Photosynthetic entrainment of the Arabidopsis thaliana circadian clock

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Circadian clocks provide a competitive advantage in an environment that is heavily influenced by the rotation of the Earth, by driving daily rhythms in behaviour, physiology and metabolism in bacteria, fungi, plants and animals. Circadian clocks comprise transcription–translation feedback loops, which are entrained by environmental signals such as light and temperature to adjust the phase of rhythms to match the local environment. The production of sugars by photosynthesis is a key metabolic output of the circadian clock in plants. Here we show that these rhythmic, endogenous oscillations in sugar levels provide metabolic feedback to the circadian oscillator through the morning-expressed gene PSEUDO-RESPONSE REGULATOR 7 (PRR7), and we identify that prr7 mutants are insensitive to the effects of sucrose on the circadian period. Thus, photosynthesis has a marked effect on the entrainment and maintenance of robust circadian rhythms in A. thaliana, demonstrating that metabolism has a crucial role in regulation of the circadian clock.

In plants, energy is derived from photosynthesis in chloroplasts by fixing CO2 into sugar in a light-dependent manner. Net carbon assimilation and starch metabolism are under circadian regulation, as are transcripts associated with chlorophyll biosynthesis and the photosynthetic apparatus, which peak at about 4 h after dawn. For A. thaliana seedlings, the addition of sucrose to the growth medium shortens the circadian period when the plants are grown in continuous light and can sustain circadian rhythms when they are grown in continuous dark. Because exogenous sugars can influence the circadian oscillator, we sought to investigate whether endogenous sugars derived from photosynthesis are part of the circadian network in plants.

To investigate whether photosynthesis can influence the core circadian clock in A. thaliana, we inhibited photosynthesis by growing seedlings in CO2-free air or in medium containing 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), an inhibitor of photosystem II, and we monitored the circadian rhythms of luciferase (LUC)-based reporters of transcription of the core clock gene promoters. We performed these experiments in continuous low light (10 μmol m⁻² s⁻¹) because we observed that exogenous sucrose markedly shortened the circadian period in low light but had only subtle effects in higher light (50 μmol m⁻² s⁻¹) (Extended Data Fig. 2a, b). CO2 depletion (Fig. 1a) or DCMU treatment (Extended Data Fig. 2c, d) increased the period of the clock reporters by a mean length of 2.9 h and 2.5 h, respectively, compared with untreated controls. Both treatments increased the activity of the PRR7 promoter:LUC construct (PRR7:LUC) and reduced the activity of the CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) promoter:LUC construct (CCA1:LUC), which then damped towards arrhythmicity over time (Fig. 1a and Extended Data Fig. 2b).

Exogenous sucrose is probably ineffective at altering the period in higher light because the response is already saturated by higher levels of endogenous sugars produced by photosynthesis, whereas complete inhibition of photosynthesis will be effective at altering the period under either set of light conditions.

In light–dark cycles, there are robust endogenous rhythms of soluble sugars, which peak at about 4–8 h after dawn (Extended Data Fig. 3a). Inhibition of photosynthesis by either CO2 depletion or DCMU treatment reduced the endogenous glucose, fructose and sucrose concentrations (Extended Data Fig. 3b, c). To test whether the effects of inhibition of photosynthesis on the circadian oscillator were due to reduced sugar production, we resupplied exogenous sucrose to CO2-depleted or DCMU-treated seedlings. The period lengthening normally induced

![Figure 1: Photosynthetically derived sugars influence the circadian clock in A. thaliana.](image)

