustain rhythmicity in constant darkness. These results indicate that TOC1 does not simply mediate the interaction between light and the circadian clock but that its function also is essential in the absence of a light input to the clock. The dual role of *TOC1* in constant darkness and in the red light–mediated control of circadian gene expression reflects the intricate connection between phototransduction pathways and the oscillator and functionally parallels some of the clock components characterized in other systems (Crosthwaite et al., 1997; Shigeyoshi et al., 1997; Allada et al., 1998; Merrow et al., 1999; Bunger et al., 2000; Iwasaki et al., 2000).

Under continuous blue light, *CAB2*::*luc* and *CCR2*::*luc* expression cycled rhythmically, with a significantly shorter period in *TOC1* RNAi and *toc1-2* plants than in wild-type plants (Figures 1 and 2). The fact that the oscillations still were rhythmic suggests that different components are involved in the functioning of the clock under red light and blue light conditions, *TOC1* being essential in the former. Other clock component candidates, including *CCA1*/*LHY* and the *TOC1*-like gene family (Matsushika et al., 2000; Strayer et al., 2000), could partially compensate for the loss of *TOC1*, explaining the clock activity in blue light.

Our results also show that increased and rhythmic *TOC1* expression delays the pace of the clock, whereas constitutive *TOC1* overexpression completely abolishes rhythmicity. These results indicate that both the expression level and the cyclic control of *TOC1* expression are crucial for clock function.

Involvement of TOC1 in the Red Light Induction of *CCA1* and *LHY* Expression

In Arabidopsis, light stimulates the formation of active forms of phytochromes, which regulate many physiological and developmental responses (Neff et al., 2000). Previous reports have provided evidence that PIF3 functions in the phytochrome signal transduction pathway by interacting directly with both phyA and phyB molecules (Ni et al., 1998). The fact that PIF3 binds to promoters of specific light-regulated genes (Martínez-García et al., 2000) suggests a short signaling pathway from phytochromes to the photoresponsive target genes that they regulate, including *CCA1* and *LHY*. The direct interaction of TOC1 with PIF3 (Makino et al., 2002; P. Más and S.A. Kay, unpublished results) suggests a possible molecular mechanism by which the red light information perceived by phytochromes is transmitted to the clock. The observation that the red light induction of *CCA1* and *LHY* is reduced in the absence of a functional TOC1 (Figure 6) or PIF3 (Martínez-García et al., 2000) protein sug-

gests that PIF3 binding to *CCA1/LHY* promoters might be modulated by its interaction with TOC1.

Role of TOC1 in the Control of Hypocotyl Elongation during Seedling Deetiolation

Our results show a strong degree of enhanced photoresponsiveness in TOC1-ox and TMG plants, whereas *toc1-2* and *TOC1* RNAi plants displayed a significant reduction in sensitivity to red light and far-red light. These data indicate that TOC1 is required for the normal phytochrome-mediated control of hypocotyl elongation during seedling deetiolation. The decreased sensitivity to red light and far-red light observed in *toc1-2* and *TOC1* RNAi plants clearly contrasts with the absence of hypocotyl phenotypes in *toc1-1* mutant plants (Figure 5) (Somers et al., 1998b). The altered hypocotyl length in some clock mutants was shown to be the result of severe circadian defects in the regulation of cell expansion rather than the result of alterations in light signaling pathways (Dowson-Day and Millar, 1999). The fact that *toc1-1* mutant plants shorten multiple circadian outputs but still have wild-type hypocotyl length suggests that hypocotyl elongation during seedling deetiolation could be controlled by different routes, dependent and independent of the clock. *toc1-1* defines a mutation with affected clock function but with a normal

