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Arabidopsis Response Regulators ARR3 and ARR4 Play Cytokinin-Independent Roles in the Control of Circadian Period [W]

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Light and temperature are potent environmental signals used to synchronize the circadian oscillator with external time and photoperiod. Phytochrome and cryptochrome photoreceptors integrate light quantity and quality to modulate the pace and phase of the clock. PHYTOCHROME B (phyB) controls period length in red light as well as the phase of the clock in white light. phyB interacts with ARABIDOPSIS RESPONSE REGULATOR4 (ARR4) in a light-dependent manner. Accordingly, we tested ARR4 and other members of the type-A ARR family for roles in clock function and show that ARR4 and its closest relative, ARR3, act redundantly in the *Arabidopsis thaliana* circadian system. Loss of ARR3 and ARR4 lengthens the period of the clock even in the absence of light, demonstrating that they do so independently of active phyB. In addition, in white light, *arr3,4* mutants show a leading phase similar to *phyB* mutants, suggesting that circadian light input is modulated by the interaction of phyB with ARR4. Although type-A ARRs are involved in cytokinin signaling, the circadian defects appear to be independent of cytokinin, as exogenous cytokinin affects the phase but not the period of the clock. Therefore, ARR3 and ARR4 are critical for proper circadian period and define an additional level of regulation of the circadian clock in *Arabidopsis*.

INTRODUCTION

Most organisms on the planet live in a diurnal environment characterized by the succession of light and dark. To synchronize cellular, physiological, and behavioral processes to the appropriate time of day, they have developed complex signaling cascades whose role is to relay the information of light availability, quality, and quantity to the master circadian system. Of course, the information provided by the light/dark cycles does not drive the circadian oscillations seen in the daily life of an organism; rather, it entrains the clock—modulates the phase of the clock to synchronize the organism with its temporal environment. In *Arabidopsis thaliana*, phytochromes are among the photoreceptors that entrain the clock (Salomé and McClung, 2005b). Mutants lacking PHYTOCHROME B (*phyB*) in particular exhibit a lengthened period of *LIGHT-HARVESTING CHLOROPHYLL a/b BINDING PROTEIN* (*LHCB*) transcription under high fluences of red light (Somers et al., 1998) and cause a leading phase for a number of rhythms in white light (Hall et al., 2002; Salomé et al., 2002). Whether the leading phase seen in these plants is a result of a direct change in the phase of the clock is not known.

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The circadian clock in *Arabidopsis* is formed by interconnected feedback loops between positive and negative elements. The two single Myb-domain transcription factors CIRCADIAN CLOCK-ASSOCIATED1 (CCA1) (Wang and Tobin, 1998) and LATE ELONGATED HYPOCOTYL (LHY) (Schaffer et al., 1998) act within the negative limb of the clock to repress the transcription of the positive factor, *TIMING OF CAB EXPRESSION1* (*TOC1*, also known as *PRR1*) (Strayer et al., 2000). TOC1 and the Myb-like transcription factor LUX ARRHYTHMO are required for high expression of *CCA1* and *LHY*, thereby closing the loop (Alabadí et al., 2001; Hazen et al., 2005). In addition, CCA1 and LHY play a positive role in the expression of the two *TOC1*-related genes *PRR7* and *PRR9* and may initiate a second loop critical for proper clock function and temperature entrainment (Farré et al., 2005; Nakamichi et al., 2005; Salomé and McClung, 2005a).

A model for light resetting of the *Arabidopsis* circadian clock was postulated to include phyB and the transcription factor PHYTOCHROME-INTERACTING FACTOR3 (PIF3) (Ni et al., 1998). PIF3 interacts strongly with the photoactivated far-red-absorbing form of phyB and to a weaker extent with phyA (Zhu et al., 2000). Because loss-of-function alleles in PIF3 lack any circadian mutant phenotype (Monte et al., 2004; Oda et al., 2004; Salomé and McClung, 2005b), the precise involvement of the transcription factor in light signaling to the clock is now in question. Quite possibly, the light-induced degradation of PIF3 by the proteasome may account for the lack of circadian defects, as the protein accumulates only in the dark (Bauer et al., 2004; Monte et al., 2004). Redundancy among family members may also obscure the exact role of PIF3 in light input to the clock (Bailey et al., 2003). Interestingly, a motif seen in the N terminus of some

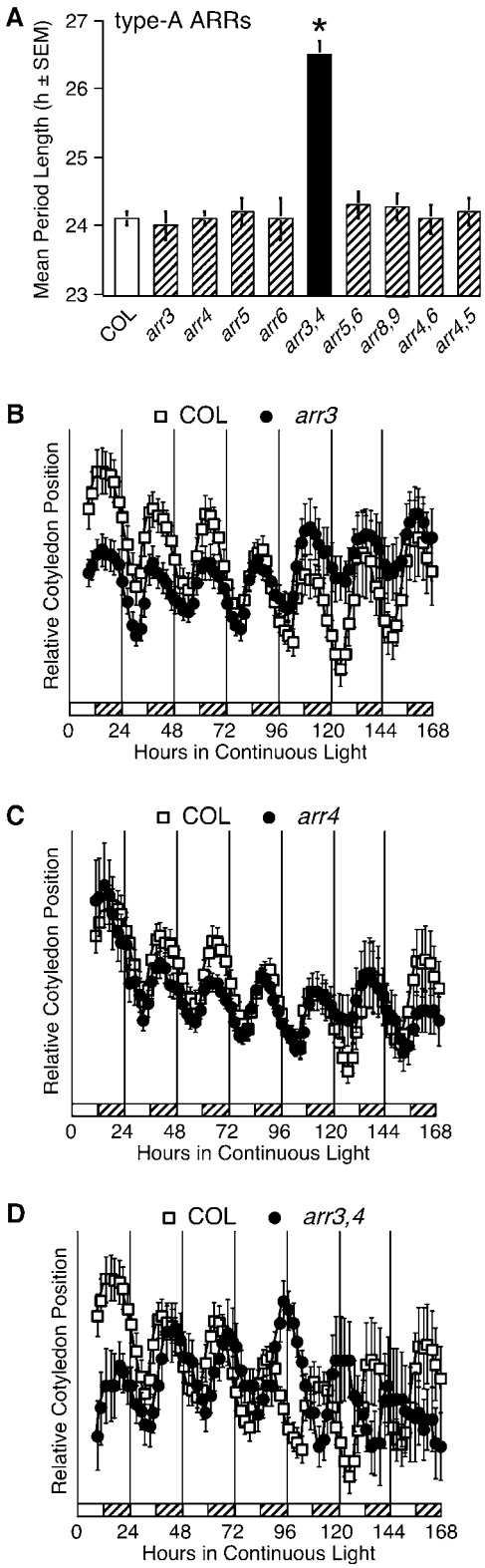

Figure 1. Cotyledon Movement Survey of Type-A ARR Loss-of-Function Seedlings.

Seedlings were grown under light/dark cycles (12 h of white light followed by 12 h of dark) for 5 d. On day 6, individual seedlings were transferred to 24-well plates and released into continuous white light. Cotyledon movement was recorded for 7 d and analyzed as described (Salomé and McClung, 2005a). The asterisk indicates a significant difference from Col ($P < 0.001$ as determined by one-way analysis of variance [ANOVA] and Duncan's multiple comparison test).

(A) Mean period length of cotyledon movement for the wild type (Col) and type-A ARR loss-of-function alleles. Error bars represent 2 SE, from 12 to 24 seedlings.

(B) to **(D)** Average cotyledon movement traces for *arr3* single **(B)**, *arr4* single **(C)**, and *arr3,4* double **(D)** mutants after entrainment by photocycles. Each trace represents the average from 12 to 24 individual cotyledons and is shown ±SE. Closed circles, mutant; open squares, Col; hatched bars, subjective night.

PIFs and PILs was found to be critical for interaction with phytochromes and may provide a biochemical signature for basic helix-loop-helix proteins involved in light signaling (Khanna et al., 2004).

We have shown previously that mutations in phyB lead to a leading circadian phase after entrainment to light/dark cycles (Salomé et al., 2002). That the leading phase is not observed after entrainment to temperature cycles suggests that light input to the clock is affected. Besides PIF3, a number of potential signaling intermediates in the phyB transduction pathway have been described. ARABIDOPSIS RESPONSE REGULATOR4 (ARR4) interacts with phyB in vitro and in vivo, and seedlings overexpressing ARR4 display a short hypocotyl consistent with the increased stability of and sustained signaling from the photoreceptor (Sweere et al., 2001). ARR4 belongs to the response regulator family, which has 23 members: 10 type-A regulators (including ARR4), 11 type-B regulators, and 2 others (Kakimoto, 2003). True response regulators are involved in signaling cascades in which an upstream cytokinin receptor kinase phosphorylates ARRs on a conserved Asp residue within their receiver domain (Schaller et al., 2002). Overexpression of type-A ARRs results in plants with reduced sensitivity to cytokinins, whereas overexpression of type-B ARRs leads to increased cytokinin sensitivity (Kiba et al., 2003; Tajima et al., 2004). Conversely, mutants lacking several type-A ARRs show an increased response to cytokinin, whereas mutants in type-B ARRs are more resistant to exogenous cytokinins (Kiba et al., 2003; To et al., 2004).

