Nature. Author manuscript; available in PMC 2014 April 30.

Published in final edited form as:
Nature. 2013 October 31; 502(7473): . doi:10.1038/nature12603.

Photosynthetic entrainment of the Arabidopsis circadian clock

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Circadian clocks provide competitive advantage in an environment that is heavily influenced by the rotation of the Earth[1,2] by driving daily rhythms in behavior, physiology and metabolism in bacteria, fungi, plants and animals[3,4]. 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[3]. Production of sugar from photosynthesis is a key metabolic output of the circadian clock in plants[2,5]. Here we show that these rhythmic endogenous sugar signals can entrain circadian rhythms in Arabidopsis by regulating circadian clock gene expression early in the photoperiod to define a 'metabolic dawn'. By inhibiting photosynthesis we demonstrate that endogenous oscillations of sugars provide metabolic feedback to the circadian oscillator through the morning-expressed *PSEUDO RESPONSE REGULATOR 7 (PRR7)* and identify that *prr7* mutants are insensitive to the effects of sugar on circadian period. Thus, photosynthesis has a profound effect on the entrainment and maintenance of robust circadian rhythms in Arabidopsis, demonstrating a critical role for metabolism in regulation of the circadian clock.

In plants, energy is derived from photosynthesis in chloroplasts by fixing CO_2 into sugar in a light-dependent manner. Net C assimilation and starch metabolism are under circadian regulation[2,6–8] as are transcripts associated with chlorophyll biosynthesis and photosynthetic apparatus, peaking ~ 4 h after dawn[5]. In Arabidopsis seedlings, addition of sucrose to the growth media shortens circadian period in continuous light[9] and can sustain circadian rhythms in continuous dark[10]. Since 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 Arabidopsis, we inhibited photosynthesis by growing seedlings in CO_2-free air or in media containing 3-(3,4-Dichlorophenyl)-1,1-dimethylurea (DCMU), an inhibitor of Photosystem II and monitored circadian rhythms of transcriptional LUCIFERASE (LUC) reporters for core clock gene promoters. We performed these experiments in continuous low light (10 μmol m^{-2} s^{-1}) because we observed that sucrose shortened circadian period strongly in low light, compared to subtle effects in higher light (50 μmol m^{-2} s^{-1}) (Extended Data Fig. 2a, b). CO_2-depletion (Fig. 1a) or DCMU treatment (Extended Data Fig. 2c, d) lengthened period of clock reporters by an average of 2.9 h and 2.5 h, respectively, compared to controls. Either treatment increased activity of *PRR7* promoter:LUC (*PRR7:*LUC) and reduced

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Author contributions M.J.H. and A.A.R.W. designed research; M.J.H., O.M., F.C.R., K.E.H. performed experiments and analyzed data; M.J.H. and A.A.R.W. prepared the manuscript.

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activity of *CIRCADIAN CLOCK ASSOCIATED1* (*CCA1*):LUC, which damped towards arrhythmia (Fig. 1a and Extended Data Fig. 2b). In contrast to exogenous sucrose, the effects of DCMU were similar in high or low light (Fig. 1b, Extended Data Fig. 2b). Exogenous sucrose is likely ineffective in altering period in higher light because the response is already saturated by higher endogenous sugars produced from photosynthesis, whereas complete inhibition of photosynthesis will be effective in either light condition.

In light-dark cycles there are robust endogenous rhythms of soluble sugars, which peak ~4-8 h after dawn[6,11] (Extended Data Fig. 3a). Inhibition of photosynthesis by either CO_2-depletion or DCMU treatment reduced endogenous sugar 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 re-supplied exogenous sucrose to CO_2-depleted or DCMU-treated seedlings. Period lengthening by either treatment was suppressed by addition of exogenous sucrose (Fig. 1c). The effects of DCMU treatment on CCA1:LUC rhythms were reversed by addition of as little as 5 mM (0.15 % w/v) exogenous sucrose to the growth media (Fig. 1d, e, Extended Data Fig. 4a). We also tested the effect of norflurazon or lincomycin, which both trigger retrograde signalling from the chloroplast to the nucleus[12]. Neither treatment lengthened circadian period of PRR7:LUC (Fig. 1c) or inhibited CCA1:LUC activity (Extended Data Fig. 4b) in the presence or absence of exogenous sucrose. Furthermore, we did not find evidence that photosynthesis might affect clock function through mechanisms associated with reactive oxygen species (ROS) production (Extended Data Fig. 5), consistent with a recent report[13].