**Figure 1** | Photosynthetically derived sugars influence the circadian clock in *A. thaliana*. a, LUC reporter activity rhythms (mean ± s.e.m.) (left) and period estimates (right) for seedlings grown in ambient air (control) or CO2-free air in continuous low light (10 μmol m⁻² s⁻¹) (n = 4). b, Period estimates for PRR7:LUC activity rhythms in continuous light (10 μmol m⁻² s⁻¹) or 50 μmol m⁻² s⁻¹) in the presence or absence of DCMU (mean ± s.d.; n = 4). c, Period estimates for PRR7:LUC activity rhythms for seedlings grown in continuous low light and treated with inhibitors in the presence or absence of exogenous sucrose (mean ± s.d.; n = 4). d, e, CCA1:LUC activity rhythms (mean ± s.e.m.) (d) and relative amplitude error of CCA1:LUC activity rhythms (e) in seedlings treated with DCMU in the presence or absence of exogenous sucrose (mean ± s.d.; n = 4). **P < 0.01; ***P < 0.001 compared with untreated controls; two-tailed Student’s t-test. n refers to number of biological replicates.
by either treatment was suppressed by the addition of exogenous sucrose (Fig. 1c). The effects of DCMU treatment on CCA1:LUC rhythms were reversed by the addition of as little as 5 mM (0.15% w/v) exogenous sucrose to the growth medium (Fig. 1d, e and Extended Data Fig. 4a). We also tested the effect of norflurazon or lincomycin, both of which trigger retrograde signalling from the chloroplast to the nucleus12. Neither treatment lengthened the circadian period of activity of PRR7:LUC (Fig. 1c) or inhibited the activity of CCA1:LUC (Extended Data Fig. 4b) in the presence or absence of exogenous sucrose. Furthermore, we found no evidence that photosynthesis might affect clock function through mechanisms associated with reactive oxygen species production (Extended Data Fig. 5), which agrees with a recent report13.

Because our data suggest that the effects of photosynthesis on the circadian clock are mediated by sugars, we investigated the role of sugars in circadian function in more detail. We first tested whether the effects of exogenous sucrose represent a general response to sugar. In continuous low light, the circadian periods of CCA1:LUC, PRR7:LUC and TIMING OF CAB EXPRESSION 1 (TOC1) promoter:LUC activity were a mean of 4.2 h shorter in seedlings grown in medium containing 90 mM (3% w/v) sucrose, glucose or fructose than in mannitol-treated controls (Fig. 2a, b). Similarly, exogenous sucrose, glucose or fructose, but not mannitol or a non-metabolizable glucose analogue (3-O-methyl glucose), were able to sustain circadian rhythms in continuous dark (Extended Data Fig. 6a). These data suggest that the effects of exogenous sucrose on circadian rhythms represent a general response to metabolically active sugars.

Oscillations of circadian reporter activity are absent or very low in continuous dark10 (Extended Data Fig. 6b). Exogenous sucrose can reinitiate the circadian oscillations of the clock output reporter CHOROPHYLL A/B BINDING PROTEIN 2 (CAB2) promoter:LUC in dark-adapted seedlings, and the phase is set to the time of sucrose addition after 72 h (subjective dawn) or 60 h (subjective dusk) in continuous dark10. We observed the same behaviour for reporters of the core circadian oscillator and confirmed that exogenous sucrose led to an increased CCA1 transcript level in dark-adapted seedlings (Extended Data Fig. 7). The phase setting of the clock indicates that sucrose is not simply amplifying damped rhythms in dark-adapted seedlings as a result of the increased availability of ATP, and this finding suggests a role for sugars in entrainment. To directly test whether sugars function in entrainment, we determined a phase-response curve (PRC) for exogenous sucrose; this curve assesses the ability of a stimulus to alter the circadian phase across a circadian cycle14. In continuous low light, the peak of the activity of CCA1:LUC and TOC1:LUC was shifted by pulses of exogenous sucrose, inducing phase advances of up to 2 h around dawn and phase delays around dusk (Fig. 2c and Extended Data Fig. 8). We observed subtle differences between reporters, similar to phase setting by temperature15. The phase shifts were not due to effects on circadian period or an osmotic signal (Extended Data Fig. 8c). These data are consistent with metabolically active sugars functioning as a Type 1 zeitgeber participating in circadian entrainment14.