We set out to characterize the circadian behavior of many single, double, and higher order mutants containing T-DNA insertions into type-A ARRs. We show here that only the double mutant *arr3,4* and the quadruple mutant *arr3,4,5,6* display a long-period phenotype as well as a leading phase characteristic of *phyB* mutants, which is obscured by the long period seen in these mutants. The effect on the pace of the clock conferred by the loss of ARR3 and ARR4 is not attributable to a change in the sensitivity of the clock to cytokinins, as exogenous application of the hormone does not lengthen the period. Finally, we demonstrate a complex genetic interaction among type-A ARRs, as the phenotype conferred by *arr3,4* can be completely suppressed by lesions in *ARR8* and *ARR9*, although the *arr8,9* double mutant has no circadian defect on its own. These findings represent an important step in the description of genes that are not essential

for the generation of rhythmicity but are critical for the proper regulation of the circadian parameters of period and phase, and they underscore the importance of characterizing whole gene families as opposed to limited sets of members.

RESULTS

Loss of the Type-A ARR3 and ARR4 Lengthens the Period of the Clock

We characterized the circadian parameters of single, double, and higher order T-DNA insertion mutants in type-A ARRs (To et al., 2004). Single or double loss-of-function mutations in most type-A ARRs did not affect the clock (Figures 1A to 1C). Among double mutant combinations between gene pairs with the highest similarity (*ARR3* and *ARR4*, *ARR5* and *ARR6*, *ARR8* and *ARR9*), only the *arr3,4* double mutant showed a striking long period (Figures 1A and 1D). No other double mutant analyzed shared this phenotype, nor did the *arr3* and *arr4* single mutants, indicating that the two genes redundantly contribute to the control of period length in cotyledon movement. Because this analysis considered only single alleles of each *ARR* gene, it remains possible that the long-period phenotype of the *arr3,4* double mutant and of higher order combinations results from a third mutation introduced along with either *arr3* or *arr4*. This hypothetical third mutation would have phenotypic consequence only in the *arr3,4* double mutant, because neither single *arr* mutant has a long period. We analyzed F2 seedlings from a cross between *arr3,4,5,6* and *arr5,6* for segregation of a long period and observed the long period in one-sixteenth of the seedlings (data not shown), consistent with segregation of two genes and ruling out the possibility of an unlinked third mutation. However, we cannot exclude the possibility of a third, linked mutation by this analysis.

LUCIFERASE (*LUC*) fusions to the promoters of the clock genes *CCA1*, *LHY*, and *TOC1* were introduced into the *arr3* and *arr4* single mutants, as well as the *arr3,4* double mutant, to determine whether the two genes act upstream of the clock, in which case clock gene oscillations would be affected, or downstream as part of an output pathway controlling cotyledon movement, in which case clock gene expression would be unaltered. After the photocycles, the period of all three clock genes was lengthened in the *arr3,4* double mutant, but it remained very close to normal in either single mutant (Figure 2). The same result was also seen after entrainment to warm/cold temperature cycles (thermocycles; data not shown), indicating that ARR3 and ARR4 likely act upstream of the clock and not along an output

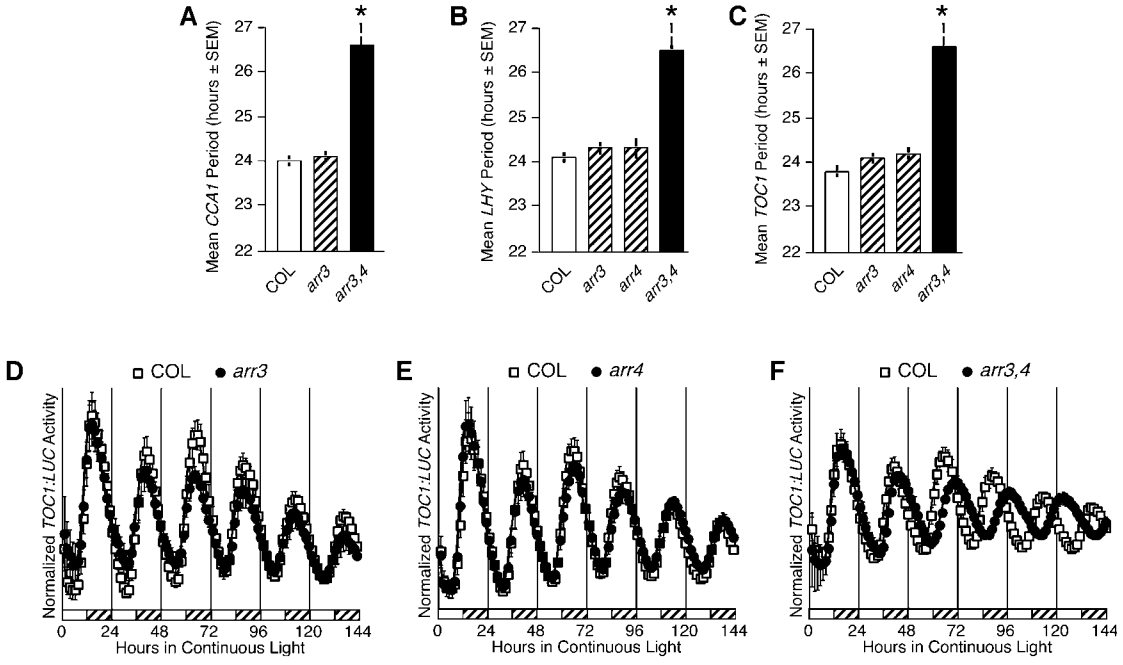

Figure 2. Loss of ARR3 and ARR4 Lengthens the Period of the Clock Genes.

All seedlings were entrained to photocycles for 10 d. On day 10, seedlings were transferred to 96-well plates containing Murashige and Skoog (MS) medium salts supplemented with 2% sucrose and 30 μL of 2.5 mM D-luciferin. After another entraining cycle on the Topcount luminometer, plates were released into continuous white light and LUC activity was recorded for 6 d. The asterisks indicate significant differences from Col ($P < 0.001$ as determined by one-way ANOVA and Duncan's multiple comparison test).

(A) to **(C)** Mean period length in the expression of the clock genes *CCA1* **(A)**, *LHY* **(B)**, and *TOC1* **(C)** in Col, *arr3*, *arr4*, and *arr3,4* seedlings. Periods are given as averages of 24 to 60 seedlings from three independent experiments ±SE.

(D) to **(F)** Representative average traces of *TOC1:LUC* expression in wild-type Col and *arr3* **(D)**, *arr4* **(E)**, and *arr3,4* **(F)** seedlings. Mean expression is shown ±SE. Closed circles, mutant; open squares, Col; hatched bars, subjective night.

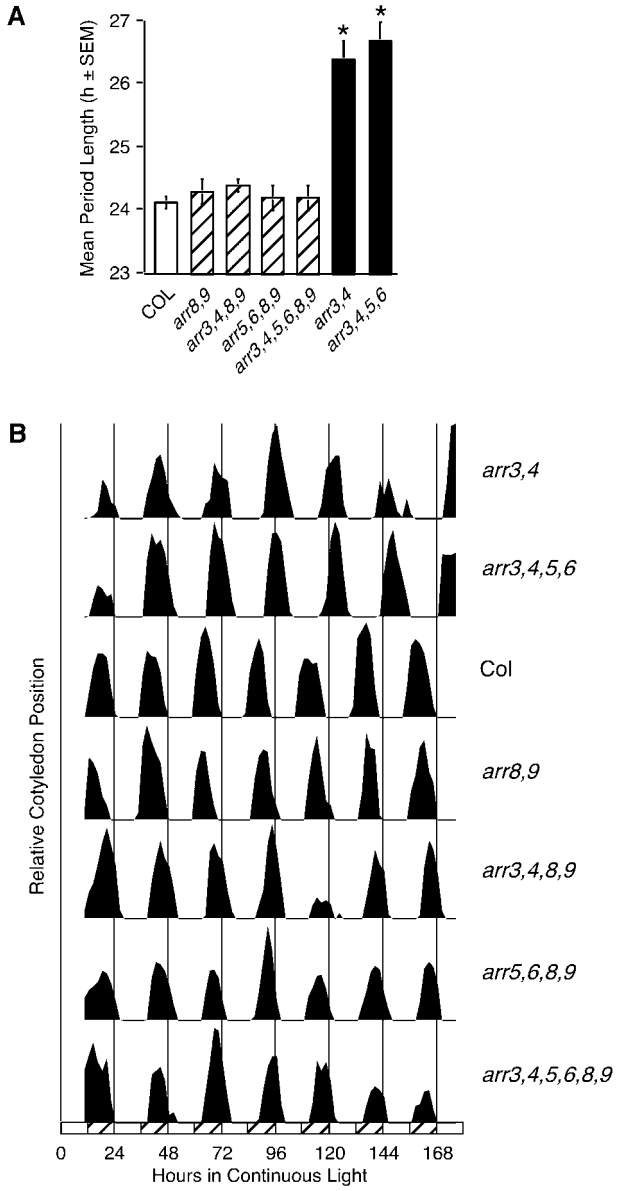

Figure 3. Loss of ARR8 and ARR9 Suppresses the Long Period of the *arr3,4* Double Mutant in Cotyledon Movement.

All seedlings were grown as described for Figure 1. The data presented here represent averages of 12 to 24 individual cotyledons from three independent experiments ± SE. The asterisks indicate significant differences from Col ($P < 0.001$ as determined by one-way ANOVA and Duncan's multiple comparison test).

