# Multiple phytohormones influence distinct parameters of the plant circadian clock

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**Circadian systems coordinate endogenous events with external signals. In mammals, hormone-clock feedbacks are a well-known integration system. Here, we investigated phytohormone effects on plant-circadian rhythms *via* the promoter:luciferase system. We report that many hormones control specific features of the plant-circadian system, and do so in distinct ways. In particular, cytokinins delay circadian phase, auxins regulate circadian amplitude and clock precision, and brassinosteroid and abscisic acid modulate circadian periodicity. We confirmed the pharmacology in hormone synthesis and perception mutants, as rhythmic expression is predictably altered in an array of hormone-related mutants. We genetically dissected one mechanism that integrates hormone signals into the clock, and showed that the hormone-activated ARABIDOPSIS RESPONSE REGULATOR 4 and the photoreceptor phytochrome B are elements in the input of the cytokinin signal to circadian phase. Furthermore, molecular-expression targets of this signal were found. Collectively, we found that plants have multiple input/output feedbacks, implying that many hormones can function on the circadian system to adjust the clock to external signals to properly maintain the clock system.**

## Introduction

Many biological processes are regulated in a rhythmic manner with a periodicity that matches the daily cycle. The circadian systems that generate these approximately 24-h rhythms exist in many organisms, both prokaryote and eukaryote, ranging from cyanobacteria to mammalian (Reppert & Weaver 2002; Eriksson & Millar 2003). Like many organisms, plants sense various environmental conditions, such as light and temperature, and integrate this information with their circadian clock to measure day length and seasonal change (Eriksson & Millar 2003; Mizuno 2004). This integration can increase fitness advantage for organisms presumably by appropriately adjusting endogenous metabolism to the ambient light-dark cycle. To synchronize the circadian system with external cues, intrinsic signaling pathways might converge with the circadian clock.

In the model plant *Arabidopsis thaliana* (Arabidopsis), several clock genes have been isolated and analyzed *via* molecular-genetic approaches (Eriksson & Millar 2003). These studies led to the first proposed mechanisms for the molecular basis for daily time keeping in plants. In this model, two morning-expressed Myb-transcription factors, *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*) and *LATE ELONGATED HYPOCOTYL* (*LHY*), and an evening-expressed pseudoresponse regulator *TIMING OF CAB2 EXPRESSION 1* (*TOC1*) are believed to be positive/negative feedback components of the circadian oscillator (Eriksson & Millar 2003). More recently, mathematical approaches extended this model to interlocked feedback loops, and the additional clock components *GIGANTEA* (*GI*) was a proposed candidate for this interconnection (Locke *et al.* 2005). A molecular model is thus emerging regarding the components of the time-keeping pacemaker. It is less clear how this timer is modulated by external cues.

Various exogenous growth conditions, such as varying light, temperature, abiotic stress and disease, regulate a set of endogenous plant hormones. In turn, these hormones regulate an extensive array of physiological processes to ensure maximal fitness over the whole plant life cycle, including the response to varying environmental conditions (Srivastava 2002). These compounds have been collectively termed the phytohormones and include cytokinin, auxin, brassinosteroid (BR), abscisic acid (ABA),

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gibberellin (GA), ethylene and salicylic acid (SA) (for a review see Srivastava 2002; Bishop & Koncz 2002; Fedoroff 2002; Guo & Ecker 2004; Martínez *et al.* 2004). A single phytohormone can act on a diverse range of biological processes. Moreover, several phytohormones often function with interactive effects. For example, alternation of light signals change the phytohormone levels of cytokinin, auxin, BR, ABA and GA. These changes lead to an altered choice in various developmental decisions. This regulation level functions from seed germination to flowering reproduction. The general notion is that phytohormones are essential for plant to sense their exogenous and endogenous conditions.

As described above, both phytohormones and the clock play important roles in integrating environmental signals and in regulating plant development and metabolism. Reports on connections between these two systems has been limited. For example, it has been reported that the clock modulates ethylene synthesis, and auxin trafficking and responsiveness (Jouve *et al.* 1999; Thain *et al.* 2004). Recently, the phytohormones cytokinin, auxin and ABA have been shown to accumulate in diurnal patterns (Nováková *et al.* 2005). Thus, aspects of phytohormone biology are under clock control. However, systematic descriptions regarding the integration of a hormonal signal into the plant-circadian system have been lacking.

Here, we investigated whether there is a hormone-clock connection in plants, as seen in the pineal hormone melatonin-clock feedback in mammals (Reppert & Weaver 2002). This is noteworthy as phytohormone signaling and the plant circadian system are generally described as distinct pathways (Blázquez *et al.* 2002). In this report, we report that a variety of phytohormones regulate distinct rhythmic parameters of the clock. These defined parameters are periodicity, phase, amplitude and clock precision (Supplementary Fig. S1 provides a brief introduction into the nature of these parameters). We further derive a molecular-genetic model for cytokinin modulation of circadian phase. Collectively, we suggest that daily physiologic responses in plants are balanced with multiple feedbacks contributed to in part by the phytohormones. In contrast to the melatonin-circadian connection present in animal systems, plants have a circadian system unexpectedly coordinated by multiple hormonal feedbacks.

## Results

### Phytohormones affect various rhythmic parameters

We directly investigated whether phytohormones regulate the circadian clock in Arabiodopsis. Classical phytohormones were exogenously added in separate experiments to unravel whether any of these compounds influence circadian rhythms, as assayed via the promoter: *luciferase* (*LUC*) system (Eriksson *et al.* 2003; Hall *et al.* 2003). Seedlings harboring a promoter:*LUC* marker were entrained under 12-h light/12-h dark cycles, and then transferred into imaging plates containing growth medium and the test phytohormone. We followed the circadian rhythms of transcription rates from the well-characterized marker genes *CHLOROPHYLL A/B-BINDING PROTEIN* (*CAB2*, also termed *LHCB1**1*) and *COLD- AND CIRCADIAN-REGULATED 2* (*CCR2*, also termed *AtGRP7*) and the putative core-oscillator gene *CCA1*. These experiments were typically under free-running constant-light conditions (LL) or in constant darkness (DD) (Fig. 1, Supplementary Figs S2–S5) (Eriksson *et al.* 2003; Hall *et al.* 2003). Phytohormone effects on the *CCR2* rhythm under free-running constant light conditions (LL) are illustrated in Fig. 1; constant darkness (DD) and the *CCA1* and *CAB* rhythms are shown in supporting information (Fig. S2–S5).