Since our data suggest that the effects of photosynthesis on the circadian clock are mediated by sugars, we investigated the role for sugars in circadian function in more detail. We first tested whether the effects of exogenous sucrose represent a general response to sugar. Circadian period of CCA1:LUC, PRR7:LUC and *TIMING OF CAB1* (*TOC1*):LUC were on average 4.2 h shorter in seedlings grown in media containing 90 mM sucrose (3% w/v), glucose or fructose compared to mannitol-treated controls in continuous low light (Fig. 2a, b). Similarly, exogenous sucrose, glucose or fructose, but not mannitol or a non-metabolisable glucose analog 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 reporters are absent or very low in continuous dark[10] (Extended Data Fig. 6b). Exogenous sucrose can reinitiate circadian oscillations of the clock output reporter *CHOROPHYLL A/B BINDING PROTEIN2 (CAB2)*: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 dark[10]. We observed the same behaviour for reporters of the core circadian oscillator and confirmed that exogenous sucrose led to increased *CCA1* transcripts 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 through increased availability of ATP and suggests a role for sugar in entrainment. To directly test whether sugars act in entrainment, we determined a phase-response curve (PRC) for exogenous sucrose, which assesses the ability of a stimulus to alter circadian phase across a circadian cycle[14]. In continuous low light, the phase of CCA1:LUC and TOC1:LUC peak activity were shifted by pulses of exogenous sucrose inducing phase advances up to 2 h around dawn and phase delays around dusk (Fig. 2c, Extended Data Fig. 8). We observed subtle differences between reporters, similar to phase-setting by temperature[15].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 sugar acting as a Type 1 *zeitgeber* participating in circadian entrainment[14].

A key feature of entrainment is variation, or 'gating', of the response to the *zeitgeber* in a time-dependent manner[3]. 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 (ZT6). These data demonstrate that input of sugar to CCA1:LUC activity is gated to be most responsive to sucrose availability early in the light period.

We next compared responses of the Arabidopsis circadian system to sucrose and light because light can act as a strong, Type 0 *zeitgeber*[3,14] and drives sugar production from photosynthesis. In dark-adapted seedlings, there was a similar transient increase in CAB2:LUC peaking ~5 h after treatment with light or sucrose (Fig. 2e). By contrast, the first circadian peak of CAB2:LUC occurred 26.9 h after onset of light compared to 22.8 h after sucrose addition, indicating a 4.1 h advanced phase set by sucrose compared to light (Fig. 2e, f). The difference in phase setting was not concentration-dependent for sucrose, or quantity-dependent for light within the range tested (Fig. 2f, Extended Data Fig. 8d). When photosynthetic sugar production was inhibited in the light by DCMU, the phase set by light was delayed by a further 2.5 to 3.5 h (Extended Data Fig. 8e). These data demonstrate that these two *zeitgebers* both act 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 highest endogenous sugar concentrations (Extended Data Fig.3). We propose that a concentration threshold of photosynthetically-derived sugar provides input to the central oscillator acting as a 'metabolic dawn' that contributes to entrainment of the Arabidopsis circadian clock (Extended Data Fig. 1).

Having established that sugars derived from photosynthesis contribute to circadian entrainment in Arabidopsis, we next investigated how this might occur. The increase in PRR7:LUC in DCMU-treated or CO_2-depleted seedlings (Fig. 1a, Extended Data Fig. 2) suggested that photosynthesis regulates *PRR7* abundance. We measured transcript levels of morning-expressed circadian clock genes in shoots of control and DCMU-treated seedlings (Fig. 3). *PRR7* transcript levels were 3.7- to 8.2-fold higher in DCMU-treated seedlings than controls between ZT10 and ZT16 and this difference was suppressed when sucrose was added to media. *PRR5* transcripts were only 1.6- to 2.9-fold higher in DCMU-treated seedlings around dawn, and *PRR9* transcript levels were unaffected. *CCA1* and *LHY* transcripts were 3.0- to 8.4-fold lower at around dawn, following the increase in *PRR7*, in DCMU-treated seedlings compared to controls. These data are consistent with the LUC reporter data (Extended Data Fig. 2) and suggest that the effect of photosynthesis is most pronounced on *PRR7*.