(A) Mean period length in cotyledon movement for the type-A ARR mutant pairs *arr3,4*, *arr5,6*, and *arr,8,9* and the higher order mutants *arr3,4,5,6*, *arr3,4,8,9*, and *arr3,4,5,6,8,9*.

(B) Representative average traces from the genotypes shown in **(A)**. Each trace is the average of 12 to 24 cotyledons from one experiment, plotted as a linear plot generated with the Chrono program (Roenneberg and Taylor, 2000). In the linear plot option, amplitudes are adjusted to be similar and any trend (downward or upward) resulting from hypocotyl elongation during the recording of the rhythms is removed. Note how the peaks from the mutants shown below Col are synchronized with the wild-type trace, whereas the peaks from *arr3,4* and *ar3,4,5,6* occur progressively later than Col, consistent with the long-period phenotype of these seedlings. Hatched bars, subjective night.

pathway. Mean *TOC1* period was lengthened slightly in the *arr3* and *arr4* single mutants, but the same was not true for mean *CCA1* and *LHY* period. Therefore, ARR3 and ARR4 are largely redundant and perform an important role in the determination of circadian period in *Arabidopsis*.

Genetic Interaction among Type-A ARRs in the Control of Period Length

ARR3 and *ARR4* belong to a minor clade within the type-A ARRs that includes *ARR5*, *ARR6*, *ARR8*, and *ARR9* (To et al., 2004). Because these four additional genes may partially compensate for the loss of *ARR3* and *ARR4*, we examined the circadian phenotypes of all quadruple mutants between gene pairs within the subclade, as well as the *arr8,9* double mutant and the *arr3,4,5,6,8,9* hextuple mutant. The *arr5,6,8,9* quadruple mutant showed a wild-type period phenotype by cotyledon movement (Figure 3). Compared with the *arr3,4* double mutant, no further period lengthening of cotyledon movement was observed in *arr3,4,5,6* (Figure 3). Remarkably, period lengthening seen in the *arr3,4* double mutant was completely suppressed by T-DNA insertion alleles of *ARR8* and *ARR9* in *arr3,4,8,9* (Figure 3). The *arr8,9* double mutant showed a normal period length by cotyledon movement, indicating that the loss of *ARR8* and *ARR9* is not itself sufficient to generate a circadian phenotype (Figure 3). The *arr3,4,5,6,8,9* hextuple mutant similarly displayed no circadian phenotype (Figure 3B). A similar complexity in the genetic interactions between type-A ARRs was seen in petiole length and rosette size (To et al., 2004).

The *arr8,9* double mutant suppressed the long period of the *arr3,4* double mutant at the level of the expression of the clock genes themselves. Indeed, mean period lengths of the *CCA1*:*LUC* and *LHY*:*LUC* reporters were normal in *arr8,9* and *arr3,4,8,9*, whereas the *arr3,4,5,6* quadruple mutant period was long, similar to the period of *arr3,4* (Figure 4).

Effects of Exogenous Cytokinin on the Expression of the Clock Genes

The expression of many type-A ARRs is induced in response to cytokinin (Kiba et al., 1999; D'Agostino et al., 2000). We wished to determine the effect of exogenous cytokinin treatment on the clock and so treated ecotype Columbia (Col) seedlings bearing a number of *LUC* fusions (*LHCB*, *CCA1*, *CAT3*, and *TOC1*) with increasing concentrations of kinetin, *trans*-zeatin, and benzyladenine (see Methods for details). Kinetin did not change period length but instead modified circadian phase in a dose-dependent manner (Figure 5A; see Supplemental Figure 1 online) for all *LUC* reporter constructs assayed. At low concentrations, kinetin resulted in a leading phase, whereas higher concentrations caused the phases of the reporters to lag behind those of untreated seedlings. That different hormone concentrations show

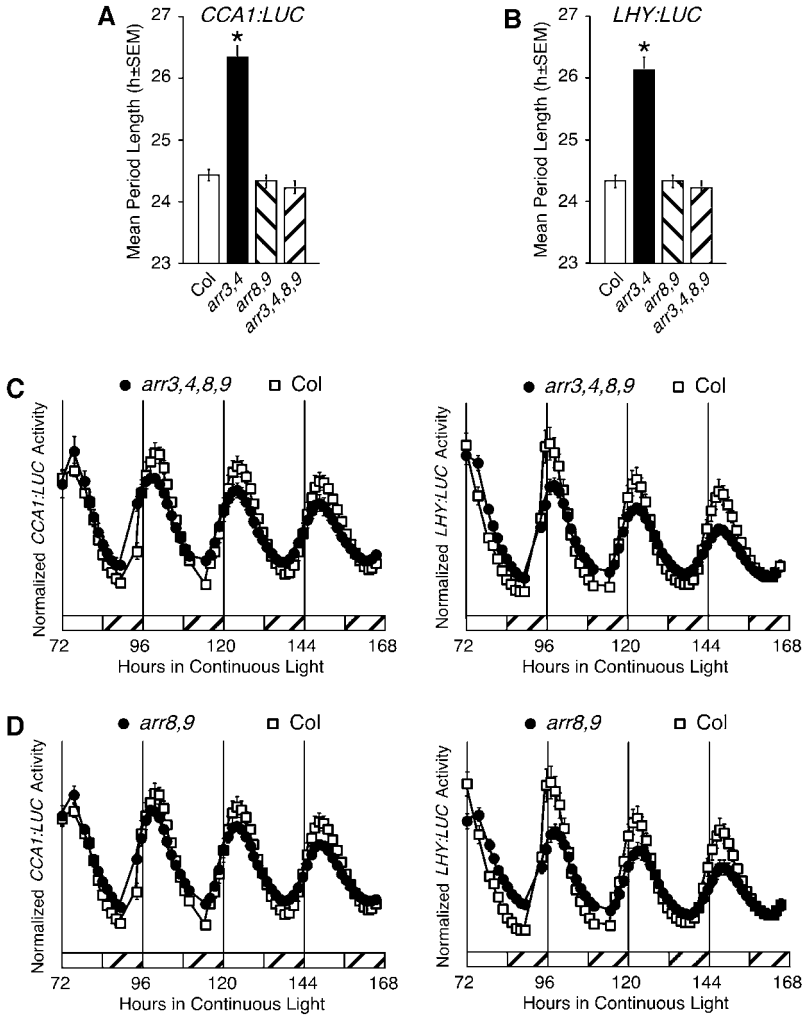

Figure 4. Loss of ARR8 and ARR9 Restores a Wild-Type Period to the *arr3,4* Mutant for the Expression of *CCA1* and *LHY*.

All seedlings were grown as described for Figure 2. Expression of *CCA1* and *LHY* was first characterized for primary transformants (4 to 24) and confirmed on four T2 lines for each construct and genotype in two independent experiments. The asterisks indicate significant differences from Col ($P < 0.001$ as determined by one-way ANOVA and Duncan's multiple comparison test). Error bars represent 2 SE from 12 (Col and *arr3,4*) and 48 (*arr8,9* and *arr3,4,8,9*) seedlings.

(A) Mean period length for *CCA1* expression in Col, *arr3,4*, *arr8,9*, and *arr3,4,8,9*.

(B) Mean period length for *LHY* expression in Col, *arr3,4*, *arr8,9*, and *arr3,4,8,9*.

(C) Representative average traces for *CCA1* (left) and *LHY* (right) expression in Col (open squares) and *arr3,4,8,9* (closed circles) ±SE. Hatched bars, subjective night.

(D) Representative average traces for *CCA1* (left) and *LHY* (right) expression in Col (open squares) and *arr8,9* (closed circles) ±SE. Hatched bars, subjective night.

opposite effects is not uncommon; for instance, low levels of auxin promote root elongation, but higher concentrations repress the same process (Evans et al., 1994). Treating seedlings with various concentrations of *trans*-zeatin or benzyladenine caused the same effects as kinetin (data not shown). It is worth noting that high concentrations of cytokinins were applied, suggesting that their action on the clock may not be physiologically relevant. Lower concentrations had no effect (data not shown). Cytokinin sensitivity assays typically use levels as low as 100 nM, but many reports use this hormone in the 1 to 5 μM range to elicit a strong response, with 100 μM the highest level tested (Higuchi et al., 2004; Nishimura et al., 2004; To et al., 2004), so the leading phase seen at 5 μM kinetin may represent a true circadian response to the hormone. The amplitude of *TOC1* expression was decreased at 50 and 100 μM kinetin (Figures 5C to 5F; see Supplemental Figure 1 online), which may suggest a toxic effect from the high kinetin levels.