Bioluminescence rhythms were mathematically surveyed to evaluate the circadian parameters of periodicity, phase, amplitude and precision (Supplementary Fig. S1 schematically illustrates these clock parameters) (Southern & Millar 2005). "Period" was defined as the time required to complete one rhythm cycle (Supplementary Fig. S1B). "Phase" referred to the given point in the cycle where rhythmic features peaked or troughed (indicated at star positions in Supplementary Fig. S1A,C). We typically used peak position as the given phase value. Phase values in reference to circadian period were indicated as the phase of Circadian Time (CT). "Amplitude" was defined as the absolute change, maxima to minima, in the activity rhythm during the cycle. Circadian rhythms have a certain "precision" (Doyle *et al.* 2002), and we defined this trait here as the error evident in a lack of robustness in curve fit. We could find a breakdown in precision being either correlated with a reduction in amplitude (pink line in Supplementary Fig. S1E) or associated with wavering periodicity and a seemingly randomized phase (blue line in Supplementary Fig. S1E). Mathematically, either effect results in an increase in the relative amplitude error (R.A.E). This R.A.E. is the error of a theoretical fit to actual data (a perfect fit, a precise rhythm, was data that absolutely matched a predicted cosine curve: R.A.E. = 0). Interestingly, many hormonally induced changes on the circadian system were observed. Cytokinins delayed circadian phase (Fig. 1A,B), auxins regulated clock precision (Fig 1C,D) and brassinosteroid and abscisic acid modulated circadian periodicity (Fig. 1E,F). We summarize below the identified roles of phytohormones in the circadian system.

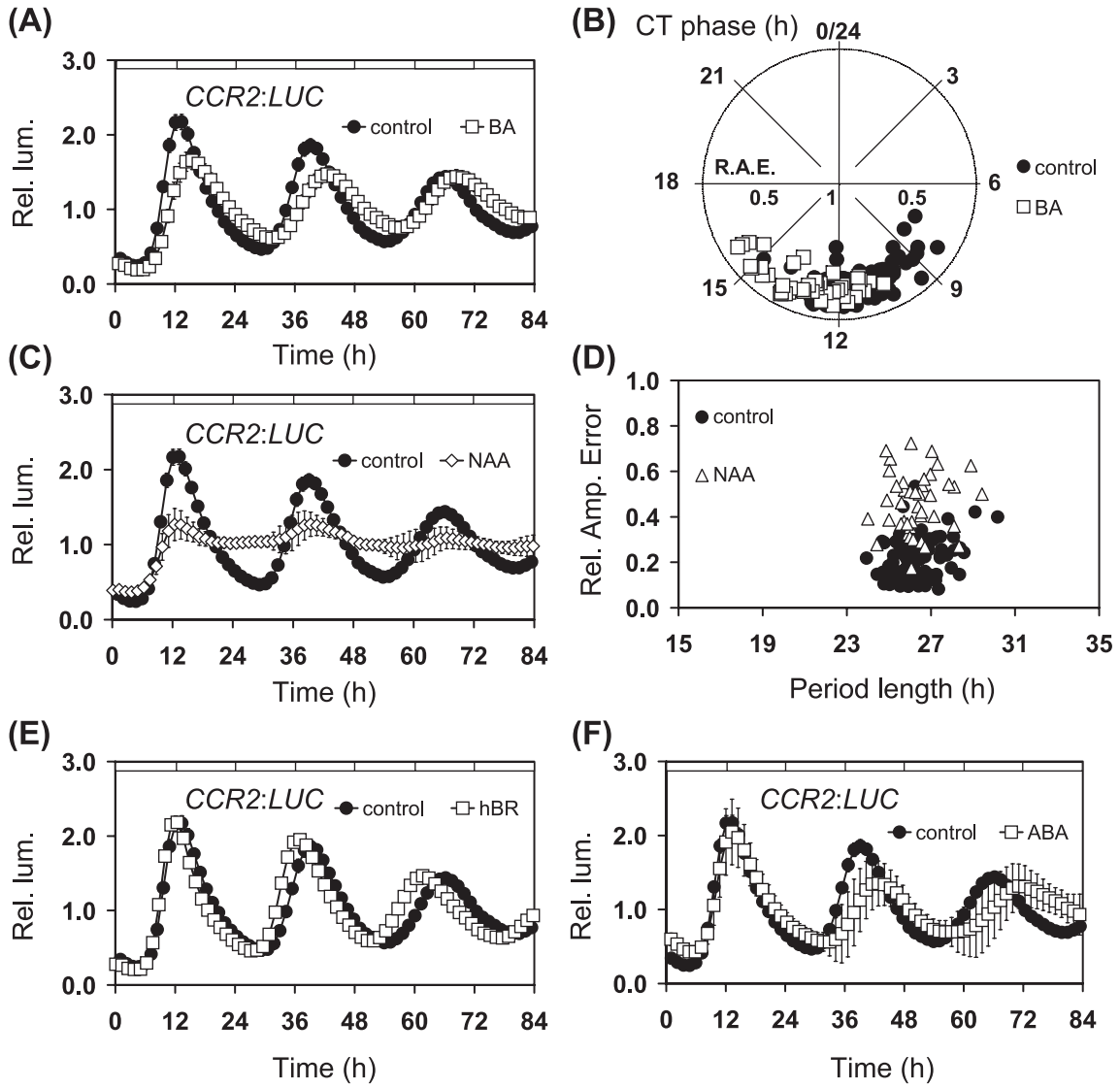


**Figure 1** Phytohormones affect various aspects of the circadian system. Seedlings harboring the *CCR2:LUC* reporter gene were transferred into microtiter plates containing 20 μM of the phytohormone indicated. Filled circles, control (0.01% DMSO). (A,B) Cytokinin application. Open squares, BA. (C,D) Auxin application. Open triangles, NAA. (E) Brassinosteroid application. Open squares, hBR. (F) ABA application. Open squares, ABA. The luminescence [counts per second (cps)] was monitored in an imaging system under LL. (A,C,E,F) The data shown represent normalized luminescence (Rel. lum.). The error bars indicate the S.E.M., which is mean of standard error from three experiments, each of which contained more than 36 measurements. (B,D) Mathematical analysis of experiments represented in (A) and (C), respectively. The circadian parameters are calculated from the rhythm traces from between 24 and 90 individual seedlings. (B) The phase angles normalized to a 24-h cycles (CT phase) are plotted with relative amplitude errors (R.A.E.), which indicate the robustness of the rhythms. The lower R.A.E. indicates the evidence of more robust rhythm. The center of circle is high R.A.E. (= 1). (D) Period length from individual seedlings is plotted against R.A.E.