A key feature of entrainment is variation, or ‘gating’, of the response to the zeitgeber in a time-dependent manner3. Sucrose application during the first subjective day of continuous low light significantly induced CCA1:LUC activity during the day but had little effect during the subjective night (Fig. 2d). This effect was most pronounced before midday (zeitgeber time 6 (ZT6)). These data demonstrate that the input of sugars to CCA1:LUC activity is gated so that the promoter is most responsive to sucrose availability early in the light period.

We next compared the responses of the A. thaliana circadian system to sucrose and light because light can act as a strong, Type 0 zeitgeber3,14 and drives sugar production from photosynthesis. In dark-adapted seedlings, there was a similar transient increase in CAB2:LUC activity, peaking at about 5 h after treatment with light or sucrose (Fig. 2e). By contrast, the first circadian peak in CAB2:LUC occurred 26.9 h after the onset of light, compared with 22.8 h after sucrose addition, indicating a 4.1 h advanced phase set by sucrose compared with light (Fig. 2e, f). The difference in phase setting did not depend on sucrose concentration or light intensity within the ranges tested (Fig. 2f and Extended Data Fig. 8d). When photosynthetic sugar production was inhibited in the light by DCMU, the phase that was set by light was delayed by a further 2.5–3.5 h (Extended Data Fig. 8e). These data demonstrate that these zeitgebers both function in discrete (non-parametric) entrainment. The difference in phase might be due to period effects but could also indicate distinct phase setting. The phase difference coincides with the delay between dawn and the highest endogenous sucrose and glucose concentrations (Extended Data Fig. 3). We propose that a concentration threshold of photosynthetically derived sugars provides input to the central oscillator, thereby functioning as a ‘metabolic dawn’ that contributes to entrainment of the A. thaliana circadian clock (Extended Data Fig. 1).

Having established that sugars derived from photosynthesis contribute to circadian entrainment in A. thaliana, we next investigated how this might occur. The increase in PRR7:LUC activity in DCMU-treated or CO2-depleted seedlings (Fig. 1a and Extended Data Fig. 2) suggested that photosynthesis regulates PRR7 transcript abundance. We measured the transcript levels of morning-expressed circadian clock genes in the shoots of control (untreated) and DCMU-treated
seedlings (Fig. 3). PRR7 transcript levels were 3.7-fold to 8.2-fold higher in DCMU-treated seedlings than in untreated plants between ZT10 and ZT16, and this difference was suppressed when sucrose was added to the medium. PRR5 transcript levels were only 1.6-fold to 2.9-fold higher in DCMU-treated seedlings around dawn (ZT0) than in untreated plants, and PRR9 transcript levels were unaffected. CCA1 and LATE ELONGATED HYPOCOTYL (LHY) transcripts were 3.0-fold to 8.4-fold lower before dawn, between ZT18 and ZT21, in DCMU-treated seedlings than in untreated material. These data are consistent with the LUC reporter data (Extended Data Fig. 2) and suggest that the effect of photosynthesis is more pronounced on PRR7 than on the other tested circadian clock genes.

These data led us to propose that photosynthetic input to the circadian oscillator might act through PRR7, a transcriptional repressor that acts on the CCA1 promoter during the night. We first tested the short-term effect of exogenous sucrose on PRR7 promoter activity. In contrast to CCA1:LUC (Fig. 2d), PRR7:LUC activity was significantly repressed during the day and the subjective night, but this repression was most pronounced during the morning (Extended Data Fig. 9a). We tested whether the induction of CCA1 depends on PRR7. CCA1:LUC induction was significantly attenuated in prr7-11 mutants compared with wild-type plants (Extended Data Fig. 9b). These data are consistent with sucrose activating CCA1 through the repression of PRR7. Next, we examined whether PRR7 contributes to the circadian period adjustment mediated by sucrose. Exogenous sucrose shortened the period of the circadian rhythm of CCA1:LUC activity by 2.7 h in wild-type plants, whereas the circadian rhythm of CCA1:LUC activity was not shortened in prr7-11 mutants exposed to exogenous sucrose (Fig. 4a). Similarly, the period of the circadian rhythm of delayed fluorescence was also shortened by exogenous sucrose exposure in wild-type plants but not prr7-11 mutants (Extended Data Fig. 9c).

