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

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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...
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 CON- STANS 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). Alabadi 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 (Alabadi 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; Mås 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 mini-gene (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 unpredictable and essential roles for TOC1 in sustaining circadian rhythmicity in the dark and in specific monocromatic 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...
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\(\mu\)mol·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, CCR2::luc was expressed arrhythmically in 85% of the toc1-2 seedlings (Figure 2E), displaying relative amplitude errors of >0.7. In constant blue light, CCR2::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 \(\mu\)mol·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 CCR2::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

<table>
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<th>Line</th>
<th>Construct</th>
<th>Arm (nucleotides)</th>
<th>Reporter</th>
<th>Period (h ± sd)</th>
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<tr>
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<td>a3s3</td>
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<td>20.98 ± 0.48</td>
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<tr>
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<td>659</td>
<td>CCR2::luc</td>
<td>20.00 ± 0.30</td>
<td>36</td>
</tr>
<tr>
<td>T3 line 69</td>
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<td>659</td>
<td>CCR2::luc</td>
<td>20.53 ± 0.28</td>
<td>36</td>
</tr>
<tr>
<td>T3 line 81</td>
<td>a2s2</td>
<td>619</td>
<td>CCR2::luc</td>
<td>20.89 ± 0.90</td>
<td>29</td>
</tr>
<tr>
<td>Columbia wild type</td>
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<td>–</td>
<td>CCR2::luc</td>
<td>24.07 ± 0.28</td>
<td>18</td>
</tr>
<tr>
<td>T3 line 10</td>
<td>a1s1</td>
<td>659</td>
<td>CAB2::luc</td>
<td>20.34 ± 0.32</td>
<td>25</td>
</tr>
<tr>
<td>T3 line 24</td>
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<td>CAB2::luc</td>
<td>19.83 ± 0.22</td>
<td>19</td>
</tr>
<tr>
<td>T3 line 57</td>
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<td>415</td>
<td>CAB2::luc</td>
<td>20.98 ± 0.85</td>
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<tr>
<td>Columbia wild type</td>
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<td>–</td>
<td>CAB2::luc</td>
<td>24.27 ± 0.17</td>
<td>15</td>
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Plants were entrained for 6 days in 12-h/12-h light/dark cycles before being released and imaged in white light (60 \(\mu\)mol·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).
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 μmol·m⁻²·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 μmol·m⁻²·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.
Role of TOC1 in the Circadian Clock

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; Alabadi 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 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 (Alabadi 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 25.9 ± 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 CRR2, 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 (Alabadi 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
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 (Martinez-Garcia et al., 2000) suggests a short signaling pathway from phytochromes to the photosensitive 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 (Martinez-Garcia et al., 2000) protein sug-
suggests 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 photosensitivity 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; Alabadi 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 (Alabadi 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 (Alabadi 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 evidence of the output feedback 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 expressions was shown to be regulated by a feedback loop mechanism. Mutations in the gene altered multiple circadian expression (Millar et al., 1995a; Somers et al., 2000). RNA was extracted using the RNeasy Plant mini-gene 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 pZP221 (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, and rDNA probes were labeled and hybridized as described previously (Kreps and Simon, 1997; Alabadi 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)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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