We also tested the *arr3* and *arr4* single, *arr3,4* double, and *arr3,4,5,6* quadruple mutants under the same conditions and found that they responded to the hormone in a manner similar to

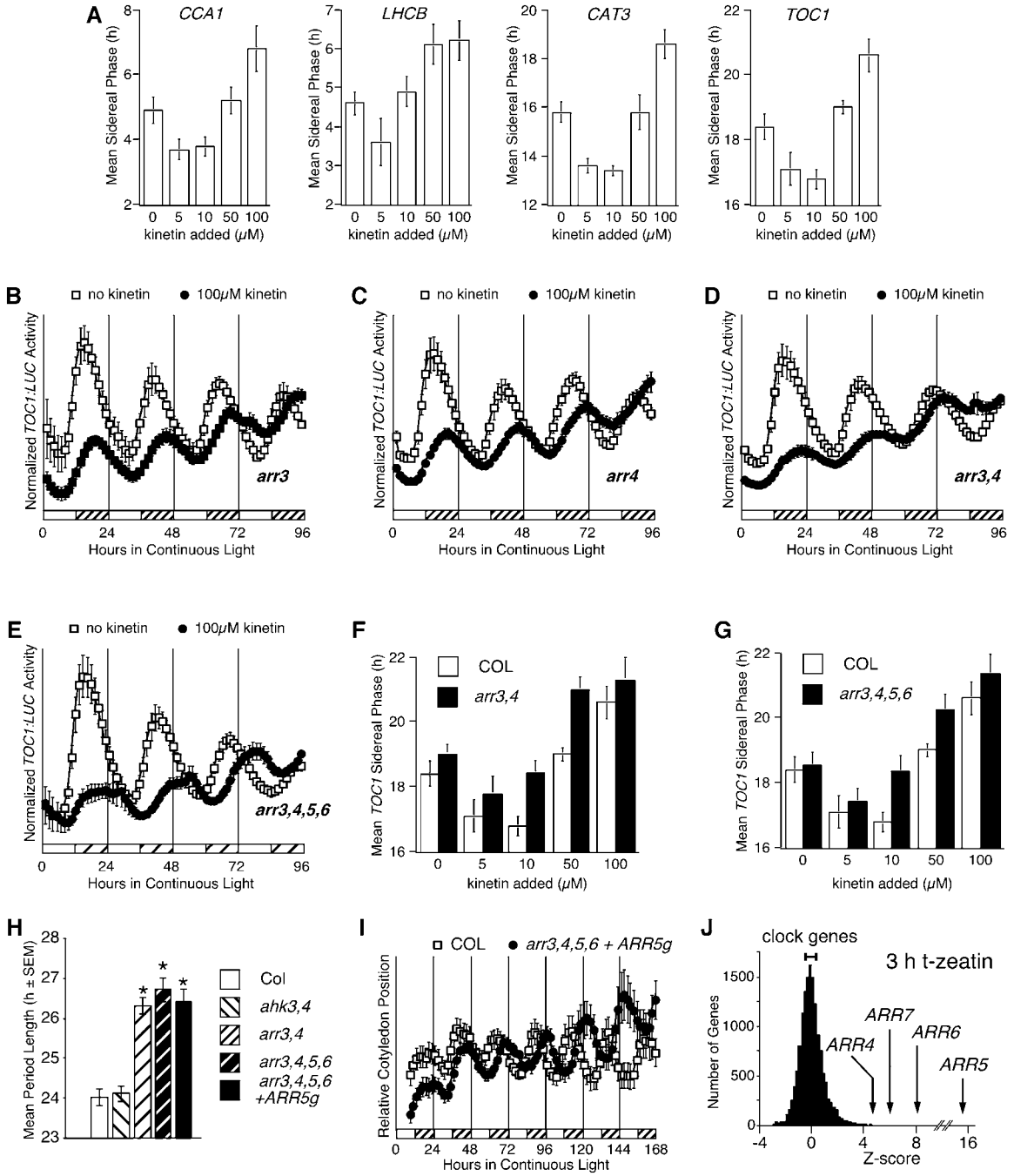

Figure 5. Lagging Circadian Phase Caused by Exogenous Cytokinin Treatment in Col and Type-A ARR Mutants.

All seedlings were grown as described for Figure 2. On day 11, the cytokinin kinetin (solubilized in slightly acidic water) was added to each well at a final concentration of 5, 10, 50, or 100 μM. Plates were entrained for an additional 1 d in light/dark cycles before being released into continuous light. LUC activity was recorded for 6 d and analyzed as described in Methods. In all panels, data are shown ±SE from 12 to 24 seedlings.

(A) Mean sidereal phase values for the clock genes *CCA1* and *TOC1* and the clock-regulated genes *LHCB* and *CAT3* in the absence and presence of kinetin. Sidereal phase represents the time of the observed peak for a given rhythm, without normalization to the endogenous period length of the rhythm.

(B) to **(E)** Representative average traces for *arr3* **(B)**, *arr4* **(C)**, *arr3,4* **(D)**, and *arr3,4,5,6* **(E)** in the absence (open squares) or presence (closed circles) of 100 μM kinetin.

(F) Mean sidereal phase for *TOC1* expression in Col and *arr3,4* in the absence and presence of increasing concentrations of kinetin.

(G) Mean sidereal phase for *TOC1* expression in Col and *arr3,4,5,6* in the absence and presence of increasing concentrations of kinetin.

wild-type seedlings, in addition to their circadian phenotype in the absence of treatment. Specifically, *arr3,4* and *arr3,4,5,6* displayed the long period characteristic of their observed phenotypes (Figures 1 and 3), but the phase of the rhythm was delayed relative to untreated mutant seedlings (Figures 5D to 5G) (To et al., 2004). If the long period seen in *arr3,4* and *arr3,4,5,6* is the result of an increased sensitivity to cytokinin, then exogenous treatment should phenocopy these mutants. Such is not the case, however, because cytokinin treatment does not lengthen period. This finding suggests that the long period observed in these mutants is not the consequence of altered cytokinin sensitivity. In support of this conclusion, *arr3,4,5,6,8,9*, which is the most cytokinin-sensitive mutant tested, exhibited a normal period (Figure 3).

Introduction of a genomic copy of *ARR5* into *arr3,4,5,6* can largely rescue the cytokinin insensitivity displayed by the quadruple mutant in the root elongation assay (To et al., 2004) but was not sufficient to eliminate the long period of the quadruple mutant (Figures 5H and 5I). In addition, *ahk3,4* seedlings, lacking two of the three cytokinin receptors, showed no period or phase phenotype when assayed by cotyledon movement (Figure 5H), although they displayed very strong resistance to cytokinin treatment for callus formation (Nishimura et al., 2004).

Finally, analysis of available microarray data sets from the AtGenExpress database (http://Arabidopsis.org/servlets/TairObject?type=expression_set&id=1007966040) revealed that none of the clock genes are strongly affected by treatment with 1 μM *trans*-zeatin (Figure 5J) (Zimmermann et al., 2004). The type-A ARRs ARR4, ARR5, ARR6, and ARR7, on the other hand, showed very strong induction in response to the hormone, as expected (Figure 5J) (Kiba et al., 1999; D'Agostino et al., 2000). We conclude that cytokinins do not influence the expression of clock-regulated genes and therefore are unlikely to be responsible for the long-period phenotype seen in *arr3,4* and a*rr3,4,5,6*.

Loss of ARR3 and ARR4 Lengthens the Period of the Clock in All Conditions

The long-period phenotype of the *arr3,4* and *arr3,4,5,6* mutants is observed after either photocycles or thermocycles, indicating that ARR3 and ARR4 do not merely participate in a light input pathway leading to the clock. We wished to determine whether the period lengthening seen in *arr3,4* and *arr3,4,5,6* was dependent on the presence of light. We entrained seedlings to photocycles for 10 d and released the seedlings in constant red light or blue light or in constant darkness. As shown in Figure 6, all light conditions tested yielded a similar period lengthening of all genes tested in the *arr3,4,5,6* mutant. The same was true for *arr3,4* (data not shown). These findings demonstrate that ARR3 and ARR4 play an important role in the determination of circadian period and that their action is not mediated through modulation of a light input pathway.

ARR4 and *ARR9* Expression Is Not under the Control of the Clock

The clock components *CCA1*, *LHY*, and *TOC1*, as well as the clock-associated genes *PRR7* and *PRR9*, all show circadian control of their expression (Schaffer et al., 1998; Wang and Tobin, 1998; Matsushika et al., 2000; Strayer et al., 2000; Salomé and McClung, 2005a). *ARR3* and *ARR4* are expressed in all tissues (To et al., 2004), but it is unknown whether they might themselves be under clock regulation. Analysis of available microarray data sets from the Nottingham Arabidopsis Stock Centre (http://affymetrix.arabidopsis.info/narrays/experimentpage.pl?experimentid=108) indicated that *ARR4* might be under clock regulation, with a peak in expression in the subjective evening, although with a very weak amplitude (Zimmermann et al., 2004). In that study, *ARR3* levels were too low to accurately determine circadian regulation. We generated translational fusions by fusing ~2800 bp of the *ARR4* promoter and ~2100 bp of the ARR9 promoter to the *LUC* reporter gene and introduced them into the Col ecotype by *Agrobacterium tumefaciens*–mediated transformation. At least 24 individual T1 seedlings were assayed for LUC activity; an average trace of these seedlings is shown in Figure 7A. *TOC1*:*LUC* data are given in Figure 7B as a reference for evening phase and amplitude expected from a known clock-regulated gene. *ARR4*:*LUC* showed a very weak oscillation in LUC activity, which coincided with peak expression of *TOC1* (Figure 7C). However, our clock gene:*LUC* fusions exhibited a much stronger amplitude in their rhythm than did *ARR4*:*LUC* (cf. amplitudes from traces shown in Figures 7A and 7B; in Figure 7C, amplitudes have been adjusted). The weak amplitude in *ARR4* expression may reflect some indirect effect, such as circadian variation in available ATP for LUC activity, rather than a true circadian regulation of *ARR4* transcription. It remains to be determined whether ARR4 protein levels cycle or whether the activity of the protein is regulated in a circadian

Figure 5. (continued).