Cytokinin has a defined role in regulating developmental events in plant growth. We tested whether cytokinins modulate clock parameters (Fig. 1A,B; Supplementary Fig. S2). In our observations, the cytokinins 6-benylaminopurine (BA), and trans-zeatin delayed by 0.8–3 h the circadian phase of marker expressions both under LL and in DD (Fig. 1A,B; Supplementary Fig. S2A–F). The effects were more subtle under LL than that seen in DD (e.g. for *CCR2* rhythm with t-zeatin, 01.54 h in DD *vs* 00.86 h under LL). Cytokinin shifted

both the phase of the first peak of *CCR2* and *CAB2* rhythms and the second peak of *CCA1* in DD (Supplementary Fig. S2B,D,E). The amplitude of gene expression was also altered. Strikingly, overt *CAB2* rhythms were observed in plants in DD that had been exposed to exogenous cytokinin, whereas the *CAB2* rhythm in wild-type plants normally damps in DD; R.A.E = 0.36 ± 0.05 with t-zeatin treatment, whereas R.A.E = 0.64 ± 0.03 without this cytokinin. The variance on periodicity of *CAB2* in DD was significantly smaller with cytokinin treatment than without it, as demonstrated by *F* statistic ($P < 0.001$) (Fig. 5A,B; Supplementary Fig. S2F) (Millar *et al*. 1995). Cytokinin also had subtle effects on periodicity of *CCR2* in DD (*CCR2* in DD: control = 27.44 ± 00.09 h; t-zeatin = 26.77 ± 00.18 h) (Fig. 2). Complicated dose–responses of periodicity in response to cytokinin application were observed (Supplementary Fig. S6). Collectively, cytokinin can have tri-functional effects on the circadian system: to shorten periodicity, to delay phase and to support rhythmicity in the dark.

Auxin is a phytohormone that often antagonizes cytokinin function. We thus tested whether auxin also interacts within the circadian system (Fig. 1C,D; Supplementary Fig. S3). The auxins 2,4-dichlorophenoxyacetic acid (2,4-D), and 1-naphthaleneacetic acid (NAA) were found to regulate clock precision under LL (Fig. 1C,D; Supplementary Fig. S3A–D). The effects were observed just before the peak of the *CCR2* rhythm. Mathematical analysis confirmed that rhythms after auxin treatment were significantly less robust than that seen in the control: the R.A.E. was 0.37 ± 0.01 for *CCR2* rhythm with NAA under LL, whereas it was 0.22 ± 0.01 in the control ($P < 0.001$). The NAA effect depended on the ambient light condition, and was not strong in DD; the R.A.E. was 0.20 ± 0.01 with NAA in DD, whereas it was 0.13 ± 0.01 in control ($P < 0.001$) (Supplementary Fig. S3E–H). Auxin is therefore important for the maintenance and precision of circadian rhythms, especially under LL.

Brassinosteroids (BRs) are known to regulate a diverse array of photomorphogenic behaviors. We investigated whether BRs affect the circadian system (Fig. 1E; Supplementary Fig. S4A–F). As a result of BR homobrassinolide (hBR) application, circadian periodicity was shortened for *CCR2*, *CAB2* and *CCA1* (1.0–2.7 h) rhythms under both LL and in DD (Fig. 2). The effects of hBR appeared stronger in DD than under LL (e.g. for *CCR2* rhythm with hBR, 01.70 h shortened in DD *vs* 01.37 h under LL). Furthermore, as was seen with cytokinin application, robust *CAB2* rhythms were observed in DD in BR-treated plants (Supplementary Fig. S4E,F); R.A.E = 0.27 ± 0.02 with hBR treatment, but R.A.E = 0.58 ± 0.04 without BR application. The variance on periodicity of *CAB2* in DD was significantly lower after BR treatment than without it, as demonstrated by an *F* statistic ($P < 0.001$). Thus, BRs play an important role in promoting periodicity.

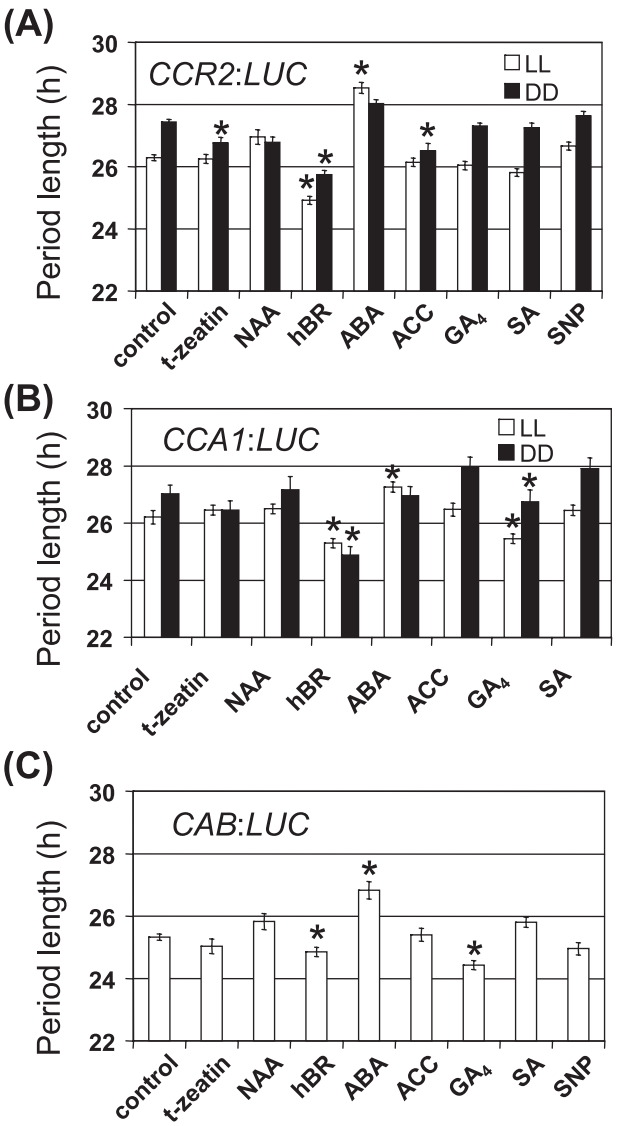


**Figure 2** Various phytohormones affect period length. Mathematical analyses of experiments represented in Fig. 1 and Supplemental Figs S1–S4 were carried out. (A) Period length of *CCR2* rhythms with each hormone indicated under LL and in DD. (B) Period length of *CCA1* rhythms under LL and in DD. (C) Period length of *CAB* rhythms under LL. In DD, the *CAB* rhythms were damped. The error bars indicate the S.E.M. ⋆$P < 0.005$.

ABA controls a diverse range of biological processes from seed dormancy to senescence, and often is a responder

to various stresses such as drought, salinity, cold and biotic stress. We therefore wondered if ABA would also integrate external signals to the circadian system (Fig. 1F; Supplementary Fig. S5). With regard to the clock, ABA application lengthened circadian periodicity under LL (e.g. the *CCR2* rhythm was a 2.2 h longer period than the control) (Fig. 2). Though, the ABA effect on periodicity was not significant in DD; $P = 0.03$ and 0.1 for *CCR2* and *CCA1* in DD, respectively (Fig. 2). Thus, ABA lengthens the circadian periodicity, and the effect appears to be light dependent.

