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A Complex Genetic Interaction Between *Arabidopsis thaliana* TOC1 and CCA1/LHY in Driving the Circadian Clock and in Output Regulation

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ABSTRACT

It has been proposed that CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY) together with TIMING OF CAB EXPRESSION 1 (TOC1) make up the central oscillator of the *Arabidopsis thaliana* circadian clock. These genes thus drive rhythmic outputs, including seasonal control of flowering and photomorphogenesis. To test various clock models and to disclose the genetic relationship between TOC1 and CCA1/LHY in floral induction and photomorphogenesis, we constructed the *cca1 lhy toc1* triple mutant and *cca1 toc1* and *lhy toc1* double mutants and tested various rhythmic responses and circadian output regulation. Here we report that rhythmic activity was dramatically attenuated in *cca1 lhy toc1*. Interestingly, we also found that TOC1 regulates the floral transition in a CCA1/LHY-dependent manner while CCA1/LHY functions upstream of TOC1 in regulating a photomorphogenic process. This suggests to us that TOC1 and CCA1/LHY participate in these two processes through different strategies. Collectively, we have used genetics to provide direct experimental support of previous modeling efforts where CCA1/LHY, along with TOC1, drives the circadian oscillator and have shown that this clock is essential for correct output regulation.

CIRCADIAN rhythms are self-sustaining biological oscillations that free run under constant conditions with a periodicity close to 24 hr. The rhythmic clock is prevalent and is found in organisms ranging from prokaryotes to eukaryotes and from animals to plants (Dunlap 1999; Barak *et al.* 2000; Harmer *et al.* 2001). This clock can be reset according to environmental cues, such as light and temperature (Liu *et al.* 1998; Collett *et al.* 2001; Young and Kay 2001; Samach and Wigge 2005; Carr *et al.* 2006). Recently, rapid strides have been made in deciphering the molecular bases of the circadian system. A recognizable pattern that is emerging is the recurring trend of autoregulatory positive/negative feedback loops (Alabadi *et al.* 2001). Further, clock models have been mathematically derived and the resulting equation principals can be applied (Locke *et al.* 2005a,b; Lakin-Thomas 2006). These models explicitly generate hypothesis-driven questions.

In *Arabidopsis thaliana*, the proposed negative repressors of the oscillator are the morning-acting myb-related factors CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY) (Schaffer *et al.* 1998; Wang and Tobin 1998; Green and Tobin 1999; Alabadi *et al.* 2002; Mizoguchi *et al.* 2002), which are partially redundant genes encoding similar DNA-binding proteins. They act on the proposed positive-activator termed TIMING OF CAB EXPRESSION 1 (TOC1); it works in the evening and encodes a protein of unknown biochemical activity (Somers *et al.* 1998a; Strayer *et al.* 2000; Alabadi *et al.* 2001). TOC1 belongs to the PRR (PSEUDO-RESPONSE REGULATOR) family of proteins, consisting of five members (PRR9, PRR7, PRR5, PRR3, and PRR1/TOC1) (Matsushika *et al.* 2000; Makino *et al.* 2002; Eriksson *et al.* 2003). TOC1 and CCA1/LHY together make up the proposed central circadian loop in Arabidopsis. It is this positive–negative feedback loop between these evening and morning factors that leads to the first genetic model of the plant clock (Alabadi *et al.* 2001). This regulatory network has consistently and continuously been placed at the core of the molecular oscillator in all published models, but it does not fully describe several experimentally defined features (Schaffer *et al.* 1998; Wang and Tobin 1998; Harmer *et al.* 2000; Alabadi *et al.* 2001; Kim *et al.* 2003). Mathematical approaches drove experimental approaches to refining a simplistic loop where only CCA1/LHY and TOC1 were the sole elements of the clock. An interlocked two-loop clock model was then proposed to describe oscillatory properties, such as entrainment and response to photoperiods (Locke *et al.* 2005b). In this model, TOC1 and CCA1/LHY form a central loop, while the flowering-time gene GIGANTEA (GI) works alongside TOC1 to

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compose a secondary loop. CCA1 and LHY mediate light signal into the clock and GI potentially provides a secondary pathway for light input into the clock (Locke *et al.* 2005b). More recently, two groups have extended this to a three/four-loop model that includes PRR9 and PRR7 as morning-acting elements in a tertiary CCA1/LHY loop. We note that none of these studies has tested whether a loop with CCA1/LHY and TOC1 is indeed core to the oscillator (Locke *et al.* 2006; Zeilinger *et al.* 2006).

The circadian clock has been reported to regulate many processes, such as daily biochemical reactions and other general metabolic aspects of the cell. This in turn coordinates most, if not all, physiological processes. These are collectively called the circadian-output pathways (Harmer *et al.* 2000). For example, both *toc1* and *cca1 lhy* have defects in flowering time and photomorphogenesis, which correlates with respective mutant circadian phenotypes (Somers *et al.* 1998b; Strayer *et al.* 2000; Mizoguchi *et al.* 2002). In *toc1*, mutant plants have an early flowering phenotype when grown under a short-day photoperiod. It was found that this phenotype is the result of clock-based misinterpretation of photoperiodic information rather than of the direct effects of *toc1* on floral-induction pathways (Somers *et al.* 1998b; Strayer *et al.* 2000). Both *cca1* and *lhy* also exhibit an early flowering phenotype under short-day conditions, and this was especially marked in the *cca1 lhy* double mutant; this double mutant is nearly insensitive to photoperiodic sensing (Mizoguchi *et al.* 2002). Although both *toc1* and *cca1 lhy* have an early flowering phenotype, they have an inverted phenotype regarding early seeding photomorphogenesis, with *toc1* displaying a long hypocotyl whereas *cca1 lhy* displays a short hypocotyl (Mas *et al.* 2003; Mizoguchi *et al.* 2005).

We sought to provide direct experimental evidence for TOC1 and CCA1/LHY as core-loop elements in the clock and to disclose the genetic relationship between TOC1 and CCA1/LHY in output regulation. For this purpose, we established all the possible double mutants and the triple mutant, tested clock responsiveness under a battery of molecular assays, and performed physiological and molecular analysis of clock outputs. We found that the triple mutant *cca1 lhy toc1* often exhibited an arrhythmic phenotype under constant light (LL) conditions, which was consistent with the predictions from current mathematical clock models. Interestingly, the triple mutant displayed some limited rhythmic behavior under certain assays. The implication from this experimental data set is that the latest three/four-loop mathematical model (Locke *et al.* 2006; Zeilinger *et al.* 2006) will need to be further refined. Also, we found that TOC1 and CCA1/LHY participate in photomorphogenesis and flowering-time promotion through distinct epistatic relationships.