To assess whether PRR7 also has a role in circadian entrainment by sugars, we determined a PRC for prr7-11 to pulses of exogenous sucrose. In contrast to wild-type plants (Fig. 2c), sucrose did not induce phase advances in prr7-11 mutants (Extended Data Fig. 9d). Because SENSITIVE TO FREEZING 6 (SFR6), a subunit of the Mediator complex, contributes to the period adjustment mediated by sucrose (by an unknown mechanism), we determined whether other previously identified pathways participate in the regulation of the circadian oscillator by sugars. We measured rhythms in a range of circadian, sugar-insensitive and light-signalling mutants. With the exception of cca1-11, all of the tested mutants had a significantly shorter circadian period in the presence of sucrose than in control media (Fig. 4b and Extended Data Fig. 10). Together, these data indicate that PRR7, acting through CCA1, has a specific role in the regulation of the circadian clock by photosynthetically derived sugars and that this might occur through a novel signalling pathway.

Our findings led us to test whether PRR7 might be more widely involved in sugar signalling. When germinated on medium containing 180 mM (6% w/v) sucrose, prr7-11 mutants were resistant to the repression of chlorophyll accumulation (Fig. 4c, d), similar to glucose insensitive 2 (hexokinase 1) (gin2-1) mutants. In addition, prr7-11 seedlings had an elevated endogenous glucose concentration around dawn compared with wild-type seedlings (Fig. 4e), suggesting a role for PRR7 in regulating endogenous sugar accumulation. This finding is consistent with previous reports of the involvement of PRR proteins in regulating chlorophyll biosynthesis and primary metabolism.

Altered feeding cycles can influence the phase of peripheral clocks in animals. Similarly, it has previously been suggested that a shoot-derived photosynthesis product might regulate a simplified circadian oscillator in A. thaliana roots. Photosynthesis also contributes to entrainment by an unknown mechanism in the green alga Chlamydomonas reinhardtii. By analysing the effects of altered photosynthesis products on free-running circadian rhythms and by examining the role of PRR7, we have demonstrated that photosynthetically derived sugars provide metabolic feedback that entrains the A. thaliana circadian clock in shoots. We propose that following light activation of PRR7 at dawn, the accumulation of endogenous sugars as a result of photosynthesis represses the PRR7 promoter, leading to de-repression of CCA1.
Thus, **PRR7** expression is coordinately modulated by light and photosynthesis, allowing **PRR7** to function as a transcriptional repressor in circadian sugar signalling (Extended Data Fig. 1). Our results define a novel metabolic feedback loop that contributes to circadian entrainment in plants.

**METHODS SUMMARY**

**Growth conditions and media.** Surface-sterilized *A. thaliana* seeds (on Col-0, Ler andWs backgrounds) were sown on half-strength Murashige and Skoog agar medium (1/2 MS) without sucrose. After 2 days at 4°C in darkness, the seedlings were grown at 19°C in a 12 h light–12 h dark cycle in 50 μmol m⁻² s⁻¹ white fluorescent light. Chemicals were added to the media as follows: 90 mM sugars, 20 μM DCMU, 5 μM norflurazon and 220 μg ml⁻¹ lincomycin. For CO₂-free air, ambient air was pumped through soda lime. Sugars were added topically to dark-adapted seedlings to a final concentration of approximately 30 mM in the media, unless otherwise indicated. For gating and PRC experiments, the seedlings were transferred to treatments 48–60 h before release into continuous light or dark conditions after 7–11 days in cycles of 12 h light and 12 h dark. Seedlings were supplied with 1–2 mM d-luciferin and released into continuous light or darkLuciferase experiments. **LUC** reporter lines were grown in clusters of 5–10 seedlings, supplied with 1–2 mM d-luciferin and released into continuous light or dark conditions after 7–11 days in cycles of 12 h light and 12 h dark. Seedlings were transferred to treatments 48–60 h before release into continuous light or dark conditions. Light was supplied from red- and blue-light-emitting diodes (LEDs) at 50 μmol m⁻² s⁻¹ (high light) or 10 μmol m⁻² s⁻¹ (low light). Circadian period and relative amplitude error estimates were determined using the Biological Rhythms Analysis Software System (BRASS). All n values represent biological replicates, and all data are representative of independently repeated experiments.