Gibberellins (GA) and ethylene are often known as a component of light signals. We therefore test applied GA and ethylene to the circadian system. Gibberellin $A_4$ ($GA_4$) did not noticeably affect *CCR2* expression rhythm; however, $GA_4$ shortened the period length and increased the amplitude of *CAB* and *CCA1* rhythms (Fig. 2). The effect of $GA_4$ is subtle and depends on the output measured. When we applied the ethylene-synthesis precursor, 1-aminocyclopropane-1-carboxylicacid (ACC) and looked for responses on the circadian system, we found modestly shortened periodicity (0.9 h) of *CCR2* rhythm, but only in DD (Fig. 2). Furthermore, we found no significant effects on the other markers measured. Thus, GA and ethylene had subtle effects on the circadian system, but the effects depend on the output measured.

The circadian system influences photoperiodic flowering induction (Hayama & Coupland 2004). Recently, the phytohormones salicylic acid (SA) and nitric oxide (NO) were reported to modulate this transition from vegetate stage to reproduction (Martínez *et al.* 2004; He *et al.* 2004). This knowledge led us to test whether these compounds also modulate circadian parameters. We tested circadian effects in response to treatment with either SA or sodium nitroprusside (SNP), a donor for NO. These compounds in our experiments did not significantly influence circadian parameters (Fig. 2). We conclude that SA and NO are not phytohormones in the tuning of the circadian system.

Auxin affects circadian precision, as described above. Further, auxin is structurally similar to the pineal hormone melatonin, as both are indolamines (Kolár & Machácková 2005). To exclude the possibility that auxin structurally mimics a melatonin-like reaction, we also tested the consequence of melatonin addition on the plant-circadian system. Here we found no significant plant responses for any circadian parameters (data not shown). This confirms other's findings that melatonin is not a circadian-acting hormone in plants (Kolár & Machácková 2005).

In summary, the phytohormones described here pharmacologically affect the circadian system in distinct ways and change the rhythmic expression of marker genes, including one of the core oscillator genes *CCA1*.

### Many phytohormone mutants exhibit aberrant clock phenotype

To confirm the above hormone pharmacology, we assayed rhythmic expression in hormone synthesis and perception mutants that harbor an introduced *CCR2:LUC* reporter; tested lines included *cytokinin hypersensitive* (*ckh1*), *constitutive and photomorphogenesis and dwarfism* (*cpd*), and *ABA-deficient 2* (*aba2*) (Fig. 3) (Kubo & Kakimoto 2000; Bishop & Koncz 2002; Fedoroff 2002). The *ckh1* mutant exhibited a short-period phenotype in DD (Fig. 3A). The *cpd* mutation displayed a 3 h long-period phenotype under LL (Fig. 3A). The *aba2* mutant exhibited the predicted shortening-period phenotype in DD (01.55 h in DD) (Fig. 3B).

Pharmacologic effects of ethylene were subtle, as described above. However, the ethylene signal is reported to converse with a cytokinin signal *via ARR2* (Hass *et al.* 2004). Thus, we also assayed *CCR2* rhythms in the ethylene mutants *ethylene receptor 1* (*etr1*), *ethylene insensitive 4* (*ein4*), and *ethylene insensitive 5* (*ein5*) (Guo & Ecker 2004). These ethylene mutants had altered circadian phase or periodicity, depending on the light conditions (Fig. 3B,C). *etr1* had a shortened-period (2 h) and a late-phase phenotype in DD (1.5 h), and *ein4* had a lengthened-period phenotype under LL (1.5 h). *ein5* exhibited a late-phase phenotype in DD (2.5 h) and had a long-period phenotype under LL (2 h).

Collectively, we found that many of the hormone mutants tested exhibited predictable clock phenotypes. A variety of ethylene mutants also exhibited conditional phenotypes in a light-dependent manner, and these phenotypes simulated cytokinin changes. We confirmed the pharmacology with these genetic data that phytohormone signals indeed modulate various parameters of the circadian clock.

### Integration of a hormone cytokinin to the clock

To genetically define a mechanism for one of the hormone-clock integrations, we focused on the cytokinin signal. In plants, light inputs shorten period length and mutations in photoreceptors alter circadian rhythms (Millar *et al.* 1995; Somers *et al.* 1998; Salomé *et al.* 2002). Recently, the cytokinin-activated ARABIDOPSIS RESPONSE REGULATOR 4 (ARR4) has been reported to modulate phytochrome B (phyB)-mediated red-light signaling (Sweere *et al.* 2001; To *et al.* 2004) (Fig. 7B). The *phyB* mutant has a short-period phenotype under LL