MATERIALS AND METHODS

Plant material and growth condition: The *cca1-11* and *lhy-21* mutant alleles have been described (Doyle *et al.* 2002; Hall *et al.* 2003; Gould *et al.* 2005). *toc1-21* in Ws-2 was derived from the same mutagenesis as above, and a graphical depiction of the mutation site is shown in supplemental Figure 1 at http://www.genetics.org/supplemental/. We introduced *CAB2::LUC* (6A), described in Hall *et al.* (2001), via manual fertilization of this line to the mutants. From the resultant segregants, we selected all the double and triple mutants. Crossing in the marker ensured a single homozygous *CAB2::LUC* locus. Seedling growth for rhythmicity experiments was, unless otherwise stated, with a fluence rate of white light at ~65 μmol m^{-2} s^{-1} at a constant temperature of 22°. Imaging was performed as described via established protocols, where the light was provided from red- and blue-light-emitting diodes at ~2 μmol m^{-2} s^{-1} (Dowson-Day and Millar 1999; Thain *et al.* 2000). Period length and relative amplitude of error were estimated using the fast Fourier transform–nonlinear least squares (FFT–NLLS) program (Plautz *et al.* 1997). Release assays (Figure 4) were conducted as described (McWatters *et al.* 2000).

Expression analysis by real-time PCR: Total RNA was extracted with the QIAGEN (Valencia, CA) RNeasy plant mini kit. From 2 μg of RNA, cDNA was synthesized using the Superscript first-strand synthesis system (Invitrogen, San Diego) with oligo(dT) primers. Real-time PCRs were performed in a 20-μl volume comprising primers, cDNA template, and SYBER Green PCR master mix in a Bio-Rad (Hercules, CA) real-time detection system. The efficiency of amplification was assessed relative to a tublin standard. Each RNA sample was assayed in triplicate. RNAs were assayed from two to three independent biological replicates. Expression levels were calculated relative to tublin using a comparative threshold (CT) cycle method method or standard curve method. Levels of samples calculated according to the CT method were normalized to the maximum level of each RNA sample, which was set to 1. The primer sequences were as described (Ding *et al.* 2007).

Measurement of flowering time: Flowering-time analysis was carried out on plants grown in a controlled-environment cabinet under SD growth (8 hr light/16 hr dark) at ~20°. Flowering time was measured by counting the number of rosette and cauline leaves. Data are presented as mean ± SE (n = 20–24). These flowering-time experiments were replicated with similar results.

Analysis of hypocotyl length: For hypocotyl-length analysis, seeds were stratified in the dark at 4° for 3 days on 3% sucrose–Murashige and Skoog plates (as used in luminescence assays) and then transferred to short-day growth conditions (8 hr light/16 hr dark). Hypocotyl length was measured after 1 week of growth, as described (Davis *et al.* 2001), and the mean value ± SE was calculated (n = 20–30 for each genotype).

RESULTS

Defective clock responses of multiple mutants between *toc1* and *cca1/lhy*: In Arabidopsis, an interlocked feedback-loop clock model has been developed to describe much of the genetic data collected from clock mutants (*e.g.*, Zeilinger *et al.* 2006). In all such models, the three genes *TOC1*, *CCA1*, and *LHY* are placed centrally within a core loop and, if this multiple-loop model is correct, then whenever all are mutated, rhythmic responses should be dramatically attenuated.

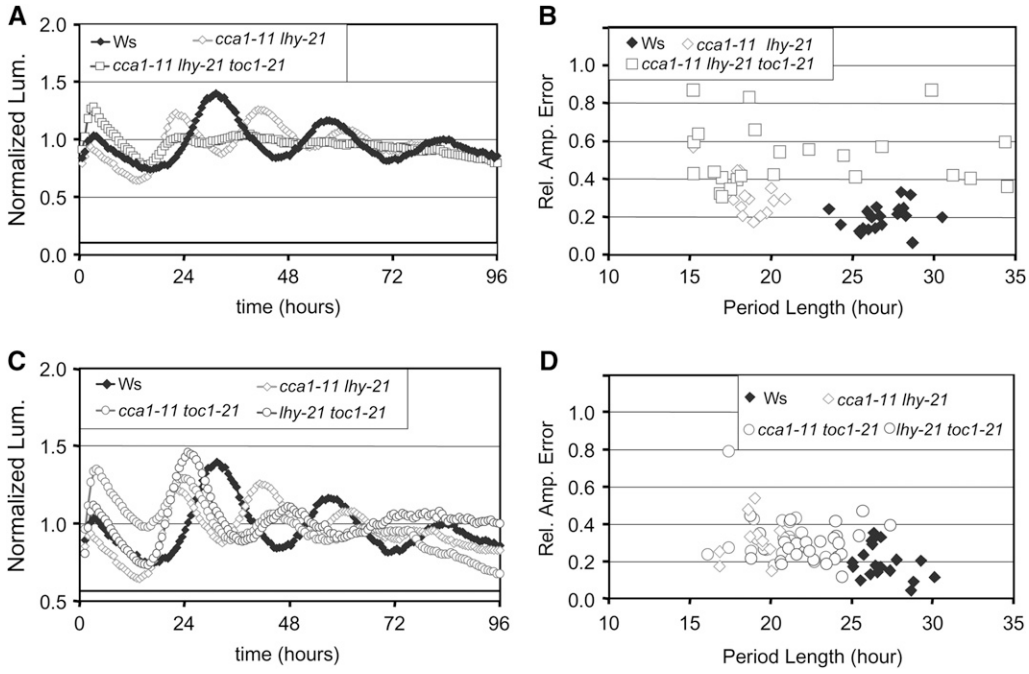

FIGURE 1.—*toc1* enhanced the circadian phenotype of *cca1* and *lhy*. Seedlings of the Ws wild type, *cca1-11 lhy-21*, *cca1-11 toc1-21*, *lhy-21 toc1-21*, and *cca1-11 lhy-21 toc1-21* were grown under standard 12L/12D conditions and then transferred to LL, and *CAB2::LUC* luminescence was monitored. (A) *CAB::LUC* bioluminescence rhythms in Ws, *cca1-11 lhy-21*, and *cca1-11 lhy-21 toc1-21* under LL. (B) Period analysis of *CAB::LUC* bioluminescence rhythms in A. (C) *CAB::LUC* bioluminescence rhythms in Ws, *cca1-11 lhy-21*, *cca1-11 toc1-21*, and *lhy-21 toc1-21* under LL. (D) Period analysis of *CAB::LUC* bioluminescence rhythms in B; the period analysis was conducted between the 20- to 96-hr interval. The data shown represent normalized luminescence from 12 seedlings. This experiment was repeated two times with similar results.