(H) Mean period length of cotyledon movement for Col, *ahk3,4*, *arr3,4*, *arr,3,4,5,6*, and *arr3,4,5,6* containing the ARR5 transgene. The asterisks indicate significant differences from Col ($P < 0.001$ as determined by one-way ANOVA and Duncan's multiple comparison test).

(I) Average cotyledon movement traces for *arr3,4,5,6* containing the *ARR5* transgene after photocycles and release into constant light. Each trace represents the average of 24 individual cotyledons. Mean cotyledon position is shown ±SE. Closed circles, *arr3,4,5,6* + *ARR5*; open squares, Col; hatched bars, subjective night.

(J) Expression of the clock genes *CCA1*, *LHY*, and *TOC1* in response to 1 μM *trans*-zeatin treatment. The data set, available from The Arabidopsis Information Resource website as part of the AtGenExpress database, was analyzed as follows. A cutoff of 50 was used, below which genes were considered not expressed. A total of 14,641 genes were considered expressed in these experiments. Then, a fold induction was calculated by dividing the expression level of each expressed gene in the presence of the hormone by the expression level in a mock-treated sample. The ratios were finally converted to *Z*-scores, and *Z*-score values were plotted using Kaleidagraph version 4.0.2 (Synergy Software). *Z*-score values for each gene are as follows: *CCA1*, −0.54; *LHY*, 0.31; *TOC1*, −0.40; *ARR4*, 4.65; *ARR5*, 15.54; *ARR6*, 8.1; *ARR7*, 5.93.

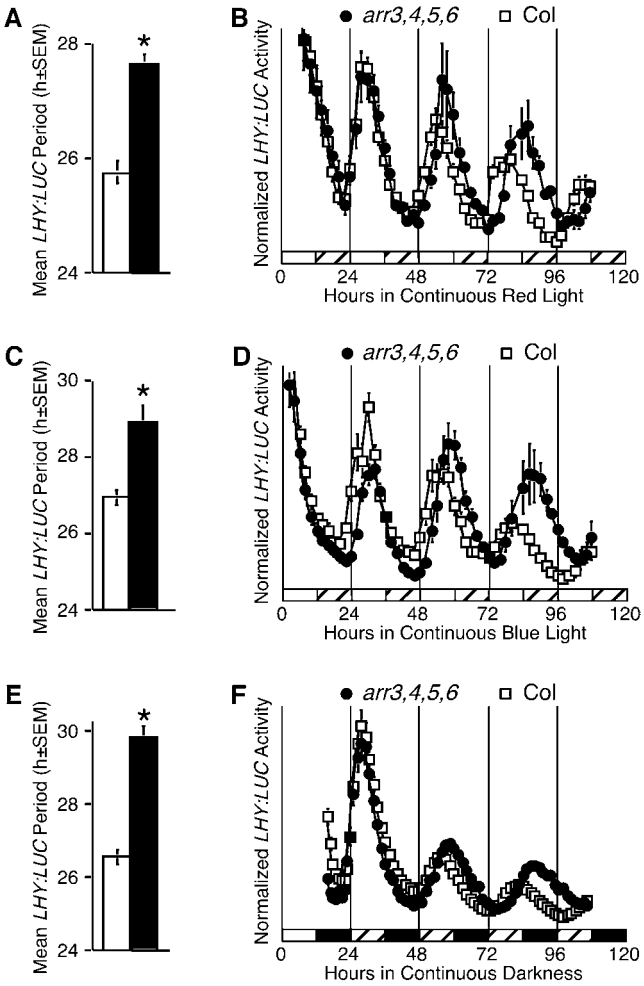

Figure 6. The Period of the Clock Gene *LHY* Is Lengthened in *arr3,4,5,6* in All Light Conditions.

All seedlings were grown as described for Figure 2. At 12 h after the onset of illumination on day 11, plates were released in 10 μmol·m^{-2}·s^{-1} continuous red light (**[A]** and **[B]**), 5 μmol·m^{-2}·s^{-1} blue light (**[C]** and **[D]**), or constant darkness (**[E]** and **[F]**). LUC activity was recorded for 5 d. The asterisks indicate significant differences from Col ($P < 0.001$ as determined by Student's heteroscedastic *t* test). Data are shown ±SE from 12 to 24 seedlings.

(A) Mean period length of *LHY* in Col and *arr3,4,5,6* in red light.

(B) Representative average trace of *LHY* expression in Col (open squares) and *arr3,4,5,6* (closed circles) in constant red light. Hatched bars, subjective night.

(C) Mean period length of *LHY* in Col and *arr3,4,5,6* in blue light.

(D) Representative average trace of *LHY* expression in Col (open squares) and *arr3,4,5,6* (closed circles) in constant blue light. Hatched bars, subjective night.

(E) Mean period length of *LHY* in Col and *arr3,4,5,6* in the dark.

(F) Representative average trace of *LHY* expression in Col (open squares) and *arr3,4,5,6* (closed circles) in the dark. Hatched bars, subjective night.

manner. ARR4 protein accumulates in white and red light, and this was dependent on active phyB (Sweere et al., 2001). The expression of *ARR9* did not appear to be under circadian control.

We conclude that the expression of *ARR4* and *ARR9*, as seen with translational fusions to their respective promoters, is unlikely to be under strong circadian control.

phyB-Like Phenotypes in *arr3,4* and *arr3,4,5,6* Mutants

We initially set out to characterize mutants defective in the type-A ARRs for circadian defects to test the hypothesis that one or more would display a leading phase phenotype similar to that of *phyB* loss-of-function mutants. However, only a long period in the *arr3,4* double and *arr3,4,5,6* quadruple mutants was observed (Figures 1 to 4). Long-period mutants normally display a lagging phase phenotype during entraining cycles, and the phase of the rhythms during the first day in free-running conditions similarly lags behind that of the wild type (Pittendrigh, 1981; Dunlap et al., 2004; Salomé and McClung, 2005a).

The sidereal phase of the rhythm represents the time of the observed peak for a given rhythm, without normalization to the free-running period length of the rhythm. Sidereal phase values for Col, *arr3,4*, and *arr3,4,5,6* were similar (Figures 8A and 8B), with *arr3,4* showing a slightly lagging phase relative to Col, but not as pronounced as would be expected given the expected phase lag of 2 h (the free-running period of the mutant was 24 h for these entraining conditions). Therefore, the *arr3,4* and *arr3,4,5,6* mutants do not behave like typical long-period mutants and do not show the expected lagging phase. In fact, when sidereal phase values were converted to circadian time phase, *arr3,4* and *arr3,4,5,6* were seen to have a leading circadian time phase (Figure 8C), similar to the phenotype seen in *phyB* mutants (Salomé et al., 2002). These results suggest that ARR3 and ARR4 may modulate phyB signaling to the clock and that the loss of both ARR proteins can generate a leading phase in gene expression. However, the ability to detect this first effect on the clock (leading phase) is obscured by the second effect on the clock (long period).

Another phenotype characteristic of *phyB* mutants is their long hypocotyl in white light and red light (Somers et al., 1991; Salomé et al., 2002). Conflicting data exist on the precise role that ARR4 plays in this process. Overexpression of ARR4 shortens the hypocotyl in red light (Sweere et al., 2001), indicating a positive role in phyB signaling. However the *arr3*, *arr4*, and *arr3,4* mutants also exhibit a shorter hypocotyl (To et al., 2004), suggesting a negative role for ARR3 and ARR4 in this signaling cascade. However, the range of fluences under which the hypocotyl phenotype is observed is distinct: low to high fluence but not very low fluence for ARR4-overexpressing plants, and very low to low fluence but not high fluence for *arr3,4*. While entraining our seedlings to photocycles, we noticed that *arr3,4* and *arr3,4,5,6* seedlings had long hypocotyls. Many mutants with circadian phenotypes show shorter or longer hypocotyls in shorter photoperiods but not in constant light (Doyle et al., 2002; Mizoguchi et al., 2002; Hall et al., 2003). When grown in short days, *arr3,4* and *arr3,4,5,6* plants have longer petioles than wild-type plants, and this phenotype is reminiscent of *phyB* mutants grown in the same conditions (To et al., 2004). We measured hypocotyl

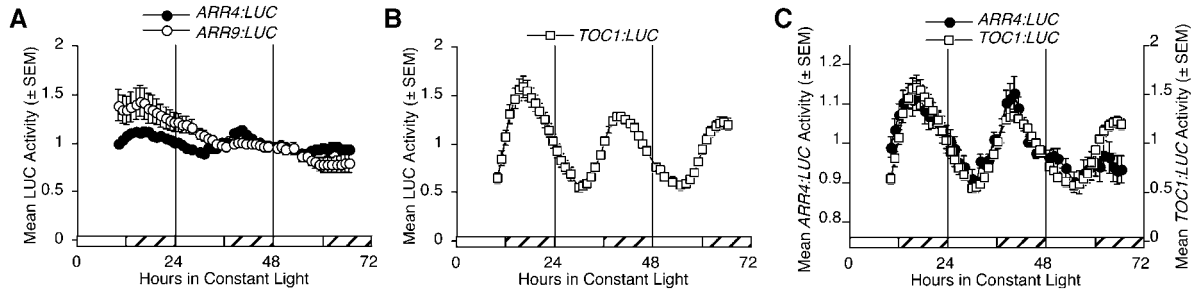

Figure 7. Expression Profile of *ARR4* and *ARR9* as Seen with Promoter:LUC Fusions.