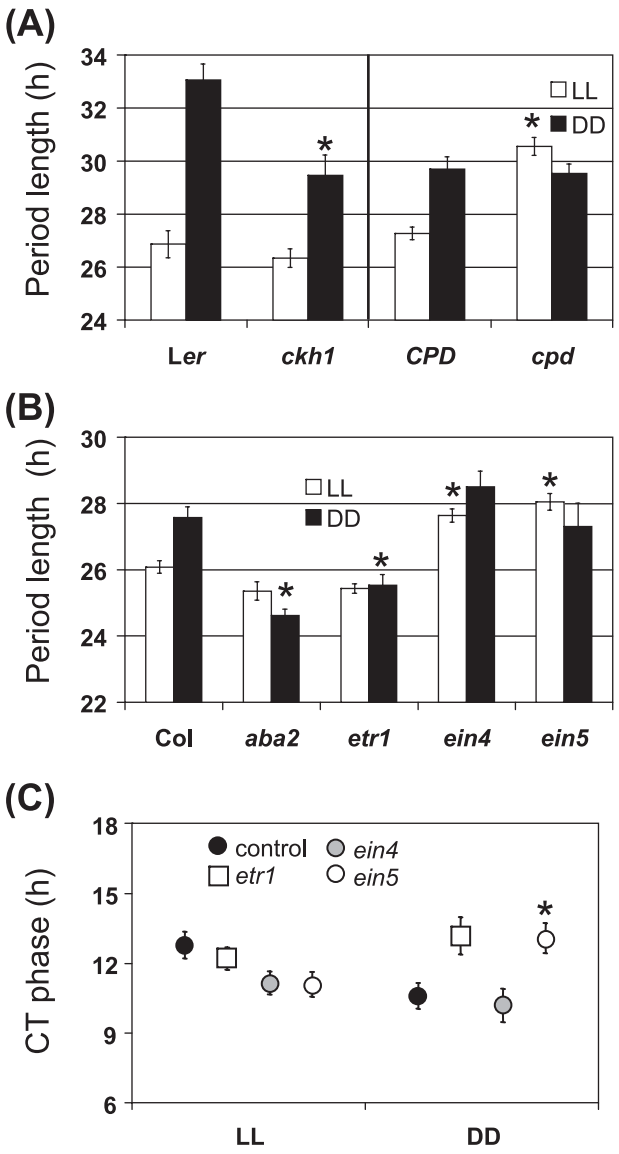


**Figure 3** *CCR2:LUC* rhythms in phytohormone mutants. Phytohormone mutants harboring *CCR2:LUC* were transferred to LL or DD, and assayed. The period length and CT phase was estimated from 24 to 48 plants. (A) Period length of *CCR2* rhythms in *ckh1* mutants [Landsberg *erecta* (L*er*) background] and *cpd* (originally L*er* background crossed to Ws *CCR2:LUC* line 3 times; segregated wild-type plant *CPD* was indicated as a background) under LL and in DD. (B) Period length of *CCR2* rhythms in *aba2*, *etr1*, *ein4* and *ein5* mutants (Columbia background) under LL and in DD. (C) CT phase of *CCR2* rhythms in *ethylene* mutants under LL and in DD. ● control; □ *etr1*; ◍ *ein4*; ○ *ein5*. The error bars indicate the S.E.M. ⋆$P < 0.005$. Note that period phenotype affects the CT phase. Thus, the phase phenotypes of *ein4* and *ein5* under LL and of *etr1* in DD ($P < 0.05$) represented the period changes.

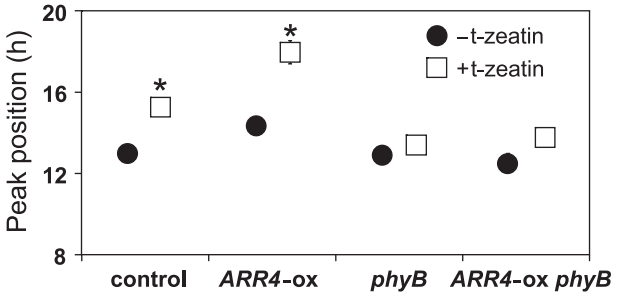


**Figure 4** Cytokinin affects phase peak positions through ARR4 and phyB under constant red light (RR). phyB, ARR4-ox, and ARR4-ox phyB plants harboring *CCR2:LUC* grown on LD cycles were transferred to constant red light (RR) and assayed on the plates with or without cytokinin t-zeatin (Supplementary Fig. S5). The first peak positions were collected from control (Ws), *ARR4*-ox, *phyB*, and *ARR4*-ox *phyB*. ● without cytokinin treatment; □ with cytokinin. The error bars indicate the S.E.M. ⋆$P < 0.005$ for the comparisons of with and without treatment.

and in DD, a long-period phenotype under continuous red (RR), and an out of phase phenotype with the rhythms of leaf movement (Somers *et al.* 1998; Salomé *et al.* 2002). Therefore, if *ARR4* and *phyB* contribute to the hormonal integration within the circadian system, the clock sensitivity to cytokinin should be altered in *ARR4* over-producing plants (*ARR4*-ox) and in *phyB* mutants. To test our hypothesis, we generated *ARR4*-ox lines, harboring the *CCR2:LUC* transgene and assayed cytokinin responses of clock parameters under constant red light (RR) (Fig. 4, Supplementary Fig. S7). Before cytokinin application, we found no strong differences between control and *ARR4*-ox lines with regard to phase positions. After cytokinin application, the *ARR4*-ox plants exhibited an ~4 h delayed-peak phenotype under RR, whereas wild-type delayed the peak by only 2 h (Fig. 4C). Thus, *ARR4*-ox lines are hypersensitive to a cytokinin input towards circadian phase. We also investigated cytokinin effects on *CCR2* and *CAB* rhythms in the *phyB* mutant (Figs 4 and 5). No significant change was detected in the peak position of the *CCR2* rhythm in the *phyB* mutant after cytokinin application (Fig. 4). Furthermore, *phyB* failed to recover clock precision of the *CAB* rhythm in DD after cytokinin application (Fig. 5A,C); R.A.E. = 0.36 ± 0.05 in control with t-zeatin treatment, whereas R.A.E. = 0.67 ± 0.05 in *phyB* with cytokinin treatment: the variance of period in *phyB* was not significantly different upon cytokinin treatment, as demonstrated by *F* statistic ($P = 0.37$; $P < 0.001$ in wild-type). Thus, the *phyB* mutant is resistant to cytokinin inputs. Furthermore, we assayed *CCR2* rhythms in plants altered for both *ARR4*-ox and *phyB*.