[Again, we note that *cca1*, *lhy*, and *toc1* mutations alone do not block clock function (SOMERS *et al.* 1998b; ALABADI *et al.* 2002; MIZOGUCHI *et al.* 2002).] To test this hypothesis, we constructed the *cca1 lhy toc1* triple mutant and assayed the free-running response using *CAB2::LUC* as a reporter. The *cca1 toc1* and the *lhy toc1* double mutants were also selected to further expand our understanding of their genetic interactions within the proposed core. A driven rhythm after release from entraining conditions was detected in *cca1 lhy toc1* in the first day under constant light conditions. This is compared to the *cca1 lhy* double that, as expected, maintained rhythmic expression of *CAB2*, albeit with an earlier phase of the first peak (Figure 1A). Through FFT–NLLS analysis, most *cca1 lhy toc1* seedlings lacked detectable rhythmicity, as these lines had a very high error compared to wild-type plants (Figure 1B). Therefore, the circadian clock in *cca1 lhy toc1* was severely disrupted. Both *cca1 toc1* and *lhy toc1* maintained a relatively strong rhythm, similar to that seen with *cca1 lhy* (Figure 1, B–D). All double-mutant lines displayed short periodicity. However, the period length in *cca1 toc1* and *lhy toc1* was ~2–3 hr longer than that in *cca1 lhy* under the same conditions (Figure 1, C and D), which suggested to us that *TOC1* is not redundant to CCA1 and LHY with regard to period-length control in the same manner as CCA1 and LHY are to each other.

To further examine the clock defects present within *cca1 lhy toc1*, we performed real-time PCR on RNA extracted from replicate time points from mutant seedlings that were released into constant light after 1-week entraining under 12 hr light/12 hr darkness (12L/12D). With this assay, we found that the expression patterns of other clock-regulated genes were disrupted. For example, *GI* exhibited an earlier peak of expression that shifted ~4 hr earlier compared to the wild type. Further, the *cca1 lhy toc1* triple mutant dramatically dampened rhythmic expression of *GI* after one very short period cycle (Figure 2A). *LUX ARRHYTHMO* (*LUX*), which is another presumably critical activator of *CCA1* and *LHY* (HAZEN *et al.* 2005), was also found to have an earlier peak of expression in *cca1 lhy toc1*, compared to the wild type, and the early phase was shifted by ~8 hr. Rhythmic expression of *LUX* was also abolished after one very short period peak (Figure 2, A and B). With regard to a morning gene mathematically important for a tertiary circadian loop (ZEILINGER *et al.* 2006), we note published work that the peak of *PRR9* expression was greatly reduced in *cca1 lhy* (FARRE *et al.* 2005). We thus examined *PRR9* expression in *cca1 lhy toc1*. We found that *PRR9* lost rhythmic expression once the plants were transferred into constant-light conditions and there was markedly low abundance of *PRR9* transcript over a circadian cycle (Figure 2C).

The collective requirement of *cca1 lhy toc1* for rhythmicity after temperature entrainment: Temperature serves as an important environmental time cue and entrainment to temperature cycles has been reported (SOMERS *et al.* 1998a,b; MICHAEL *et al.* 2003). We sought to test the roles of TOC1 and CCA1/LHY with regard to perception of temperature entrainment. We tested the driven responsiveness and circadian behavior under LL of genotypes grown under LL at 22° after entrainment for 1 week to 12 hr warm 22°/12 hr cool 18° (WC) cycles.

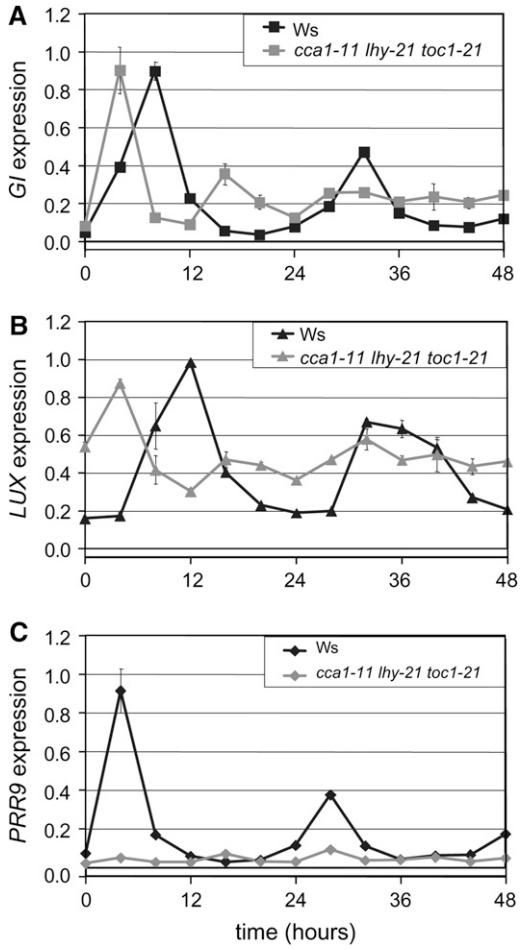

FIGURE 2.—Disrupted rhythms in the *cca1 lhy toc1* triple mutant. Ws and *cca1-11 lhy-21 toc1-21* mutant seedlings were grown for 7 days in standard 12L/12D conditions, and then entrained seedlings were transferred into constant light conditions and harvested every 4 hr. Total RNA was assayed by real-time PCR and the accumulation of *GI*, *LUX*, and *PRR9* was measured relative to an internal tubulin control. The maximum level in the wild type was set to 1 for each experiment. *GI*, *LUX*, and *PRR9* expression in Ws is represented by triangles, squares, and diamonds, respectively. *GI*, *LUX*, and *PRR9* expression in *cca1-11 lhy-21 toc1-21* were represented with triangles, squares, and diamonds, respectively. This experiment was repeated two times with similar results.

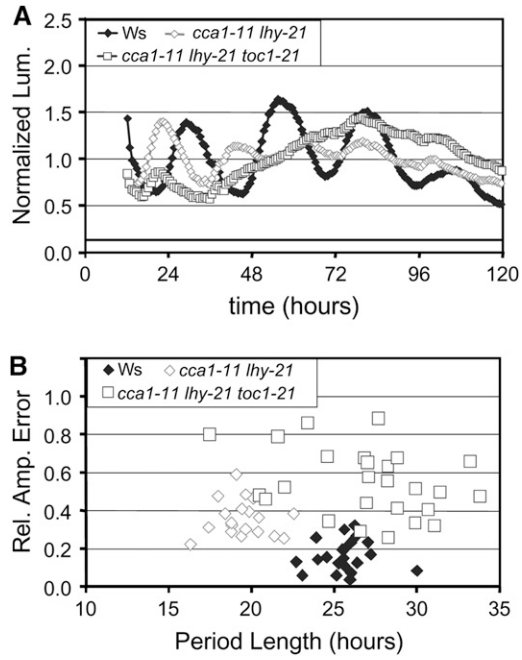

FIGURE 3.—In plants entrained to temperature cycles, rhythms are severely disrupted in *cca1 lhy toc1*. (A) Wild-type, the *cca1-11 lhy-21* double mutant, and the *cca1-11 lhy-21 toc1-21* triple-mutant seedlings were grown for 1 week in a temperature-entraining regimen consisting of 12 hr at 22°, followed by 12 hr at 18°, all under continuous light. At the end of the 10th day (at 22°, similar to dusk), seedlings were released into continuous light and temperature of 22° and assayed. The traces represent *CAB::LUC* expression. Ws, *cca1-11 lhy-21*, and *cca1-11 lhy-21 toc1-21* are represented by solid diamonds, open diamonds, and open squares, respectively. (B) Period analysis of *CAB::LUC* bioluminescence rhythms shown in Figure 1A between hours 24 and 120. This experiment was repeated two times with similar results.