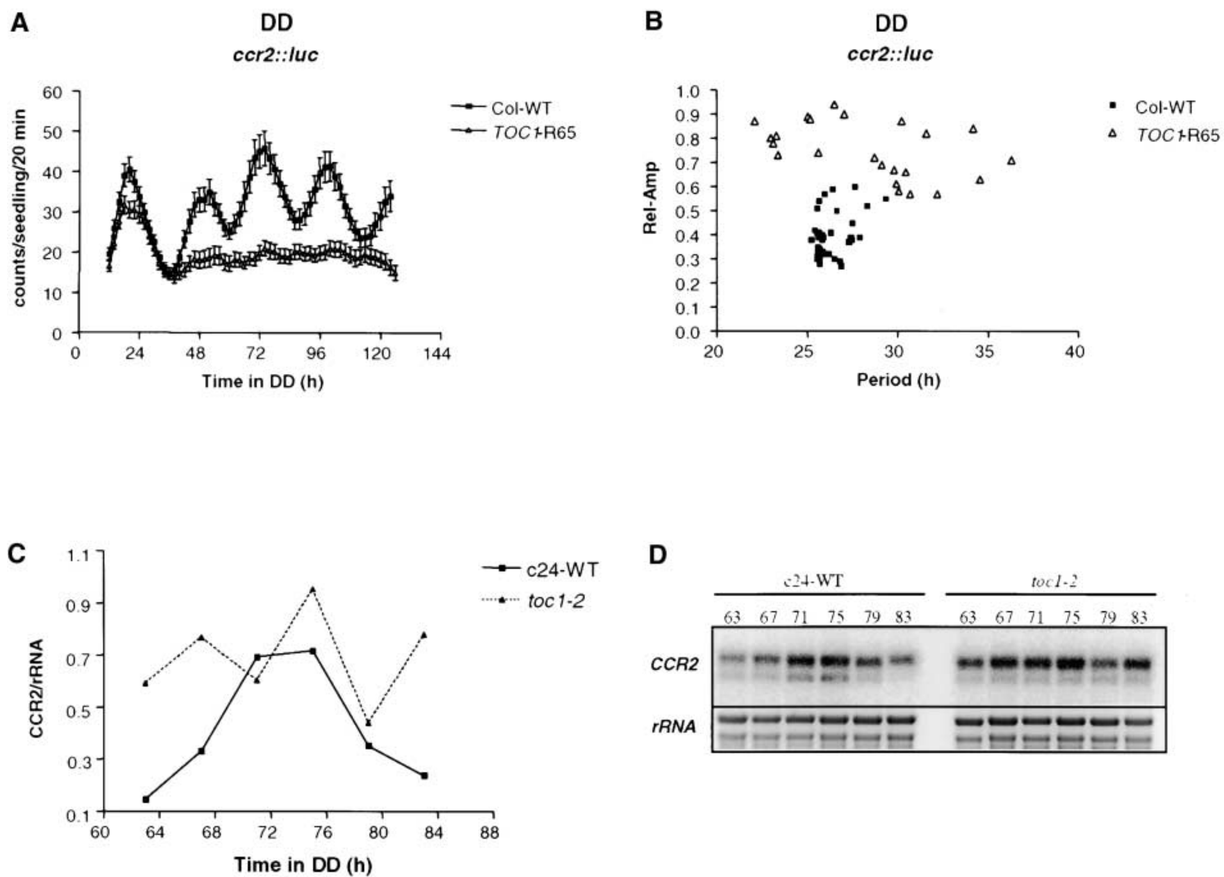

Figure 7. Arrhythmic Gene Expression in *TOC1* RNAi and *toc1-2* Plants in Constant Darkness.

(A) Bioluminescence analysis of *CCR2*::*luc* expression in wild-type and *TOC1* RNAi plants that were grown in 12-h/12-h light/dark cycles for 6 days before being transferred to constant darkness (DD). Traces represent averages of 30 to 40 seedlings. The experiment was repeated three times with similar results in all cases. WT, wild type

(B) Scatterplot of period estimates for the individual wild-type and *TOC1* RNAi traces shown in **(A)**. Period estimates were analyzed using the fast Fourier transform nonlinear least-squares best-fit algorithm as described (Millar et al., 1995b; Plautz et al., 1997). Relative amplitude error values of $>$0.7 represent very altered circadian rhythms (Dowson-Day and Millar, 1999).

(C) *CCR2* levels of expression in wild-type and *toc1-2* plants. The seedlings were entrained for 7 days in light/dark cycles and maintained for another 2 days in constant darkness before RNA was extracted every 4 h during the next 24 h. RNA was extracted and analyzed by RNA gel blotting as described (Somers et al., 2000; Alabadí et al., 2001).

light-mediated deetiolation response. These conclusions are in agreement with the fact that under constant white light, *TOC1* RNAi and *toc1-2* plants displayed both shortened circadian expression (Figure 1) and long hypocotyls (data not shown). These results exclude the possibility that the long-hypocotyl phenotypes are attributable to arrhythmicity.

CCA1-overexpressing plants exhibited a reduced responsiveness to red light signals in the control of hypocotyl growth during deetiolation (see supplemental data online). Conversely, *cca1-1* mutant plants and *cca1-1*/lhy-RNAi plants (Alabadí et al., 2002) showed a hypersensitive response to red light (see supplemental data online). These results are consistent with the idea that appropriate TOC1 levels are important in the phytochrome-mediated control of hypocotyl elongation during deetiolation. Low TOC1 mRNA levels in CCA1-overexpressing (Alabadí et al., 2002), TOC1 RNAi, and *toc1-2* plants correlates with long hypocotyl phenotypes, whereas high TOC1 levels in TMG, TOC1-ox, and *cca1-1*/lhy-12 plants (Mizoguchi et al., 2002) are associated with short hypocotyl phenotypes (see supplemental data online).