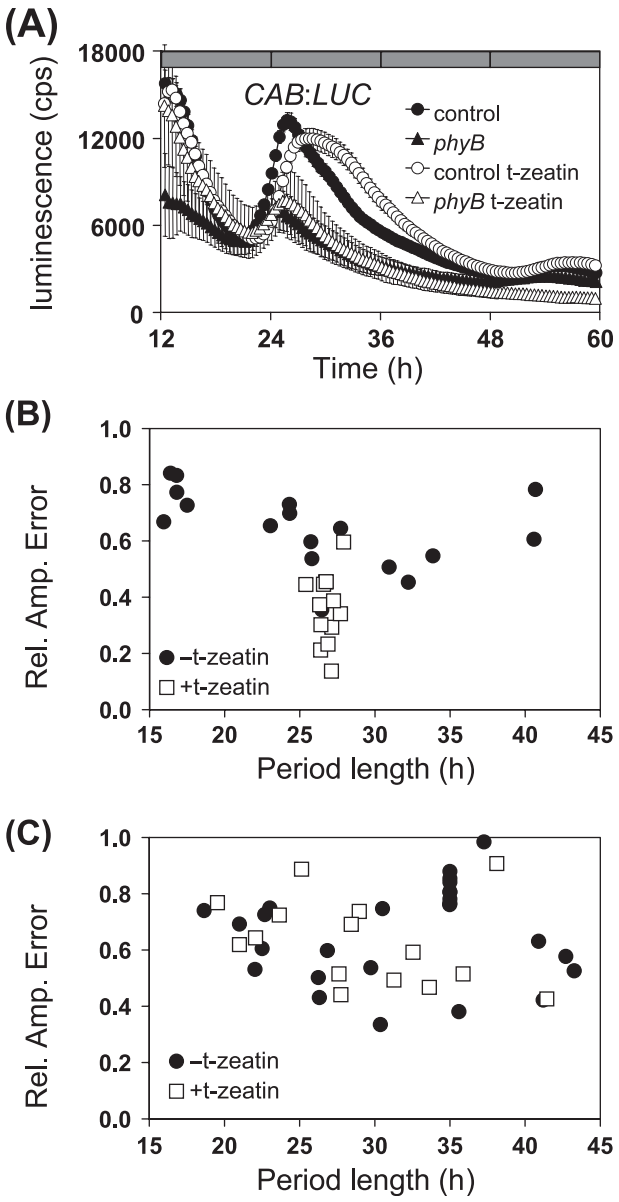


**Figure 5** *phyB* lack the ability to recover robust *CAB* rhythm in constant darkness (DD). (A) *CAB* rhythms were measured in DD with or without cytokinin t-zeatin in wild-type (Ws) plants and *phyB* mutant. (B) Circadian precision of *CAB* rhythm with cytokinin application in wild-type plants. Circadian parameters of *CAB:LUC* were calculated from the data (A). Relative amplitude error (R.A.E.) was plotted against period length from more than 24 seedlings. Some of the plots are out of range, indicating no detectable rhythmicity. As *CAB* rhythms normally damps in DD, this data set cannot be "fit" to a cosine curve. Therefore, high R.A.E. is represented on the data from wild type. However, the data on cytokinin t-zeatin treatment represent lower R.A.E. with the condensed period length, suggesting increased levels of cytokinin produce robust rhythms. (C) Circadian precision of *CAB* rhythm with cytokinin application in *phyB*. Period lengths are plotted against R.A.E. from individual seedlings. A total of 72 *phyB* mutants were analyzed. Rhythmicity in wild-type was recovered after the cytokinin application (R.A.E. ≤ 0.6), whereas for this circadian parameter, most of the *phyB* mutants were out of the range of detection (90% ≥ 0.6, 70% > 1.0). This indicated that phyB was arrhythmic in DD, with or without cytokinin.

This double "mutant" had a phenotype similar to the *phyB* single mutant (Fig. 4; Supplementary Fig. S7). Mathematical analyses confirmed that, in addition to peak-position changes, the CT phase delay was cytokinin dependent: the CT phase delays were 01.43 h in control, 02.35 h in *ARR4*-ox, and were not significant in *phyB* or *ARR4*-ox *phyB*. *phyB* is thus epistatic to *ARR4*-mediated cytokinin input to circadian phase. With regard to the phase of circadian rhythm, it is clear that *ARR4*-ox exhibited a cytokinin hypersensitive phenotype and the *phyB* mutation eliminated this sensitivity.

To dissect the molecular link between hormonal signal and the circadian system, we analyzed transient induction of expression of the clock-associated genes *CCA1*, *TOC1* and *GI*. Wild-type plants were treated with the indicated phytohormones for 1 h during either the morning or the evening, and these samples were then harvested for RNA isolation and expression analyses. In these analyses, phytohormone applications resulted in significant expression changes (Fig. 6). Cytokinin t-zeatin application induced *TOC1* and *GI* in the morning, and *CCA1* in the evening. After auxin NAA application, *CCA1* were repressed in the morning. ABA reduced *CCA1* gene expression. Thus, phytohormones rapidly lead to a change in clock gene expression, and altered the balance of clock components.

## Discussion

We provide here evidence of a reciprocal interaction between hormone signaling and the circadian clock: hormone synthesis is circadian-regulated (Jouve *et al.* 1999; Thain *et al.* 2004; Nováková *et al.* 2005), and many phytohormones control various clock parameters (Fig. 7A). Circadian and/or diurnal changes of the phytohormone cytokinin, auxin, ABA, and ethylene, have been previously reported (Thain *et al.* 2004; Nováková *et al.* 2005). In direct hormone measurements, it was reported that cytokinin and auxin accumulation and ethylene release oscillate with a peak in the middle of day, and that ABA increases after dusk (Nováková *et al.* 2005). Here we found that BR and ABA function specifically on periodicity, that cytokinin acts modestly on periodicity and strongly on phase, and that the action of auxin controls amplitude and clock-precision (Fig. 7A).

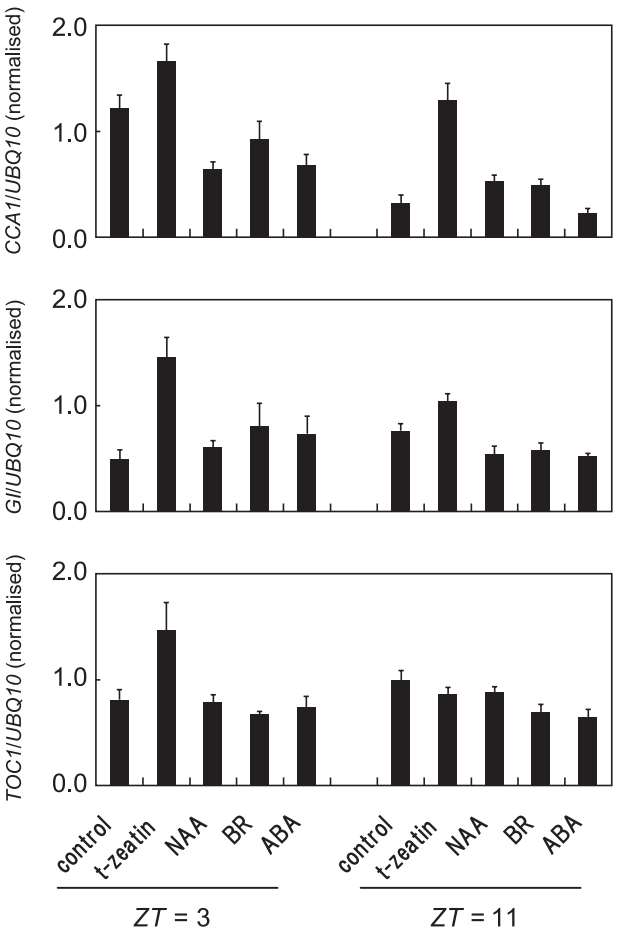


**Figure 6** Phytohormones regulate mRNA accumulation of clock component genes. Seedlings treated with phytohormones for 1-h at specific phases of the day were harvested. Total RNA was the substrate for RT-PCR against the coding regions of the core-clock genes *CCA1*, *GI*, and *TOC1*, and as a control, *UBQ10*. Results are presented as proportion against the medium value after normalization with respect to poly ubiqitin (*UBQ10*).

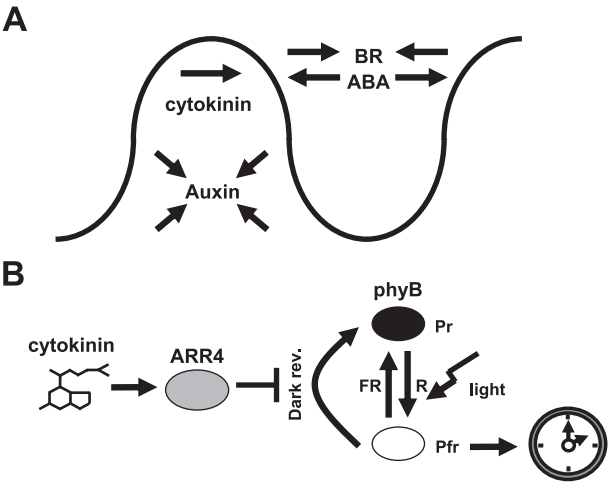


**Figure 7** Hormone-input models. (A) Summary for phytohormone effects on the circadian system. Cytokinin delays phase, auxin maintains precision and regulates amplitude, BR promotes periodicity, and ABA represses periodicity. (B) A model for the integration of the cytokinin signal to the clock. Cytokinin activates *ARR4*. When ARR4 is over-expressed, dark-conversion of phyB *Pfr*-active form is inhibited and the *Pfr*-active form extends the time light signal enter the clock.