We first noted that, for WC-cycle entrained seedlings, both *cca1 lhy* and wild-type plants maintained strong rhythmic expression. In contrast, *cca1 lhy toc1* lost its driven rhythm after one cycle at 22° under LL (Figure 3A). Although rhythms could be fit to ~50% of triple-mutant seedlings with FFT–NLLS analysis, most had a very high error compared to the wild type (Figure 3B). The residual one cycle of *CAB2* expression suggested to us that, under constant light conditions, the *cca1 lhy toc1* phenotype could be partially rescued when plants were exposed to WC cycles. However, the free-running rhythm under LL after a WC entrainment was arrested within a day after transfer to constant conditions (Figure 3A). Collectively, we interpret these data as the *cca1 lhy toc1* triple mutant being capable of both light/dark and warm/cool perception.

A diurnally entrained clock stops in the middle of a circadian day in *cca1 lhy toc1*: Since the clock in *cca1 lhy toc1* was apparently disrupted, we sought to define the arrest time point of this triple mutant. To this end, we performed a release assay (McWATTERS *et al.* 2000). For this, we grew seedlings under 12L/12D before transfer to LL at subjective dawn (0 hr). Replicate samples were then transferred to darkness every 2 hr for imaging of the first peak of CAB2 expression. The average time of the first peak of *CAB2* expression in constant darkness (DD) was plotted against the duration of the preceding light interval. We found, as previously reported (HALL *et al.* 2003), that the circadian clock in wild-type plants continued to oscillate and that the peak phase was only marginally affected by the single light–dark transition. Note that the peak of *CAB2* expression occurred close to the phase predicted from the discontinued light–dark (LD) cycle at 24–30 hr or 46–56 hr after the last dark–light transition at 0 hr (Figure 4A). In this assay, the *cca1*

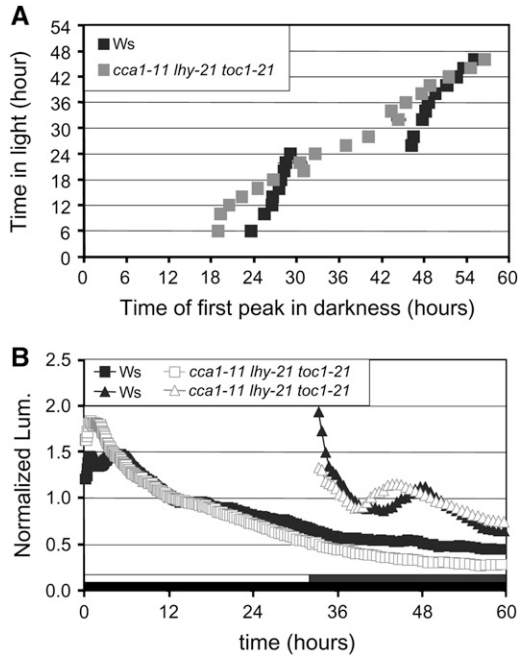

FIGURE 4.—The clock in *cca1 lhy toc1* stops in the middle of the day Wild-type and *cca1-11 lhy-21 toc1-21* seedlings were entrained for 7 days to 12L/12D cycles at a constant 22°. (A) At 0 hr (subjective dawn), all seedlings were transferred to LL and 22° (in an open area). Replicate samples of 40–60 seedlings were transferred to DD and 22° at 2-hr intervals, and *CAB:LUC* luminescence was monitored. Data shown represent the mean phase of peak luminescence (error bars are smaller than the symbols). (B) One example of wild type and *cca1-11 lhy-21 toc1-21* exposed to LL for 34 hr before transferring to DD before monitoring luminescence. Wild-type plants and *cca1-11 lhy-21 toc1-21* seedlings that were transferred into DD at 0 hr (subjective dawn) were used as the control.

lhy toc1 triple displayed no evidence of gating and thus no evidence of circadian regulation after seedlings where exposed to light for any duration >10 hr. After this point, the peak time of *CAB2* expression was clearly set by the final light–dark transition, whatever the duration, and not by the entraining LD cycle (Figure 4B). Since *CAB2* expression in *cca1 lhy toc1* was arrhythmic under LL (Figures 1 and 2), we did not expect to detect a clock-regulated peak of *CAB2* after anything more than a day of continuous light. However, even seedlings that had been exposed to LL for >30 hr were still found to maintain a circadian-inducible *CAB2* peak, which was not a result from an acute response to light (Figure 4B). We suggest that the *cca1 lhy toc1* triple mutant could restart the clock making use of the dusk signal. This implies that an as-of-yet-undescribed component(s) of a residual clock is present in the *cca1 lhy toc1* triple mutant and that *cca1 lhy toc1* is sensitive to the "light-off" signal.

***toc1* enhanced the inhibitory effect of *cca1* and *lhy* on *PRR9* and less so on *PRR7*:** It was reported that both *PRR9* and *PRR7* expression levels were reduced in the *cca1 lhy* double mutant (FARRE *et al.* 2005). To test if *TOC1* also participates in regulating *PRR9* and *PRR7* expression, as indirectly implied by a three/four-loop model, we performed real-time PCR on RNA extracted from replicate time points from wild type and *cca1*, *lhy*, *toc1*, and all double- and triple-mutant seedlings grown under 8L/16D cycles and examined evident *PRR9* and *PRR7* transcript abundance. With this assay, we did not detect any differences in *PRR9* expression between wild-type plants and *toc1* (Figure 5, A and B). In the *cca1* single mutant, the peak of *PRR9* expression was reduced, consistent with published data (FARRE *et al.* 2005). Although the transcript level of *PRR9* in *lhy* was also reduced, its expression was higher than that in *cca1* (Figure 5, A and B). Accordingly, the expression of *PRR9* was higher in *lhy toc1* than that in *cca1 toc1*, which had a similarly low-level *PRR9* transcript as that of *cca1 lhy* and *cca1 lhy toc1* (Figure 5, A and B). In both the *cca1 toc1* double mutant and the *cca1 lhy toc1* triple mutant, the peak of *PRR9* accumulation was reduced to a level similar to that seen in the *cca1 lhy* double mutant. We suggest that *TOC1* activates *PRR9* expression through a pathway controlled by *CCA1* and *LHY* (Figure 5, A and B). Mutations in *CCA1* and *LHY* did not influence *PRR7* expression as strongly as the effect on *PRR9*. The peak of *PRR7* transcript accumulation was only slightly reduced in the double mutants *cca1 lhy* and *cca1 toc1* and in the *cca1 lhy toc1* triple mutant (Figure 5, C and D). It was also noted that no major difference in *PPR7* was detected between wild-type plants and any single mutant assayed (Figure 5, C and D).