Perspectives

The conceptual idea of differentiated input and output pathways and a central oscillator clearly is an oversimplified theoretical description of the components of the circadian clock. We now know that the limits between each of these components are diffuse, with elements of the output feeding back into the clock and input components oscillating themselves (Harmer et al., 2001). Previous results provided evidence that TOC1 satisfied some of the criteria expected for a component of the core of the oscillator. Its rhythmic expression was shown to be regulated by a feedback loop mechanism. Mutations in the gene altered multiple circadian outputs independently of light and temperature conditions, and its reciprocal regulation with *CCA1/LHY* provided a mechanistic framework to explain circadian rhythmicity in plants (Somers et al., 1998b; Strayer et al., 2000; Alabadí et al., 2001). Our results showing that TOC1 is crucial for a functional clock under free-running conditions in constant darkness excludes the possibility that TOC1 is only a part of the light input to the clock. The results presented here and in previous publications are consistent with the hypothesis that TOC1 is essential for clock function, with a dual role in the dark and in the control of clock outputs, by integrating the reception of red light signals.

METHODS

Plasmid Construction

Three different regions of *TOC1* cDNA were ligated in the sense and antisense orientations into pHANNIBAL vector (Smith et al., 2000). The construct a1s1 (Table 1) comprised 659 bp (from 119 bp upstream of the ATG to 540 bp downstream of the ATG); construct a2s2 comprised 619 bp of TOC1 open reading frame (nucleotides 960 to 1580); and construct a3s3 corresponded to 415 bp (from nucleotide 1720 of the TOC1 open reading frame to 278 bp downstream of the stop codon). The constructs were subcloned as NotI fragments into the binary vectors pMLBART27 (Basta resistance; Gleave, 1992) and pART27 (kanamycin resistance; Wesley et al., 2001). *TOC1* minigene plasmid was constructed using the *TOC1* genomic sequence (from 2354 bp upstream of the ATG to 410 bp downstream of the stop codon) fused to the FLAG epitope, which was used as a tag. The TOC1-overexpressing plasmid was constructed by subcloning the coding region of the *TOC1* cDNA fused to yellow fluorescent protein (Clontech, Palo Alto, CA) into the pRTL2 vector (Carrington et al., 1991) downstream of the 35S promoter of *Cauliflower mosaic virus*. The *TOC1* minigene and TOC1-overexpressing constructs were subcloned into the binary vector pPZP221 (Hajdukiewicz et al., 1994).

Bioluminescence Assays and Hypocotyl Length Analyses

Arabidopsis plants transformed by *Agrobacterium tumefaciens*–mediated DNA transfer (Clough and Bent, 1998) were selected on Murashige and Skoog (1962) agar plates under 12-h/12-h light/dark cycles for 6 days and transferred to media without selection. Analyses of bioluminescence rhythms under continuous white light (60 μmol·m^{-2}·s^{-1}) or in different intensities of red and blue light were performed as described (Millar et al., 1995a; Somers et al., 1998b). Expression of *CCR2*::*luc* in constant darkness was imaged and analyzed using the Night Owl imaging system and WinLight software (Perkin-Elmer). Bioluminescence from individual traces was examined by fast Fourier transform nonlinear least-squares analysis to estimate rhythmic cycles and period lengths (Millar et al., 1995b; Plautz et al., 1997).

For hypocotyl length analysis, seeds were stratified on 3% Suc–Murashige and Skoog (1962) plates in the dark at 4°C for 4 days, exposed to white light (50 μmol·m^{-2}·s^{-1}) for 6 h, and kept in the dark for 18 h before exposure at the appropriate light quality and fluence rate for 6 days. Hypocotyl length was measured with a ruler.

Reverse Transcriptase–Mediated PCR and RNA Gel Blot Analysis

RNA gel blot analysis of RNA levels was performed as described (Somers et al., 2000). RNA was extracted using the RNeasy Plant Mini Kit according to the manufacturer's recommendations (Qiagen, Valencia, CA). Total RNA (6 μg/lane) was separated on 1.2% formaldehyde gels and blotted onto nitrocellulose membranes (Micron Separations, Westborough, MA). The *TOC1*, *CCA1*, *LHY*, *CCR2*, and rDNA probes were labeled and hybridized as described previously (Kreps and Simon, 1997; Alabadí et al., 2001). Quantitation of the RNA gel blot data and analysis of the images was performed on a PhosphorImager and using ImageQuant Software (Molecular Dynamics, Sunnyvale, CA).

For detection of the different members of the *TOC1* family, plants were maintained in 12-h/12-h light/dark cycles for 6 days before samples were harvested every 4 h during one light/dark cycle. RNA was extracted using the RNeasy Plant Mini Kit according to the manufacturer's recommendations (Qiagen). SuperScript II RNase H^{-} re-

verse transcriptase (Gibco BRL) was used to synthesize the first-strand cDNA with oligo(dT_{12-18}) primer (Gibco BRL) from 1 μg of total RNA at 42°C for 50 min. Two microliters of the cDNAs from each time point was combined in one tube, and 1 μL of the mixture was used for PCR amplification using specific primers for each member of the *TOC1* family (Makino et al., 2002). Samples were run on 1.2% agarose gels, transferred to nitrocellulose membranes, and subjected to hybridization according to standard protocols.

Upon request, all novel materials described in this article will be made available in a timely manner for noncommercial research purposes.

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