**Real-time PCR.** RNA was extracted from three biological replicates of 12-day-old seedlings growing in a cycle of 12 h light and 12 h dark from 37–58 h after transfer to treatments at dusk. cDNA was synthesized from DNease-treated RNA with oligo(dT) primers. Gene-specific primers (see Methods) were used to amplify products on a Rotor-Gene 6000 Real-Time PCR machine.

**Online Content** Any additional Methods, Extended Data display items and Source Data are available in the online version of the paper; references unique to these sections appear only in the online paper.

**Received 27 February; accepted 23 August 2013.**

**Published online 23 October 2013.**

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**Acknowledgements** This work was supported by BBSRC grant BB/H006826/1. We thank J. D Neill, J. Davies and J. Hibberd for comments on the manuscript.

**Author Contributions** M.J.H. and A.A.R.W. designed the research. M.J.H., O.M., F.C.R. and K.E.H. performed the experiments and analysed the data. M.J.H. and A.A.R.W. prepared the manuscript.

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was introduced into the Ler ecotype and the following mutants by CCA1:LUC detection for 800 s at each time point with an HRPCS4 (Photek) or an LB 985 into continuous light after 7–11 days of light–dark cycles. Luminescence was water into a sealed growth plate with an outlet. The CO2 concentration of the air spindly release into continuous conditions. CO2-free air was produced by pumping ambient and equal variance, were performed in Microsoft Excel. The power of 0.9. Two-sided statistical tests, including assessments of normal distribution and equal variance, were performed in independently repeated experiments. Tests were justified by determining the minimum difference of means with a power of 0.9. Two-sided statistical tests, including assessments of normal distribution and equal variance, were performed in Microsoft Excel. The gining-1(Ca-0) line was used for sugar-insensitivity experiments to allow direct comparison with pprR mutants and Col-0. Surface-sterilized seeds were sown on half-strength Murashige and Skoog media (1/2 MS), pH 5.7, without sucrose and solidified with 0.8% (w/v) Bacto agar. After sowing, seeds were kept at 4°C in darkness for 2 days, then grown in 12 h light–12 h dark cycles under 50 μmol m−2 s−1 cool fluorescent white light at a constant temperature of 19°C.

Photon-counting experiments. Clusters of five to ten seedlings were grown on 1/2 MS agar medium and entrained in 12 h light and 12 h dark cycles (50 μmol m−2 s−1 light). For LUC measurement, seedlings were dosed twice with 1–2 mM lincomycin. Seedlings were transferred to treatments 48–60 h before sampling. Chlorophyll measurements, fresh tissue was extracted in methanol, and the concentrations were determined as described previously43.