Thus, we closed the hormone-clock interconnectivity loop in plants by reporting the discovery that many phytohormones alter the circadian parameters of periodicity, phase, amplitude, and precision.

In several animal systems, classes of hormones that are rhythmically secreted from the suprachiasmatic nuclei (SCN) affect the circadian system to organize their oscillations with the various cells (Reppert & Weaver 2002). However, in contrast to the SCN seen in animal systems, there is no evidence for such a pacemaker or a master clock tissue in plants. Further, the rhythms of one plant organ have been reported to be independent from others, suggesting that the plant circadian clock is coupled more weakly than that of animal systems (Thain *et al.* 2000, 2002). These "uncoupled" clocks could be coupled through hormonal mediation, as phytohormones are trafficked molecules throughout the plant. This could suggest that hormone-clock connections function not only to synchronize oscillators in different tissues, but also functions to integrate external signals ensuring robust maintenance of the clock thought the whole organism. Remarkably, phytohormones can influence each other, and sometimes antagonize each other's function in plant development (Bishop & Koncz 2002; Fedoroff 2002; Srivastava 2002; Guo & Ecker 2004; Martínez *et al.* 2004). These actions are differential and cannot solely be *via* light signaling.

In our observation, cytokinin mediates circadian parameters *via* light signaling through ARR4 and phyB. Based on our results, we propose a genetic mechanism that describes the cytokinin-input to the clock (Fig. 7B): (i) cytokinin activates *ARR4* (and perhaps, other response regulators), (ii) ARR4 alters phyB *Pfr*-stability, which is required for phyB-mediated light signaling (Sweere *et al.* 2001) and (iii) activated phyB changes the gene expression of the core-clock genes in a phase-dependent manner. A further effect of cytokinin on periodicity and phase responses were recently described (Salomé *et al.* 2006). In our observations, cytokinin alters phase more dominantly over periodicity. Perhaps, the high concentration

of cytokinin used in the Salomé *et al.* (2006) report continuously delayed phase, and this manifested as lengthened-periodicity. This is exactly what we found in our detailed dose–response analysis (Supplementary Fig. S6). Similar complex cytokinin responses have been genetically observed (Smalle *et al.* 2002). Furthermore, the *arr4* loss-of-function mutant was described as a long-period mutant only in the context of an *arr3* mutation; however, over-expression of *ARRs* did not affect circadian rhythms in the previous report (Salomé *et al.* 2006). In our experiments, consistent with the report by Salomé *et al.* (2006), we found no strong changes of circadian parameters in *ARR4*-ox lines that were not treated with cytokinin, *ARR4*-ox plants exhibit delayed-phase phenotype only upon cytokinin treatment. A percieved inconsistency between the phenotype of the *arr4* loss-of-function mutant and the over-expression lines has also been reported in light-related growth (Sweere *et al.* 2001; To *et al.* 2004). Moreover, we noticed a natural-variation difference of ecotypes used between our study and that of Salomé *et al.* (2006). In particular, the Col-0 ecotype used by Salomé *et al.* (2006) has an atypical response to the Ws ecotype used in our study (data not shown) These natural-variation differences are currently not understood. Additionally, we also report cytokinin-induced expression of the clock genes *CCA1*, *GI* and *TOC1*. It has been reported that other pseudoresponse regulator genes, *PRR9* and *PRR5*, were also regulated by the hormone cytokinin (Brenner *et al.* 2005). These genes also play roles for circadian system and red light response (Eriksson *et al.* 2003; Mizuno 2004).

Auxin application eventually breaks clock precision under LL. To our knowledge, this is the first finding of phytohormone that disrupts circadian oscillation. Similar effects on clock precision have been observed in the circadian-gating mutants *elf3* and *elf4* (McWatters *et al.* 2000; Doyle *et al.* 2002). Auxin thus phenocopies the lack of precise and circadian maintenance seen in these gating mutants. Interestingly, auxin application in the morning repressed *CCA1* gene expression, a molecular phenotype seen in *elf3* and *elf4*. It is plausible that auxin modulates the circadian-gating mechanism.

Here, we showed that BR shortened clock periodicity on *CCR2*, *CCA1*, and *CAB* rhythms, and the BR-defect *cpd* mutants exhibited lengthened-period phenotype on *CCR2* rhythms. Curiously, another BR-deficient mutant *det2* was reported to shorten the period of *CAB* rhythm in DD (Millar *et al.* 1995). We confirmed this short-period phenotype in *cpd* harboring the *CAB:LUC* marker (data not shown). Moreover, though cytokinin antagonizes the BR function in hypocotyl elongation, both phytohormones mimic light signals in distinct but not opposite way with regard to the clock. Our findings imply new interactions among the hormone signaling. Thus, BRs indeed play an important role in clock periodicity, but understanding the detailed functions remains.

In classical studies, ABA application was reported to decrease circadian amplitude and to change phase, but not to alter the circadian periodicity in the leaf movement rhythm in *Oxalis regnellii* (Skrove *et al.* 1982). Here, in our precise molecular analysis, we found that ABA clearly lengthens periodicity. In the ABA treatment of leaf movement rhythms in *Oxalis regnelli*, the decreased amplitude might have masked periodicity changes. Another point of interest is the currently unclear mechanism by which ABA regulates circadian periodicity. Aspects of this mechanism might include the ABA signaling factor ABI3. It was previously shown to bind to the clock component TOC1 (Kurup *et al.* 2000). It is interesting that ABA reduced *CCA1* mRNA. The interaction between ABA signal and ABI3–TOC1 interactions would be worthy of investigation.

Previous studies reported that ethylene does not affect circadian parameters, even though ethylene levels are rhythmic (Thain *et al.* 2004). Although these studies were clearly confirmed in our experiments, we did find that mutations altering ethylene synthesis and signaling can exhibit aberrant, albeit conditional, clock phenotypes. The phenotype of ethylene signaling mutants could exhibit cytokinin-like phenotypes. We conclude that, although ethylene itself is not a key compound for regulation of the circadian system, ethylene signaling can influence circadian processes.