Genetic interactions between TOC1 and CCA1/LHY in the control of flowering time: It was previously reported that, under short days, both the *lhy* and the *cca1* single mutants flowered earlier than wild-type plants and that the *cca1 lhy* double mutant flowered significantly earlier than either of the two single mutants (MIZOGUCHI *et al.* 2002). The *toc1-1* allele was also found to have an earlier-flowering phenotype under short-day conditions, which resulted from the circadian defect (SOMERS *et al.* 1998b; STRAYER *et al.* 2000). To disclose a relationship between TOC1 and CCA1/LHY in flowering-time control, we measured flowering time of the double mutants *cca1 lhy*, *cca1 toc1*, and *lhy toc1*, as well as the triple mutant *cca1 lhy toc1* and all respective single mutants under short-day conditions. All single mutants were marginally early flowering, and the *cca1 lhy* double mutant was significantly early flowering (Figure 6A), in agreement with published findings (ALABADI *et al.* 2001; MIZOGUCHI *et al.* 2002, 2005). Any double-mutant combination with *toc1* flowered earlier than any single mutant and, strikingly, the *cca1 lhy toc1* triple mutant flowered similarly as the *cca1 lhy* double mutant (Figure 6A).

A previous report indicted that the earlier-flowering phenotype of *cca1 lhy* was the result of an early phase of *GI* expression. This in turn resulted in a higher expression of *FT* (MIZOGUCHI *et al.* 2005). To detect if the

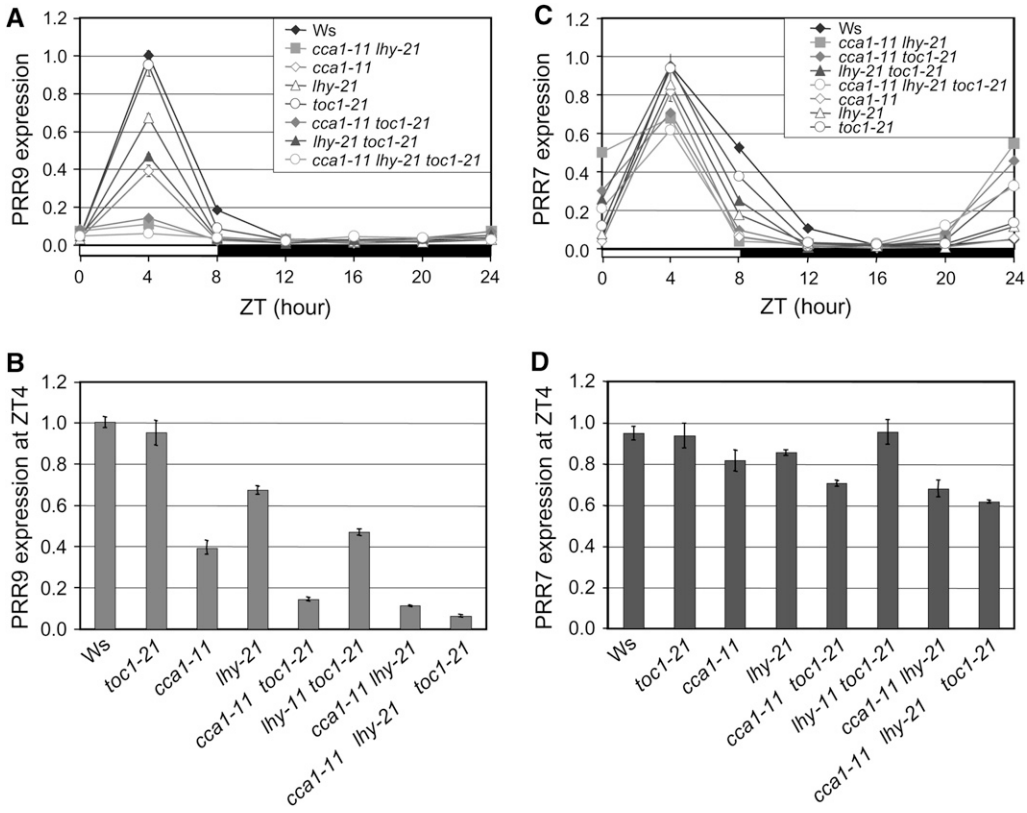

FIGURE 5.—TOC1 regulates *PRR9* expression through CCA1 and LHY and has a smaller effect on *PRR7*. Seedlings of the Ws wild type, *cca1-11*, *lhy-21*, *toc1-21*, *cca1-11 lhy-21*, *cca1-11 toc1-21*, *lhy-21 toc1-21*, and *cca1-11 lhy-21 toc1-21* were grown under standard 8L/16D conditions for 1 week, and then replicate seedling samples were harvested every 4 hr. Extracted total RNA was assayed by real-time PCR and the accumulation of *PRR9* and *PRR7* was measured relative to an internal tubulin control. The maximum level in the wild type was set to 1 for each experiment. *PRR9* and *PRR7* expression in the Ws wild type, *cca1-11*, *lhy-21*, *toc1-21*, *cca1-11 lhy-21*, *cca1-11 toc1-21*, *lhy-21 toc1-21*, and *cca1-11 lhy-21 toc1-21* was represented. This experiment was repeated two times with similar results. (A) *PRR9* expression in Ws wild type, *cca1-11*, *lhy-21*, *toc1-21*, *cca1-11 lhy-21*, *cca1-11 toc1-21*, *lhy-21 toc1-21*, and *cca1-11 lhy-21 toc1-21* was induced under standard 8L/16D conditions. (B) *PRR7* expression in Ws wild type, *cca1-11*, *lhy-21*, *toc1-21*, *cca1-11 lhy-21*, *cca1-11 toc1-21*, *lhy-21 toc1-21*, and *cca1-11 lhy-21 toc1-21* was induced under standard 8L/16D conditions. (C) *PRR9* expression at ZT4 in Ws wild type, *cca1-11*, *lhy-21*, *toc1-21*, *cca1-11 lhy-21*, *cca1-11 toc1-21*, *lhy-21 toc1-21*, and *cca1-11 lhy-21 toc1-21*, which were grown under standard 8L/16D conditions. (D) *PRR7* expression at ZT4 in Ws wild type, *cca1-11*, *lhy-21*, *toc1-21*, *cca1-11 lhy-21*, *cca1-11 toc1-21*, *lhy-21 toc1-21*, and *cca1-11 lhy-21 toc1-21*, which were grown under standard 8L/16D conditions.