METHODS
Plant materials and growth methods. A. thaliana of the Col-0 ecotype was transformed with CCA1:LUC, TOC1:LUC, PRR7:LUC or CCR21:LUC. A. thaliana of the Wa ecotype was transformed with GLLuc, PRR9:LUC or CAB21:LUC. CCA1:LUC was introduced into the Ler ecotype and the following mutants by crossing: prr5-11, prr7-11, prr9-10 (ref. 26), prr3-1 (ref. 27), gigantea (gi-2)33, zeiluppe (ze1-3)36, gin2-1 (ref. 18), fructose-insensitive-1 (fis-1)33, abscisic acid deficient 2 (aba2-1; gin1)42, abac-1 (gin5)32, abscisic acid insensitive 1 (abi1)-1, constitutive triple response 1 (ctr1-2; gin4)32, hookless-1 (hsl1)-1, phytochrome A Phytochrome B (phy2-191; phy2-5)33, cryochrome 1 (cry1)-1, long hypocotyl 5 (lhy-215)36, constitutive photomorphogenic 1 (cpl-1)-4, far-red elongated hypocotyl 3 (fes1-1)36, phytochrome interacting factor 3 (fis-3)-33 and spindly (spy-3)-42. Ler/CCA1:LUC and gin2-1/CCA1:LUC were backcrossed to Ler or CCA1:LUC, respectively. The gin2-1(Ca-0) line was used for sugar-insensitivity experiments to allow direct comparison with pprR mutants and Col-0. Surface-sterilized seeds were sown on half-strength Murashige and Skoog media (1/2 MS), pH 5.7, without sucrose and solidified with 0.8% (w/v) Bacto agar. After sowing, seeds were kept at 4°C in darkness for 2 days, then grown in 12 h light–12 h dark cycles under 50 μmol m−2 s−1 cool fluorescent white light at a constant temperature of 19°C.

Real-time PCR. Ten-day-old seedlings growing in 12 h light–12 h dark cycles (50 μmol m−2 s−1) were transferred to treatments at dusk, and leaf tissue was collected at 3 h intervals 37–58 h later. Total RNA was extracted from three biological replicates of frozen leaf tissue using the RNeasy Plant Mini Kit (QIAGEN) and RnaFree DNase on-column treatment (QIAGEN). cDNA was synthesized from 1 μg RNA with the RevertAid First Strand cDNA Synthesis Kit (Fermentas) using oligo(dT) primers. Technical replicates of gene-specific products were amplified in 10 μl reactions using the Rotor-Gene SYBR Green PCR Kit on a Rotor-Gene 6000 Real-Time PCR machine fitted with a Rotor-Disc 100 (QIAGEN). The primer sequences were as follows: UBQ10-F, 5′-GCGCTGTGATAAATCTCTGTA GATAATCT-3′; UBQ10-R, 5′-AAAGCAGATAAGGAGAACGAAATGACT-3′; CCA1-F, 5′-GTGATTGTGTGAGGGCTGATG-3′; CCA1-R, 5′-TGGTGGTTACTGACCTGGAAG-3′; HY5-F, 5′-AGGAAACAGCTAAGTGGCAGCATT-3′; HY5-R, 5′-TGGGAAACATTGAGACCCGTG-3′; PRR7-F, 5′-CCACACGAAAGAACAAAGAACA-3′; PRR7-R, 5′-CGAGGGGCCTTCTGCTG-3′; PRR9-F, 5′-CCGATTAGGCAGGAGACGCA-3′; PRR9-R, 5′-GGATGGATGCTTCAAGG-3′. Relative transcript levels were determined by incorporating PCR efficiencies as described previously33.

Sodium and chlorophyll measurements. For soluble sugar measurements, 50–100 mg frozen tissue was extracted twice in 80% (v/v) ethanol and used immediately to determine sugar concentrations with the Sucrose/Fructose/o-Glucose assay kit (Megazyme). For chlorophyll measurements, fresh tissue was extracted in methanol, and the concentrations were determined as described previously43.