All seedlings were grown as described for Figure 2. At 12 h after the onset of illumination on day 11, plates were released in constant white light, and LUC activity was recorded over 5 d. Data represent average values ± SE of 24 primary transformants (for *ARR4* and *ARR9*) and 12 seedlings from a T3 line (*TOC1*).

(A) Average traces for *ARR4*:*LUC* and *ARR9*:*LUC* under the same conditions. Closed circles, *ARR4*:*LUC*; open circles, *ARR9*:*LUC*. The scale on the *y* axis for **(A)** and **(B)** is identical; therefore, relative amplitude can be compared directly.

(B) Average trace for *TOC1*:*LUC* in constant white light. Open squares, *TOC1*:*LUC*.

(C) Average traces for *ARR4*:*LUC* and *TOC1*:*LUC*, redrawn from **(A)** and **(B)** with adjusted amplitudes. Open squares, *TOC1*:*LUC*; closed circles, *ARR4*:*LUC*; hatched bars, subjective night.

elongation in constant white light and light/dark cycles of white light. We observed long hypocotyls in the mutants under light/dark cycles but not in constant light (Figure 8D). However, we note that hypocotyl lengthening is not as pronounced as in the photoreceptor null mutants *phyB-9* and *cry1-304*. Therefore, we conclude that ARR3 and ARR4 play a role in the control of hypocotyl length under light/dark cycles, possibly acting on phyB stability. However, their contribution in white light can only partially explain the long hypocotyl of *phyB* mutants.

Overexpression of ARRs Does Not Change Circadian Period

Overexpression of single genes is very often used to determine whether the activity of a gene is limiting. In the case of clock genes, overexpression of *CCA1*, *LHY*, *TOC1*, or *ZTL* leads to arrhythmicity (Schaffer et al., 1998; Wang and Tobin, 1998; Más et al., 2003a; Somers et al., 2004). Redundancy between *CCA1* and *LHY* is evident, as either single mutant only shows a short period but the *cca1 lhy* double mutant becomes arrhythmic when released into constant light (Alabadí et al., 2002; Mizoguchi et al., 2002). We entrained seedlings overexpressing ARR4, ARR5, ARR6, or ARR9 (driven from the strong constitutive cauliflower mosaic virus 35S promoter) to photocycles and measured cotyledon movement after transfer into constant light for 7 d. As shown in Figure 9, all overexpressing lines displayed a normal period length and circadian phase when assayed in white light. Although these results do not rule out a role for these ARRs in clock function, they do demonstrate that normal ARR activity is not limiting to clock function and that simple overexpression is insufficient to disrupt clock function. The amplitude of cotyledon movement was slightly affected during the first 3 d upon transfer into constant light, but this probably reflects the effect of ARR overexpression on cotyledon and petiole growth and not on the amplitude of the oscillator itself. Overexpression of phyB similarly causes a decreased amplitude in cotyledon movement, and petiole length in phyB-overexpressing plants is greatly shortened (data not shown) (Wester et al., 1994).

DISCUSSION

Light is one of the most potent environmental cues for the entrainment of circadian clocks. In *Arabidopsis*, phyB signaling is critical for the proper determination of circadian period (Somers et al., 1998) and phase (Salomé et al., 2002). Because the response regulator ARR4 interacts with phyB to positively modulate red light signaling (Sweere et al., 2001), we wished to determine whether ARR4 and other response regulators played a role in light signaling to the clock. Our results show that loss of ARR4 function is insufficient to impair clock function, probably because of the redundancy of ARR4 with ARR3. Indeed, the *arr3,4* double mutant is altered in its circadian rhythms. Two distinct phenotypes were observed in *arr3,4*, of which one may be attributed to an effect on phyB activity. *arr3,4* seedlings exhibit a long period in either red or blue light. By contrast, loss of *PHYB* lengthens the period of the clock under high fluence rates of red light but not blue light (Somers et al., 1998). In addition, the *arr3,4* long period is seen even in the absence of light, when phyB is not active. Therefore, we do not think that the circadian phenotype of long period of the *arr3,4* double mutant can be explained solely through interactions of ARR3 and ARR4 with phyB.

We hypothesize that the second circadian defect seen in *arr3,4* seedlings is related to decreased phyB activity. Loss of phyB function results in a leading phase in white light (Salomé et al., 2002). If ARR3 and ARR4 positively modulate phyB signaling to the clock, one would predict that the *arr3,4* mutant would have reduced phyB signaling, which would confer a leading phase. Indeed, when we recalculated the sidereal phases of *arr3,4* and *arr3,4,5,6* mutants in circadian time, which normalizes for the long period, we observed a leading phase relative to the wild type. Mutants with altered period length normally display a circadian phase defect: a long-period mutant will show a lagging phase, whereas a short-period mutant will exhibit a leading phase (Dunlap et al., 2004). Thus, the long period of *arr3,4* would be predicted to also confer a lagging phase when determined in sidereal time. If the leading phase resulting from reduced phyB

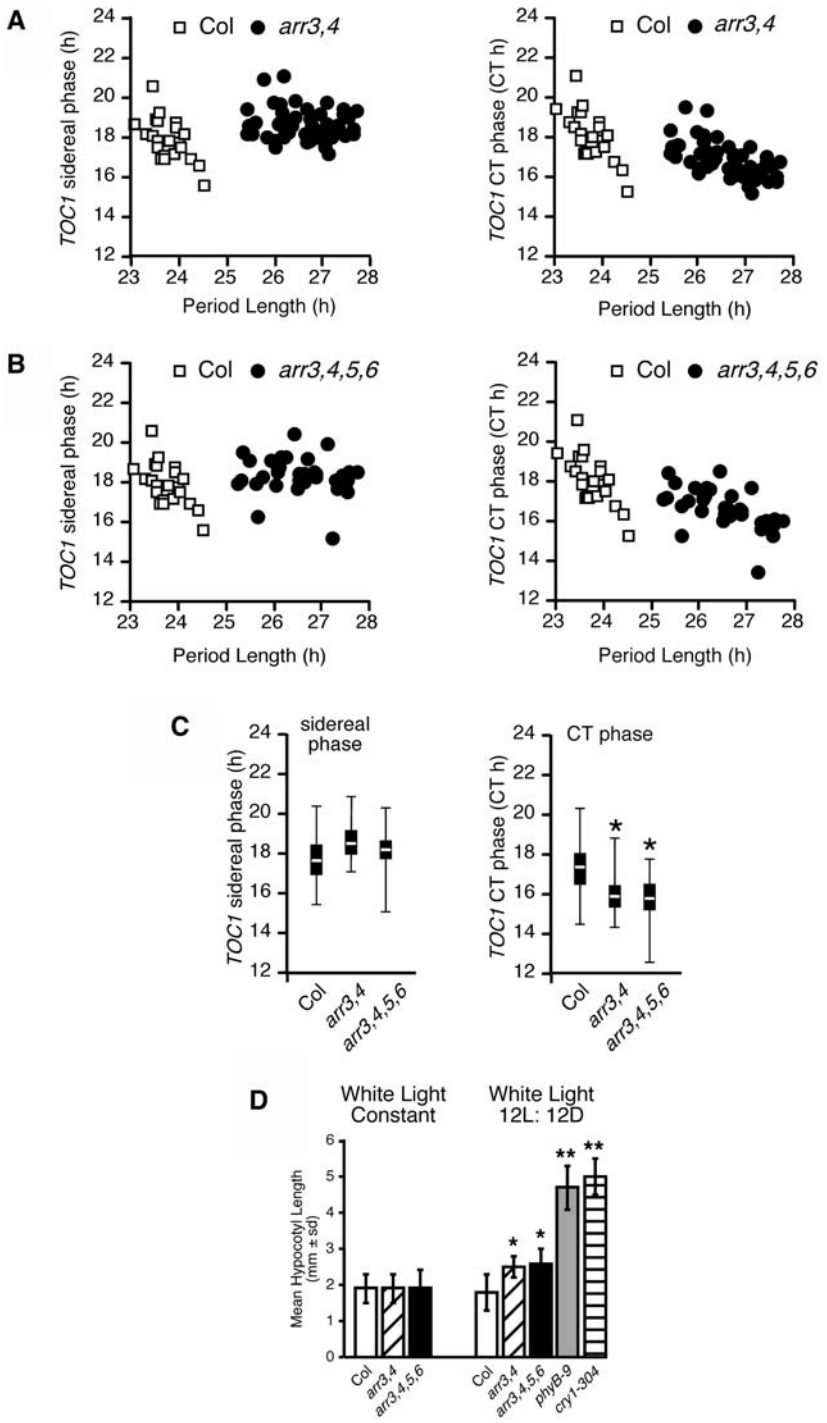

Figure 8. The *arr3,4* and *arr3,4,5,6* Mutants Share *phyB*-Like Phenotypes.

The period and phase values for Col, *arr3,4*, and *arr3,4,5,6* shown in Figure 2 are replotted in **(A)** and **(B)** as scatterplots of period against phase. Circadian time (CT) phase values were obtained by dividing sidereal phase values by the individual's period length and then multiplying the value by 24 [CT = (phase/period) × 24].