early flowering phenotype of the *cca1 toc1* and the *lhy toc1* double mutants and the *cca1 lhy toc1* triple mutant was also a result from earlier phased expression of *GI* and the resultant increased *FT*, we examined *GI* and *FT* expression by real-time PCR from RNA extracted from plants grown under 8L/16D. In wild-type plants, *GI* had a peak of expression ~8 hr after lights were turned on. In contrast, the peak expression of *GI* shifted earlier by 4 hr in *cca1 toc1, lhy toc1,* and *cca1 lhy toc1*, and, as expected, *cca1 lhy* (Figure 6B). Accordingly, *FT* expression was increased compared to the wild type in *cca1 toc1*, *lhy toc1*, and *cca1 lhy toc1*, as it was in *cca1 lhy* (MIZOGUCHI *et al.* 2005).

***TOC1* is required for the short-hypocotyl phenotype of the *cca1 lhy* double mutant:** *TOC1* is a proposed positive factor in the light-mediated repression of hypocotyl elongation during seedling deetiolation (MAS *et al.* 2003). The *cca1 lhy* double mutant under short-day growth conditions displays a short hypocotyl compared to the wild type. This has led to the suggestion that *CCA1* and *LHY* are negative regulators in seedling deetiolation (MIZOGUCHI *et al.* 2005). To test the epistatic relationship between *toc1* and *cca1/lhy* in the photomorphogenic response, we measured hypocotyl lengths of the *cca1 toc1* and *lhy toc1* double mutants and the *cca1 lhy toc1* triple mutant under short-day conditions. We found that the *cca1 toc1* and the *lhy toc1* double mutants and the *cca1 lhy toc1* triple mutant exhibited a hypocotyl length similar to that seen with the *toc1* mutant. All displayed a much longer hypocotyl than that of *cca1*, *lhy*, *cca1 lhy*, and wild-type plants (Figure 7). Therefore, the short-hypocotyl phenotype of the *lhy cca1* double mutant under short-day conditions was dependent on TOC1 activity.

***toc1* coupled with *cca1* or *lhy* is capable of light detection:** It was shown before that the transcript of *PRR9* rapidly and transiently accumulated when etiolated seedlings were exposed to white light and that the light-dependent acute response of *PRR9* is a phytochrome-mediated event (MAKINO *et al.* 2001; ITO *et al.* 2003). Since the *cca1 lhy toc1* mutant was hyposensitive to light under the photomorphogenic assay (Figure 7), it was plausible that light-induced *PRR9* expression might be accordingly affected. To this end, we examined *PRR9* expression through real-time PCR from RNA extracted from wild-type and mutant seedlings grown in darkness that were harvested after being exposed to a 1-hr white-light treatment or, as the control,