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Extended Data Figure 1 | A model for entrainment of the *A. thaliana* circadian clock by photosynthetically derived sugars. From dawn, light activates PRR7 and drives photosynthesis. The concentrations of simple sugars produced by photosynthesis accumulate within the plant during the day (red dashed line), peaking around 4–8 h after dawn. High endogenous sugar concentrations lead to suppression of the PRR7 promoter, contributing to the phase of PRR7 rhythms. PRR7 is a transcriptional repressor of the circadian clock component CCA1. Thus, the rhythms of endogenous sugars derived from photosynthesis contribute to circadian entrainment through PRR7. We propose that the timing of these events represents a 'metabolic dawn'. Dawn is a time-dependent gradient of light intensity, whereas 'metabolic dawn' represents a gradient of increasing metabolite concentration. The metabolic gradient lags behind that of light and contributes to the setting of the circadian clock. In the model, previously established relationships are shown by black connectors, and novel relationships proposed in this study are shown by orange connectors.
Extended Data Figure 2 | Effects of exogenous sucrose and photosynthesis inhibition on circadian rhythms. a, b, Period estimates for the rhythms of the promoter:LUC reporters in continuous low light (a) or continuous light (b) in plants grown in media with or without sucrose (mean ± s.d.; n = 4).

c, d, Promoter:LUC reporter rhythms (mean ± s.e.m.) and relative amplitude error versus period plots for seedlings in media in the presence or absence of DCMU in continuous low light (c) or continuous light (d) (n = 4). * P < 0.05; ** P < 0.01; *** P < 0.001; compared with untreated plants by two-tailed Student’s t-test. n refers to number of biological replicates.
Extended Data Figure 3 | The rhythms of endogenous sugars peak in the morning and are reduced by inhibition of photosynthesis. a, Leaf sucrose and glucose concentrations in 10-day-old seedlings growing in 12 h light and 12 h dark cycles (mean ± s.d.; n = 3). b, Glucose, fructose and sucrose concentrations 4 h after subjective dawn in 13-day-old seedlings grown in CO₂-free air or ambient air in continuous low light for 5 days (mean ± s.d.; n = 3). c, Glucose concentration in 10-day-old seedlings growing in a light–dark cycle at 12–36 h after transfer to DCMU or control media at dusk (mean ± s.d.; n = 3). * P < 0.05; ** P < 0.01 by two-tailed Student’s t-test compared with ZT0 in a and compared with control conditions in b and c. n refers to number of biological replicates.
Extended Data Figure 4 | Effects of DCMU, norflurazon or lincomycin on \( CCA1: \text{LUC} \) rhythms in the presence or absence of exogenous sucrose.

**a**, \( CCA1: \text{LUC} \) rhythms in continuous low light for seedlings transferred to media containing DCMU in the presence of the indicated exogenous sucrose concentrations compared with control media (mean ± s.e.m.; \( n = 4 \)).

**b**, \( CCA1: \text{LUC} \) rhythms in continuous light for seedlings transferred to media containing DCMU, norflurazon or lincomycin in the absence (left) or presence (right) of exogenous sucrose (mean ± s.e.m.; \( n = 4 \)). \( n \) refers to number of biological replicates.
Extended Data Figure 5 | Altering reactive oxygen species (ROS) production does not influence circadian rhythms. 

a, CAB2:LUC rhythms in seedlings transferred to continuous light and treated with 1 mM glutathione or 5 mM ascorbate. The short-period mutant toc1-1 and long-period mutant ztl-1 were included as positive controls (means ± s.d.; n = 2–3).

b, Relative amplitude error versus period plot for leaf movement rhythms in wild-type plants and NADPH oxidase rbohD,F mutants in continuous light (mean ± s.e.m.).

c, Promoter:LUC rhythms and relative amplitude error versus period plots for seedlings grown in continuous light or continuous low light and treated with 10 μM diphenyleneiodonium (DPI) or 0.1% (v/v) dimethylsulphoxide (DMSO) at 0 h (mean ± s.e.m.; n = 4). n refers to number of biological replicates.
Extended Data Figure 6 | Metabolically active sugars sustain circadian rhythms in darkness. a, CCA1:LUC rhythms in continuous dark in seedlings grown in media containing the indicated sugars or control treatments (mean ± s.e.m.; n = 4). b, Promoter:LUC rhythms (mean ± s.e.m.; n = 4) and relative amplitude error versus period plots (n = 4–8) for seedlings grown in continuous dark in media with or without sucrose. Note that rhythms could not be detected in seedlings grown without sucrose for the morning-expressed CCA1:LUC or PRR9:LUC but could be detected for the evening-expressed GI:LUC and TOC1:LUC, despite the small amplitude. n refers to number of biological replicates.
Extended Data Figure 7 | Exogenous sugar can set the circadian phase in dark-adapted seedlings. 