(A) Sidereal phase and CT phase values for Col and *arr3,4*.

(B) Sidereal phase and CT phase values for Col and *arr3,4,5,6*.

(C) Box plot graph of sidereal phase and CT phase for Col, *arr3,4*, and *arr3,4,5,6*. The black box portion of the plot includes 50% of the data, with the white line representing the median. The error bars extend to the minimum and maximum data values. The asterisks indicate significant differences from Col ($P < 0.001$ as determined by Student's heteroscedastic *t* test).

(D) Hypocotyl elongation of Col, *arr3,4*, and *arr3,4,5,6* in constant white light (left) or photocycles (12 h of light and 12 h of dark; right). The single asterisks indicate significant differences from Col ($P < 0.001$ as determined by Student's heteroscedastic *t* test). The double asterisks indicate significant differences from Col and *arr3,4,5,6*. Data shown are ±SD.

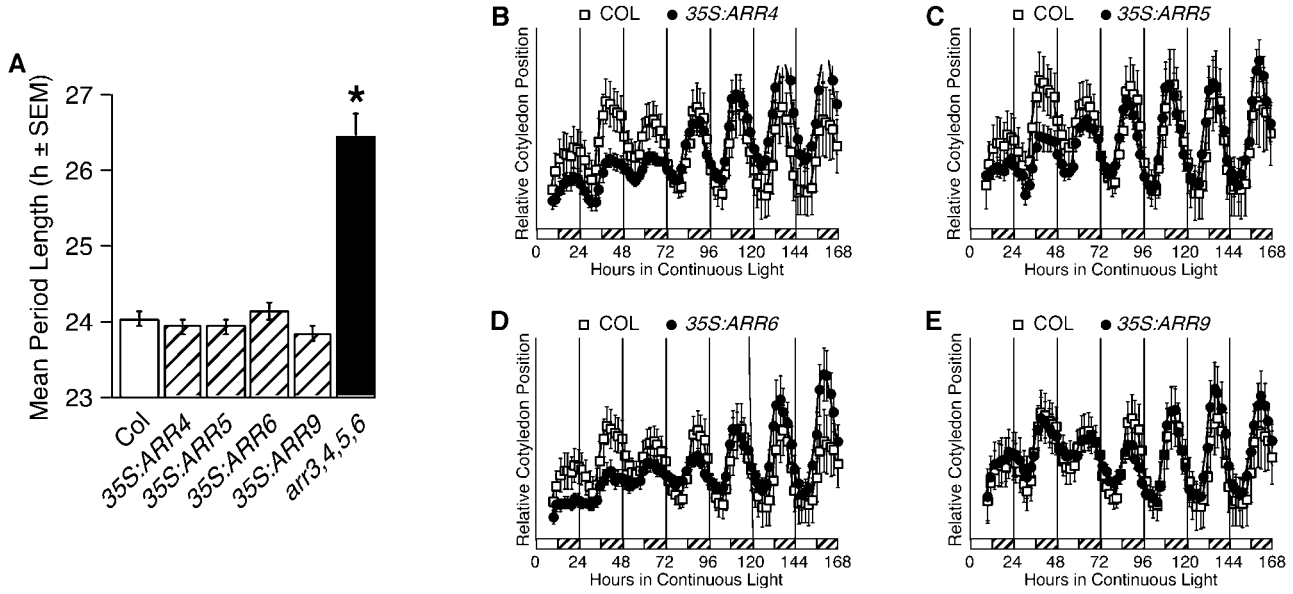

Figure 9. Overexpression of Type-A ARRs Does Not Affect the Clock.

All seedlings were grown as described for Figure 1. The data presented here represent averages ± SE of 30 to 48 individual cotyledons from one representative experiment, repeated twice with identical results. The asterisk indicates a significant difference from Col ($P < 0.001$ as determined by one-way ANOVA and Duncan's multiple comparison test).

(A) Mean period length in cotyledon movement in Col and seedlings overexpressing ARR4, ARR5, ARR6, or ARR9. The mean period for *arr3,4,5,6* is replotted here from Figure 1 for reference.

(B) to **(E)** Average cotyledon movement traces for *35S:ARR4* **(B)**, *35S:ARR5* **(C)**, *35S:ARR6* **(D)**, and *35S:ARR9* **(E)** after entrainment by light/dark cycles. Each trace represents the average from 30 to 48 individual cotyledons and is shown ±SE. Closed circles, mutant; open squares, Col; hatched bars, subjective night.

signaling and the lagging phase associated with the long period were quantitatively similar, although opposite in sign, the resulting phase would appear normal, consistent with the observed result.

Thus, we conclude that simultaneous loss of ARR3 and ARR4 results in two separable defects. First, their loss attenuates phyB signaling to the clock. Independently, loss of ARR3 and ARR4 results in a long period, although the mechanism by which this occurs is not yet known (Figure 10).

Both type-A and type-B ARRs are involved in cytokinin signaling. Does the role of ARR3 and ARR4 in modulating clock function suggest that cytokinin signaling itself might regulate clock function? Several lines of evidence suggest that this is not the case. First, there is a gradual increase in cytokinin sensitivity with the progressive loss of more ARRs seen from *arr3,4* through *arr3,4,5,6* to *arr3,4,5,6,8,9* mutants (Figures 1 to 4) (To et al., 2004). By contrast, the long period is similar in *arr3,4* and *arr3,4,5,6*, whereas the period is wild type in *arr3,4,5,6,8,9*, *arr5,6,8,9*, and *ahk3,4*. Clearly, the period phenotype does not correlate with cytokinin sensitivity. Similarly, the phase alterations in response to increasing exogenous cytokinin treatments are qualitatively different from the long-period phenotype seen in the cytokinin-hypersensitive mutants *arr3,4* and *arr3,4,5,6*. The long period of *arr3,4,5,6* is not rescued by the introduction of a genomic copy of ARR5 (Figure 5H), although ARR5 rescues the cytokinin sensitivity of root elongation in *arr3,4,5,6* (Figure 5H) (To et al., 2004). Moreover, the long period seen in these mutants is not exaggerated by cytokinin treatment, as period remains identical in treated and untreated seedlings, although circadian phase is delayed in the mutants to the same extent as seen in wild-type seedlings (Figure 5). Finally, we note that the hormone concentrations used here to produce an effect are quite high, which suggests that the lagging phase observed in the presence of exogenous hormone may not be physiologically relevant. Thus, we propose that the two type-A ARRs possess an as yet undescribed function that modulates the pace of the clock (Figure 10). This function is independent of cytokinin action, as exogenous applications of the hormone, albeit at high concentration, leads to a distinct clock response, that of lagging phase, in wild-type and mutant seedlings. Although *ARR3* and *ARR4* are expressed in most tissues of the plant, *ARR3* mRNA levels are much lower than those of *ARR4* (see Supplemental Figure 2 online). Nonetheless, *ARR3* fully compensates for the loss of *ARR4* for clock function; it is possible that *ARR3* expression increases in the *arr4* mutant, although this has not been tested. The triple cytokinin receptor mutant maintains a basal level of *ARR5*, although it is no longer induced by exogenous cytokinin (Higuchi et al., 2004). These findings, together with the lack of circadian defect upon overexpression of ARR4, suggest a model in which low, basal levels of ARR3 and ARR4 maintain proper circadian pace (Figure 10). Induction of ARR3 and ARR4 by cytokinin only increases their expression above basal levels but does not further affect the period of the oscillator.

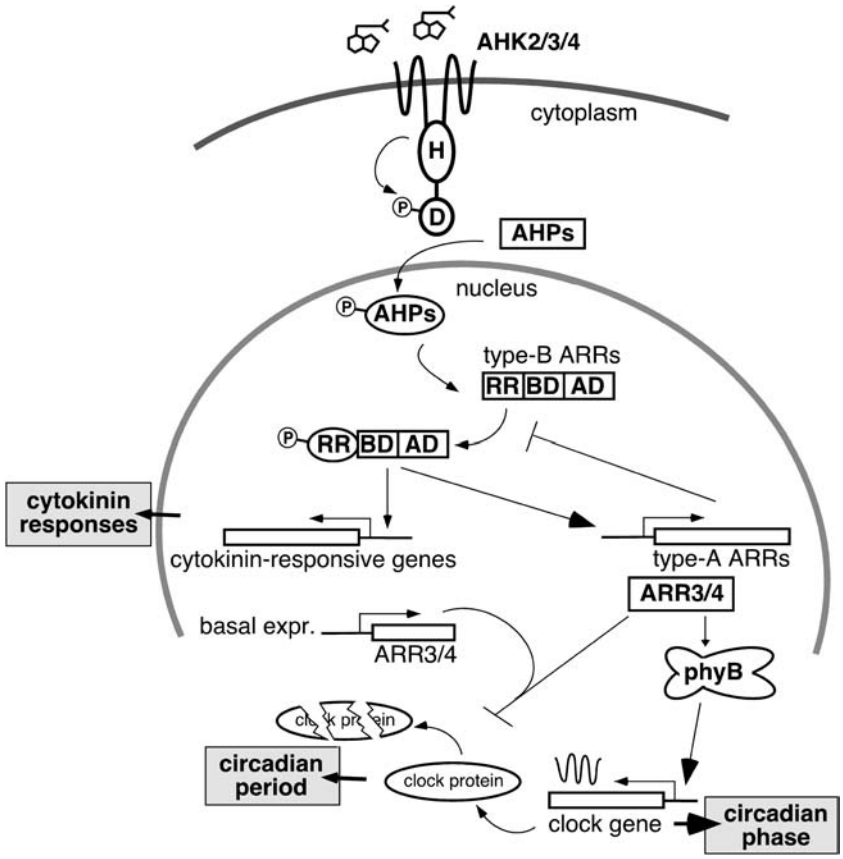

Figure 10. Tentative Model for ARR3 and ARR4 Function in and out of the Circadian Clock.