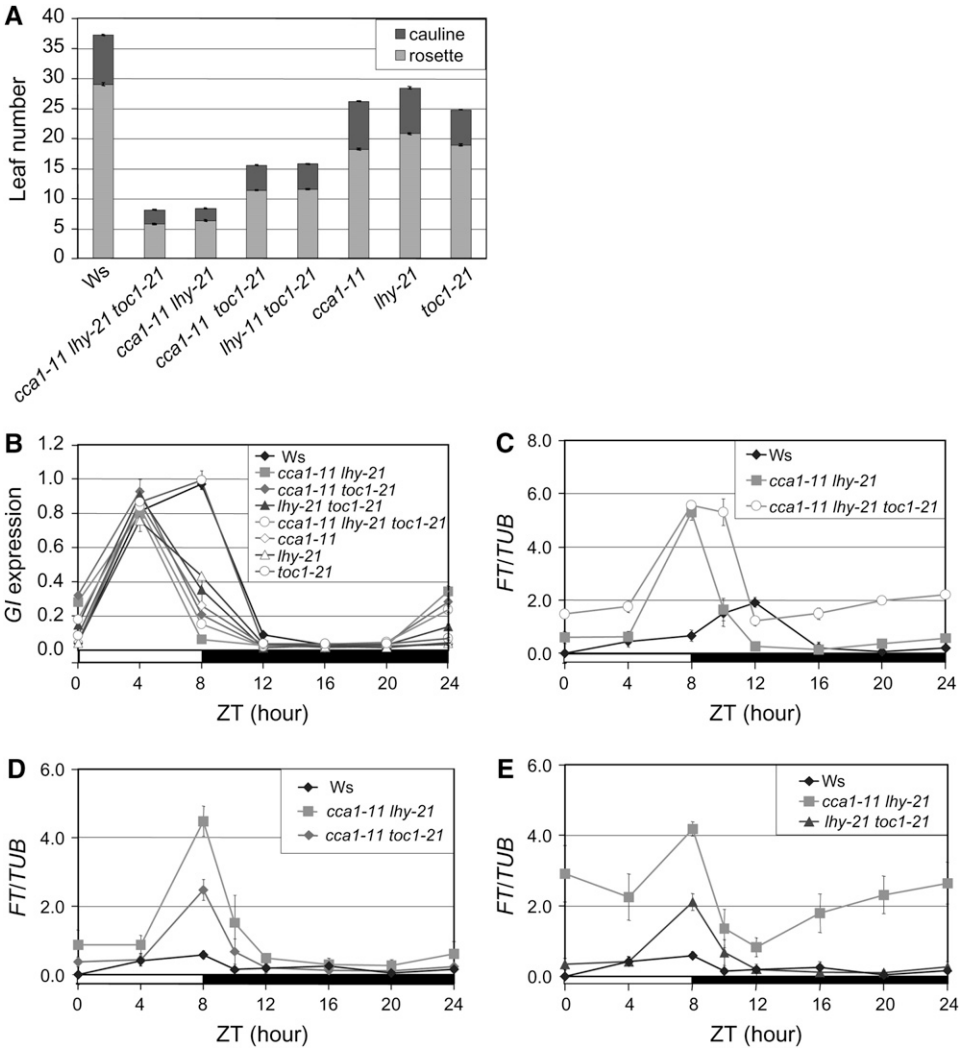

FIGURE 6.—Interactions between TOC1 and CCA1/LHY in the control of flowering time. (A) Flowering time of Ws wild type, *cca1-11*, *lhy-21*, *toc1-21*, *cca1-11 lhy-21*, *cca1-11 toc1-21*, *lhy-21 toc1-21*, and *cca1-11 lhy-21 toc1-21* was measured under standard 8L/16D conditions. Mean leaf number is shown ±SE (n = 30–40). (B) *GI* expression in Ws wild type, *cca1-11*, *lhy-21*, *toc1-21*, *cca1-11 lhy-21*, *cca1-11 toc1-21*, *lhy-21 toc1-21*, and *cca1-11 lhy-21 toc1-21*, which were grown under standard 8L/16D conditions; The RNA samples were prepared as in Figure 4. (C) *FT* expression in Ws wild type, *cca1-11 lhy-21*, and *cca1-11 lhy-21 toc1-21*, which were grown under standard 8L/16D conditions. The RNA samples were prepared as in Figure 4. (D) *FT* expression in Ws, *cca1-11 toc1-21*, and *cca1-11 lhy-21*, which were grown under standard 8L/16D conditions. The RNA samples were prepared as in Figure 4. (E) *FT* expression in Ws *lhy-21 toc1-21* and *cca1-11 lhy-21*, which were grown under standard 8L/16D conditions. The RNA samples were prepared as in Figure 4. All above experiments were repeated two times with similar results.

without such a light pulse. Interestingly, we found that light-induced *PRR9* expression was not reduced in *cca1 lhy toc1*. On the contrary, it was marginally increased (Figure 8). Moreover, *PRR9* levels were slightly increased in *cca1*, *lhy*, *toc1*, *cca1 toc1*, *lhy toc1*, and *cca1 lhy* mutants (Figure 8). The increased *PRR9* expression in *toc1* was consistent with the low expression level of *PRR9* in *TOC1* overexpression lines (ITO *et al.* 2003). What is clear is that any combination of mutations involving *cca1*, *lhy*, and *toc1* is *per se* capable of light perception.

DISCUSSION

Previous research revealed a reciprocal regulation between TOC1 and LHY/CCA1 within the Arabidopsis circadian clock. The myb-related transcription factors LHY/CCA1 act as negative elements that repress *TOC1* expression, and, conversely, TOC1 appears to be a positive element for *LHY* and *CCA1* expression (ALABADI *et al.* 2001). The data presented here provide further evidence that TOC1 and CCA1/LHY interact in a complicated network in driving clock regulation and in output control. The disrupted clock function of *cca1 lhy toc1* under constant light conditions provides direct experimental support for elements of the clock model proposed by LOCKE *et al.* (2005b), and its extensions (LOCKE *et al.* 2006; ZEILINGER *et al.* 2006). Interestingly, the requirement of *TOC1* in the floral transition was found to be a *CCA1/LHY*-dependent mechanism, whereas the *CCA1/LHY* requirement for photomorphogenesis was found to require *TOC1*. This collectively demonstrates that *TOC1* and *CCA1/LHY* participate in a complex epistatic manner perhaps consistent with their action as a loop.

We found drastic rhythm disruptions for *CAB2::LUC* reporter activity in *cca1 lhy toc1* after one 24-hr cycle under constant light (Figure 1A). Our real-time PCR data also revealed the rapid loss of rhythmicity in *cca1 lhy toc1*. Both *GI* and *LUX*, evening-expressed regulators of *CCA1/LHY* (FOWLER *et al.* 1999; HAZEN *et al.* 2005), lost rhythmic amplitude in the triple mutant after one constant-light cycle (Figure 2, A and B). Similarly, *PRR9*, a morning-clock gene (MATSUSHIKA *et al.* 2000; MAKINO *et al.* 2002; ERIKSSON *et al.* 2003), exhibited markedly

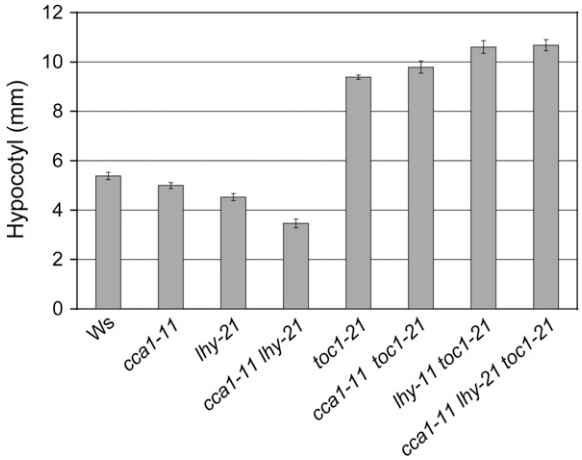

FIGURE 7.—*TOC1* is required for short hypocotyl length phenotype in the *cca1 lhy* double mutant. Hypocotyl elongation after 6 days of growth under standard 8L/16D conditions. Hypocotyl lengths are mean ±SE (n = 20–30). This experiment was repeated two times with similar results.

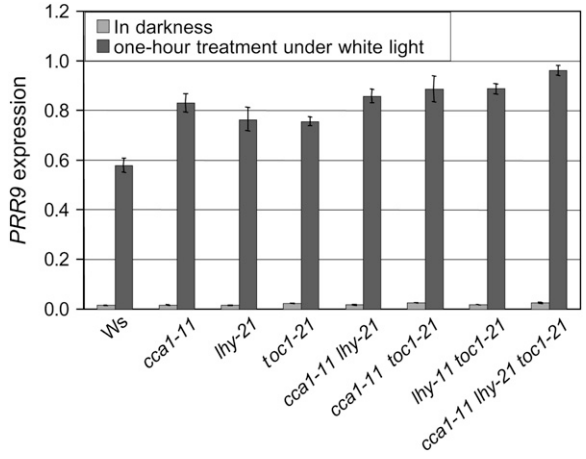

FIGURE 8.—Light-induced *PRR9* accumulation was regulated by TOC1 and CCA1/LHY. Five-day-old etiolated seedlings of Ws wild type, *cca1-11*, *lhy-21*, *toc1-21*, *cca1-11 lhy-21*, *cca1-11 toc1-21*, *lhy-21 toc1-21*, and *cca1-11 lhy-21 toc1-21* were exposed to white light, and then seedlings were harvested for each genetic background with seedlings without light treatment as the control. Total RNA was assayed by real-time PCR and the accumulation of *PRR9* was measured relative to an internal tubulin control.

dampened expression in *cca1 lhy toc1*. Under constant-light conditions, *PRR9* expression in *cca1 lhy toc1* was arrhythmic and virtually undetectable (Figure 2C), and evident defects were also detected during a diurnal light–dark cycle (Figure 5A). Collectively, we found circadian disruptions in both morning and evening clock-controlled genes in *cca1 lhy toc1*.