**a**, Time to the first circadian peak of promoter:LUC reporters in seedlings treated with sucrose after 72 h (subjective dawn, CT0) or 84 h (subjective dusk, CT12) in continuous dark (mean ± s.d.; n = 4).

**b**, Promoter:LUC rhythms of seedlings after sucrose or mannitol treatment as in **a** (mean ± s.e.m.; n = 4). 

**c**, CCA1 transcript level relative to UBQ10 in seedlings treated with sucrose or mannitol after 72 h in continuous dark (mean ± s.d.; n = 3). **P** < 0.01; ***P** < 0.001 by two-tailed Student’s t-test. n refers to number of biological replicates.
Extended Data Figure 8 | Phase setting by sugar and light. a, Change in the period of CCA1:LUC after pulses of sucrose compared with control seedlings in continuous low light (mean ± s.d.; n = 8). b, Phase response of TOC1:LUC to pulses of sucrose for seedlings in continuous low light (mean ± s.d.; n = 8). c, Phase response of CCA1:LUC to pulses of mannitol (mean ± s.d.; n = 8). d, LUC reporter rhythms (mean ± s.e.m.), time to the circadian peak (mean ± s.d.) and period estimates (mean ± s.d.) in seedlings grown in continuous darkness for 72 h then transferred to continuous light or continuous low light (n = 4). e, CCA1:LUC rhythms (mean ± s.e.m.) and time to the circadian peak in seedlings following transfer to continuous light or continuous low light in control media, medium containing DCMU, or medium containing DCMU and sucrose after 72 h in continuous dark (n = 4). * P < 0.05; ** P < 0.001 by two-tailed Student’s t-test. n refers to number of biological replicates.
Extended Data Figure 9 | Regulation of the circadian clock by sugar requires PRR7. **a**, Change in PRR7::LUC luminescence after 3 h treatment with sucrose relative to untreated plants (mean ± s.d.; n = 4). The data were normalized across the time series, and the change relative to untreated plants was plotted.

**b**, Change in CCA1::LUC luminescence in wild-type plants and prr7-11 mutants after 3 h treatment with sucrose relative to untreated plants (mean ± s.d.; n = 8). The data were normalized across the time series, and the change relative to untreated plants of the appropriate genotype was plotted.

c, Period estimates of rhythms of delayed fluorescence in wild-type and mutant seedlings in continuous low light in media with or without exogenous sucrose (mean ± s.d.; n = 4).

d, Phase response of CCA1::LUC to pulses of sucrose in prr7-11 seedlings in continuous low light (mean ± s.d.; n = 8). Compare this with the sucrose PRC for CCA1::LUC in wild-type seedlings in Fig. 2c.

* P < 0.05; ** P < 0.01; *** P < 0.001 by Student’s two-tailed t-test compared with controls in a and c and compared with wild-type plants in b. n refers to number of biological replicates.
Extended Data Figure 10 | Effect of exogenous sucrose on circadian period in circadian, sugar-insensitive and light-signalling mutants. LUC reporter rhythms in circadian, sugar-insensitive and light-signalling mutants in continuous low light in media with or without exogenous sucrose (mean ± s.e.m.; n = 4). The reporter is CCA1:Luc in all lines except for Ws, cca1-11 (CAB2:Luc) and tocl-21 (CCR2:LUC). Period estimates are shown in blue (control) and red (sucrose) for each line (mean ± s.d.; n = 8). n refers to number of biological replicates.