This model was adapted from Hwang and Sheen (2001). The cytokinin receptors AHK2, AHK3, and AHK4 perceive endogenous cytokinins and initiate a signal transduction cascade that leads to the phosphorylation and activation of type-B ARRs, which then induce the expression of primary cytokinin-responsive genes as well as of the type-A ARRs ARR3/4, ARR5/6, and ARR8/9. Type-A ARRs feed back to inhibit further type-B ARR activity. In addition, low, basal expression of ARR3 and ARR4 maintains the pace of the clock by acting on the expression, protein activity, and/or protein stability of a clock protein and modulates circadian phase by acting through phyB signaling into the clock. AHP, *Arabidopsis* histidine phosphotransmitter; RR, receiver domain of response regulator; BD, DNA binding domain; AD, transcription activation domain; P, phosphate.

Little is known of the mechanism of ARR function, especially with respect to roles in the clock. ARRs are evolutionarily related to the clock component TOC1, a member of the Pseudo Response Regulator family (Schaller et al., 2002). All five *PRR* genes play some role in the clock, because loss-of-function alleles in any affect the proper function of the circadian oscillator (Salomé and McClung, 2004). In contrast with type-B ARRs, which possess a DNA binding domain in their C terminus, type-A ARRs are not thought to directly regulate gene expression. Among type-A ARRs, only ARR3 and ARR4 show long, acidic, and Ser/Thr/Pro-rich C-terminal extensions. This C-terminal extension of ARR4 shows no obvious DNA binding motifs and is not able to replace the transactivation domain of GAL4 in yeast, suggesting that ARR4 lacks both DNA binding and transactivation functions (D'Agostino et al., 2000). Overexpression of ARR4 results in no circadian defects in white light, indicating that ARR4 activity is not limiting for proper clock function or, alternatively, that overexpression of both ARR3 and ARR4 may be required to change the pace of the clock. One possible explanation is that either protein alone is insufficient to mimic the activity of an ARR3–ARR4 complex. Similarly, the pseudoresponse regulators PRR7 and PRR9 are both important for clock function, and the *prr7,9* double mutant exhibits a very long period, yet overexpression of PRR9 alone only results in slight period shortening (Matsushika et al., 2002; Farré et al., 2005; Salomé and McClung, 2005a).

If ARR3 and ARR4 do not regulate the clock at the level of transcription, they may regulate abundance and/or activity of the clock proteins. Period lengthening may be accomplished through a delay in the degradation or inactivation of a positive component of the clock, such as TOC1. Such period lengthening is seen in lines carrying the *TOC1* minigene and in *ztl* mutants (Más et al., 2003a, 2003b). The two response regulators may be negative regulators of TOC1 and might modulate the rate of translation, activation, or degradation of TOC1. ARR3 and ARR4 could compete with TOC1 for interaction with ZTL and therefore indirectly increase TOC1 protein levels and lengthen period length. An alternative explanation is that ARR3 and ARR4 are negative regulators of negative components of the clock, such as

the transcription factors CCA1 and LHY: in the absence of ARR3 and ARR4, increased abundance of activity of CCA1/LHY could lead to a longer period, as the repression of *TOC1* expression would be maintained over a longer period of time, delaying the onset of the next cycle. Another attractive possibility calls upon the recently identified gene *LUX ARRHYTHMO*, a Myb-like transcription factor sharing high sequence similarity with the DNA binding domain of type-B ARRs (Hazen et al., 2005). LUX, like TOC1, is required for high expression of *CCA1* and *LHY*. Thus, ARR3 and ARR4 might be positive regulators of LUX. Unfortunately, none of these possible models readily explains why the long period is suppressed when loss of ARR3 and ARR4 is combined with loss of ARR8 and ARR9.

That the simultaneous loss of ARR8 and ARR9 does not lead to a circadian defect, yet this double mutant combination suppresses the circadian phenotypes of *arr3,4*, is intriguing but difficult to explain. To et al. (2004) similarly observed complex interactions among ARR3, ARR4, ARR8, and ARR9. Consistent with our observations, loss of ARR8 and ARR9 suppressed the increased red light sensitivity seen in *arr3,4*. Similarly, overexpression of ARR4 and ARR8 had opposite effects on cytokinin sensitivity, suggesting that ARR4 is a positive regulator and ARR8 is a negative regulator of cytokinin signaling (Osakabe et al., 2002). At this time, however, we lack sufficient knowledge of the mechanisms by which any ARR functions in the clock to incorporate these functions into a detailed model. Perhaps they impinge on a common target, having opposite effects.

The long-period phenotype of *arr3,4* seedlings is seen in all conditions tested (after light/dark and temperature cycles, in constant white, red, or blue light, and in the dark), indicating that ARR3 and ARR4 may target a protein that acts very close to, or even within, the clock itself. Two-hybrid screens have been performed with ARR4, but no known clock-related protein other than phyB was identified as a candidate interactor (Yamada et al., 1998). This leaves the exciting prospect of discovering a novel clock component on the basis of its interaction with ARR3 and ARR4.

METHODS

Plant Genotypes

All *Arabidopsis thaliana* genotypes (*arr3*, *arr4*, *arr3,4*, *arr3,4,5,6*, *arr8,9*, and *arr3,4,8,9*) were transformed with LUC constructs bearing translational fusions to the promoters of *CCA1*, *LHY*, and *TOC1* (Salomé and McClung, 2005a), and primary transformants were selected as described (Salomé and McClung, 2005a). Resistant seedlings were allowed to self, and T2 or T3 seeds were analyzed.

Cotyledon Movement and LUC Assays

All rhythm assays were performed as described (Salomé et al., 2002; Salomé and McClung, 2005a). For cotyledon movement, seedlings were entrained for 5 d in photocycles (12 h of light followed by 12 h of dark). For LUC activity measurements, seedlings were entrained for 10 d in photocycles or thermocycles (12 h at 22°C followed by 12 h at 12°C). All rhythms were analyzed by fast-Fourier transform nonlinear least-square technique (Plautz et al., 1997).

For hormone treatments, all seedlings were entrained for 10 d in photocycles in the absence of hormone and were transferred to 96-well plates containing each hormone (kinetin, *trans*-zeatin, or benzyladenine) or 0.01% DMSO as a control carrier for untreated seedlings in the case of *trans*-zeatin and benzyladenine. Seedlings were further entrained in photocycles for an additional 1 d before being released into constant light. The addition of DMSO had no effect on clock period or phase. Kinetin was solubilized in slightly acidic water.

Generation of Constructs and Transgenic Plants

The *ARR4* and *ARR9* promoters were amplified by PCR from genomic DNA and cloned into pCR8/GW/TOPO (Invitrogen). The *ARR4* promoter fusion includes 2824 bp of promoter sequence (from –2824 to the ATG), whereas the *ARR9* promoter fusion contains 2081 bp of promoter sequence (from –2081 to the ATG). The promoter was then recombined into the LUC vector pZPBAR-DONR as described (Salomé and McClung, 2005a). The resulting binary vectors were introduced into *Agrobacterium tumefaciens* strain ASE1. Ecotype Columbia plants were transformed as described (Salomé and McClung, 2005a). Primary transformants were selected on MS plates supplemented with 2% sucrose and containing 12.5 μg/mL BASTA and 500 μg/mL carbenicillin.

For ARR-overexpressing lines, full-length cDNAs of *ARR4*, *ARR5*, *ARR6*, and *ARR9* were amplified by PCR from a cDNA library made from wild-type Col light-grown seedlings, cloned into pENTR/D-TOPO vector (Invitrogen), and subcloned into the Gateway binary vector pGWB18 (Research Institute of Molecular Genetics) by LR recombination (Invitrogen). Each of the resulting constructs carried the constitutive cauliflower mosaic virus 35S promoter driving the expression of an *ARR* cDNA with a 4X myc tag on the N terminus. The constructs were introduced into Col by *Agrobacterium*-mediated transformation. Transformants were selected on MS medium supplemented with 50 μg/mL kanamycin. Transgene expression was confirmed in homozygous kanamycin-resistant T3 seedlings by protein gel blotting of whole seedling protein extracts and detecting with anti-c-myc antibody (Roche Applied Science). One line per construct with high levels of protein expression was selected for cotyledon movement assays.

Accession Numbers

Arabidopsis Genome Initiative locus identifiers for the genes mentioned in this study are as follows: *ARR3* (At1g59940), *ARR4* (At1g10470), *ARR5* (At3g48100), *ARR6* (At5g62920), *ARR8* (At2g41310), *ARR9* (At3g57040), phyB (At2g18790), *AHK3* (At1g27320), and *AHK4* (At2g01830).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Exogenous Cytokinin Treatments Decrease the Amplitude of *TOC1*:*LUC* Expression Only at High Levels.

Supplemental Figure 2. Virtual RNA Gel Blot of ARR3 and ARR4.

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