It is notable that not all phytohormones have strong input responses to the clock. This supports the notion that different hormone-signaling pathways have alternative inputs to circadian parameters. For example, both GA treatments and an analysis of *ga1* mutation did not reveal a strong effect of GA on the circadian system. This is in contrast with the genetic work on the *SPY* locus, a negative regulator of GA signaling. The *spy* mutant was previously shown to have a lengthen-periodicity phenotype (Tseng *et al.* 2004). These differences highlight overlapping and convergent phytohormone signals on the circadian system, as seen in the cytokinin-ethylene connection (Hass *et al.* 2004). GA signaling may have more than two pathways, and some of these pathways may mask the *SPY* effect. Alternatively, *SPY* might be a pleiotropic clock regulator and its effect on the clock is independent of GA action. With regard to hormones without an apparent clock-response, both SA and NO belong to this category. This is of interest, as these hormones regulate flowering time in Arabidopsis (He *et al.* 2004; Martínez *et al.* 2004). Perhaps, the SA and NO

effects on flowering time are independent of the circadian system. In addition, application of the mammalian clock-related hormone melatonin caused no significant changes in circadian parameters. Plants thus integrate external hormonal signals to the clock in a different fashion (and use different hormones).

Unexpectedly, we found that many phytohormones control various aspects of the plant circadian system. The clock system is doubtless balanced amongst these hormones, whose levels change in response to altering environmental conditions (Srivastava 2002). Furthermore, in addition to the pseudo-response regulator TOC1/PRR family (Mizuno 2004), an authentic response regulator(s) is also an element in the circadian system. The two-component system of plants might be one of the integrators that connects environmental signals to the circadian clock. Collectively, when compared to the clock systems of cyanobacteria, fungi and animals, plants appear to have a flexible clock system(s), fine-tuned by multiple hormonal feedbacks. This is an assistant of clock flexibility to coordinate plant development and metabolism with ever changing environmental conditions. It will be of great interest to see how widely these fine-tuning mechanisms and multiple feedbacks are conserved among other organisms.

## Experimental procedures

### Plant materials and chemicals

All experiments were carried out in *Arabidopsis thaliana* ecotype *Wassilewskija* (Ws), except for the mutant tests and their respective wild-type backgrounds. Most mutant seed was provided by the Arabidopsis Biological Resource Center (ABRC, USA) and the Nottingham Arabidopsis Stock Center (NASC, UK). *phyB-464–19* is in the Ws background (Reed *et al.* 1993), *Agrobacterium tumefaciens* and transgenic plants harboring promoter; *luciferase* (*LUC*) constructs were kindly provided by Prof Andrew Millar (University of Warwick, UK). The *luciferase* constructs reporting *CCR2* rhythms were introduced into various mutants by fertilization. *cpd* mutants in L*er* background were crossed to Ws plants harboring *CCR2*:*LUC* reporter genes 3 times. To generate *ARR4*-ox constructs, the *ARR4* coding region was amplified by polymerase chain reaction (PCR), using the primer sequences 5′-GGGG-*att*B1-CTATGGCCAGAGACGGTGGTG-3′ and 5′-GGGG-*att*B2-CTAATCTAATCCGGGACTCCTCA-3′, and this was subcloned into pDONR207 (Invitrogen, Karlsruhe, Germany). The *ARR4* coding region in pDONR207 was transferred into the pJAN33 vector, kindly provided by Dr Marc Jacoby and Prof Bernd Weisshaar (MPIZ, Germany). The pJAN33 vector harbors the CaMV 35S promoter to drive in plants high-level expression of the introduced gene. The *ARR4*-ox construct was introduced into *Arabidopsis* harboring the *CCR2*:*LUC* reporter transgene by the floral-dip method (Clough & Bent 1998). *ARR4* transcript elevation was confirmed by RT-PCR analysis of the *ARR4* mRNA in these over-expression lines (data not shown). The *ARR4*-ox plant harboring *CCR2*:*LUC* reporter transgene was crossed with the *phyB* mutant to generate the double "mutant" of this genotype. All chemicals and phytohormones were purchased from Sigma (Germany), except for homobrassinolide (Rose Scientific Ltd. Canada). Most hormones were dissolved into a stock solution using a dimethyl sulfoxide (DMSO) solvent.

### Luminescence assays

Prior to measurements, seedlings were entrained at 22 °C under 12 h white light/12 h dark cycles (LD) while growing on Murashige-Skoog (MS) 3% sucrose–1% agar plates (pH 5.7) without phytohormone under cool-white light, 10 μmol $m^{-2}$ $s^{-1}$ for 7 days. Each seedling was transferred into imaging microtiter plates (Perkin Elmer, Juegesheim, Germany) containing MS 3% sucrose–1% agar media, pH 5.7, with 20 μM phytohormones or 0.01% DMSO as a control solvent for untreated plants. Such a hormone concentration is within experimental ranges commonly used pharmacologically for a given phytohormone, and is probably saturating, but within a physiologic (non-toxic) range. 0.01% DMSO had no significant effects on any parameter of the circadian clock (data not shown). Auxins were dissolved in $dH_2O$. Afterwards, 5 mM luciferin was added on to the plants, and then the seedlings were entrained to another LD cycle. The luminescence rhythms were monitored using a luminescence scintillation counter, TOPCount NXT (Perkin Elmer), with or without custom constructed red and blue LEDs (~2 μmol $m^{-2}$ $s^{-1}$) (Southern & Millar 2005). The luminescence rhythms, containing 3–4 cycles, were mathematically analyzed by the Microsoft Excel macro BRASS, as previously described (Southern & Millar 2005). Statistical tests of multiple independent replicates were carried out with one-factor ANOVA followed by Bonferroni multiple *t*-test.

### RNA isolation and reverse transcriptase-PCR

Seedlings grown for 1 week under LD cycles were transferred to MS plates containing 20 μM of the phytohormone indicated in Fig. 6, or 0.01% DMSO as a control, at zeitgeber time (ZT) = 2 or ZT = 10, were incubated for 1 h, and then were harvested at ZT = 3 or ZT = 11. Total RNA was isolated from the seedlings using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). The total RNA was treated with DNase I before reverse transcription. Reverse transcription was performed on 1.0 μg of total RNA with SuperscriptII (Invitrogen). Quantitative PCR were performed with iQ5 real-time PCR system (BIO-LAD). Gene-specific primers were previously described: *CCA1* and *TOC1* (Hall *et al.* 2003), *GI* (Mizoguchi *et al.* 2005), and *UBQ10* (Blázquez & Weigel 1999).

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## Supplementary materials

The following supplementary material is available for this article online:

**Figure S1** Definition of circadian parameters.

**Figure S2** Cytokinins alter circadian phase.

**Figure S3** Auxins affect clock precision under LL.

**Figure S4** Brassinosteroid shortens the periodicity and recovers *CAB* rhythm in DD.

**Figure S5** ABA lengthens the periodicity under LL.

**Figure S6** Dose–response of phytohormone effects on *CCR2* periodicity.

**Figure S7** *ARR4*-ox, *phyB*, *ARR4*-ox *phyB* mutants under constant red light (RR) with or without cytokinin.