Our studies lead us to suggest that the *cca1 lhy toc1* triple mutant exhibited a clock phenotype similar to that of the *cca1 lhy gi* triple mutant. The latter was key in providing experimental evidence to support a relationship between a core loop and a secondary loop, as extended from the latest mathematical model (LOCKE *et al.* 2006). According to this three-loop clock model, and even when one considers a four-loop model (ZEILINGER *et al.* 2006), after *CCA1*, *LHY*, and *GI* were mutated, all loops would be "opened." This could explain the collapsed clock in the *cca1 lhy gi* triple mutant. Correspondingly, since all the loops would be "opened" in *cca lhy toc1*, it is not difficult to understand why *cca1 lhy toc1* also had the same strong clock defect phenotype as that in *cca1 lhy gi*. What is further obvious is that the *cca1 lhy toc1* mutant displays one driven oscillation in response to the last light-to-dark transition (Figures 1 and 2). As such, the secondary and tertiary loops derived from the three/four-loop mathematical models either must have interconnections between them or there is another "complete" loop(s) yet to be discovered that does not require CCA1/LHY and/or TOC1 as components. The nature of such a hypothetical partial loop is as of yet unknown.

Both CCA1 and LHY were reported to have a positive effect on *PRR9* transcript accumulation. In the *cca1 lhy* double mutant, *PRR9* expression was dramatically reduced (FARRE *et al.* 2005). As negative repressors in the clock, *CCA1* and *LHY* can also be activated by TOC1, as both overexpression of either myb gene resulted in reduced expression of *TOC1* (ALABADI *et al.* 2001). Therefore, TOC1 perhaps indirectly regulates *PRR9* expression. Interestingly, we did not detect any difference in *PRR9* accumulation when comparing *toc1* and the wild type (Figure 5, A and B). However, we found that *toc1* could enhance the inhibitory effect of *cca1* and *lhy* on *PRR9* accumulation (Figure 5, A and B). Although *PRR9* expression was greatly reduced in *cca1* (FARRE *et al.* 2005), *lhy* had a higher peak of *PRR9* expression compared to *cca1*. Accordingly, *lhy toc1* also had a higher peak of *PRR9* expression compared to *cca1 toc1* (Figure 5, A and B).

It was reported that the peak of *PRR7* expression was modestly reduced in *cca1 lhy* (FARRE *et al.* 2005). We found that both *cca1 toc1* and *cca1 lhy toc1* displayed similar peak levels of *PRR7*. Further, no detectable differences were found in *PRR7* expression between *toc1* and wild-type plants (Figure 5C). However, we found no clear difference in the peak of *PRR7* expression among *cca1*, *lhy*, and wild-type plants, which was contradictory to a previous report (FARRE *et al.* 2005). This might be result of the different growth conditions, as we grew our plants under short-day conditions. Under our conditions, we found the peak of *PRR7* expression was ~4 hr after the transition to light, whereas it was previously reported the peak of *PRR7* expression was 8 hr after the transition to light during growth under 12L/12D condition (Figure 5, C and D) (FARRE *et al.* 2005). Alternatively, or in addition to, this mild discrepancy could be due to an accession difference (as the wild type, we report Ws-2 whereas FARRE *et al.* reported Col-0). In addition, *LHY* was found to have a reduced effect

on *PRR7* expression, as major differences were not found regarding the peak of *PRR7* expression between *lhy toc1* and wild-type plants (Figure 5, C and D). Collectively, our studies lead us to propose that TOC1 was also involved in the activation of *PRR9* and perhaps of *PRR7*. Here, this regulation was dependent on CCA1/LHY action. Our data thus provide direct experimental support for the latest three/four-loop clock models (Locke *et al.* 2006; Zeilinger *et al.* 2006). Accordingly to these models, after *TOC1* is mutated, *CCA1* and *LHY* expression would be reduced, and this results in the indirect decrease of *PRR9* and *PRR7* expression.

The *cca1 lhy* double mutant was revealed to have an early flowering phenotype under short-day conditions (Mizoguchi *et al.* 2005). In our studies, we found that the *cca1 lhy toc1* triple mutant flowered identically to the *cca1 lhy* double mutant (Figure 5A). The double *cca1 toc1* and *lhy toc1* flowered slightly later than *cca1 lhy* and *cca1 lhy toc1* under short-day conditions, whereas both double mutants flowered much earlier than the *cca1*, the *lhy*, and the *toc1* single mutant (Figure 5A). These single mutants also exhibited an earlier flowering phenotype under short-day conditions (Alabadi *et al.* 2001; Mizoguchi *et al.* 2005). In addition, we observed that in *cca1 lhy toc1*, *cca1 toc1*, and *lhy toc1*, the phase of *GI* expression was shifted earlier, resulting in a correlative increase in *FT* expression level (Figure 5, B–E). Taken together, our results support a model where the early flowering phenotype of *toc1* is a result of the low expression of *CCA1* and *LHY*, which, in turn, leads to a phase shift of *GI* and an increase in *FT* (Mizoguchi *et al.* 2005).

Our epistatic studies revealed that under short-day conditions the *cca1 lhy toc1* triple mutant had a hypocotyl length similar to that of *toc1*. Moreover, both *cca1 toc1* and *lhy toc1* were found to have a long hypocotyl, similar to that of *toc1* and *cca1 lhy toc1* (Figure 7). As the *cca1 lhy* double mutant exhibited a short-hypocotyl length, compared to wild-type plants under short-day conditions (Mizoguchi *et al.* 2005), our observation led us to suggest that TOC1 functions downstream of CCA1/LHY in this photomorphogenic process. In addition, we also observed that light-induced *PRR9* expression in *cca1*, *lhy*, *toc1*, and all combinations of double and triple mutants was slightly increased compared to wild-type plants (Figure 7). Clearly, etiolated combinations of these clock mutations had full light sensitivity for acute induction of *PRR9* transcript. The increased *PRR9* expression level with the light induction in *toc1* was consistent with the low expression level of *PRR9* in *TOC1* overexpression lines (Ito *et al.* 2005). Accordingly, the slightly increased light-induced *PRR9* expression in *cca1 toc1*, *lhy toc1*, and *cca1 lhy toc1* could be explained by downstream regulation of *TOC1* on *CCA1* and *LHY*. However, this observation is contradictory to the long hypocotyl of *toc1*, *cca1 toc1*, *lhy toc1*, and *cca1 lhy toc1*. This lends even further support to the idea that the hypocotyl defects in these lines were due to an underlying clock phenotype, and not to a light-perception defect *per se*. This collectively implies that *TOC1* has a negative role in this photomorphogenic response and that the interaction between TOC1 and CCA1/LHY in this response is clock driven.

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