These data led us to hypothesise that photosynthetic input to the circadian oscillator might act through PRR7, a transcriptional repressor that acts on the *CCA1* promoter during the night[16]. 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 subjective night, but this was most pronounced during the morning (Extended Data Fig. 9a). We tested whether induction of *CCA1* depends on PRR7. CCA1:LUC induction was significantly attenuated in *prr7*-11 mutants compared to wild type (Extended Data Fig. 9b). These data are consistent with sugars activating *CCA1* through repression of *PRR7*. Next we examined whether PRR7 contributes to circadian period adjustment by sucrose. Exogenous sucrose shortened the period of circadian rhythms of CCA1:LUC by 2.7 h in wild-type whereas rhythms in *prr7*-11 mutants were not shortened by exogenous sucrose (Fig. 4a). Similarly, the period of circadian rhythms of delayed fluorescence[17] was also shortened by exogenous sucrose in the wild type, but not in *prr7*-11 (Extended Data Fig. 9c). To assess whether there is also a role for PRR7 in circadian

entrainment by sugars, we determined a PRC for *prr7*-11 to pulses of exogenous sucrose. In contrast to the wild type (Fig. 2c), sucrose did not induce phase advances in *prr7*-11 mutants (Extended Data Fig. 9d). Since SENSITIVE TO FREEZING 6 (SFR6), a subunit of the mediator complex, contributes to period adjustment by sucrose by an unknown mechanism[9], we determined whether other previously identified pathways participate in the regulation of the circadian oscillator by sugar. 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 significantly shorter circadian period in the presence of sucrose compared to control media (Fig. 4b, Extended Data Fig. 10). Together, these data indicate a specific role for PRR7, acting through CCA1, in regulation of the circadian clock by photosynthetically-derived sugars, and that this might occur through a novel signaling pathway.

Our findings led us to test whether *PRR7* might be more widely involved in sugar signalling. When germinated on media containing 180 mM (6% w/v) sucrose, *prr7*-11 mutants were resistant to repression of chlorophyll accumulation (Fig 4c, d), similar to that observed in *glucose insensitive2/hexokinase1* (*gin2*-1) mutants[18]. In addition, *prr7*-11 contained elevated endogenous sugar concentrations around dawn (Fig. 4e) suggesting a role for PRR7 in regulating endogenous sugar accumulation. This is consistent with previous reports of involvement of PRR proteins in regulating chlorophyll biosynthesis and primary metabolism[19–21].

Altered feeding cycles can influence phase of peripheral clocks in animals[22,23]. Similarly, it was previously suggested that a shoot-derived photosynthate might regulate a simplified circadian oscillator in Arabidopsis roots[24]. Photosynthesis contributes to entrainment by an unknown mechanism in the green algae, *Chlamydomonas reinhardtii*[25]. From analysis of the effects of altered photosynthates on free-running circadian rhythms and examining the role of PRR7, we have demonstrated that photosynthetically-derived sugars act to provide metabolic feedback that entrains the Arabidopsis circadian clock in shoots. We propose that following light-activation of *PRR7* at dawn, accumulation of endogenous sugars from photosynthesis repress the *PRR7* promoter, leading to de-repression of *CCA1*. Thus, *PRR7* expression is coordinately modulated by light and photosynthesis, permitting PRR7 to act as a transcriptional repressor in circadian sugar signaling (Extended Data Fig. 1). This defines a novel metabolic feedback loop that contributes to circadian entrainment in plants.

Methods

Plant materials and growth methods

CCA1:*LUC*, *TOC1*:*LUC, PRR7*:*LUC* and *CCR2*:*LUC* are in Col-0 ecotype, *GI*:*LUC*, *PRR9*:*LUC* and *CAB2*:*LUC* are in Ws ecotype. *CCA1:LUC* was introduced into Ler and *prr5*-11, *prr7*-11, *prr9*-10[26], *prr3*-1[27], *gigantea (gi*-2)[28], *zeitlupe* (*ztl*-3)[29], *gin2-1*[18], *fructose insensitive 1 (fins1*-1)[30], *abscisic acid deficient* (*aba2-1/gin1*)[31], *aba3-1/gin5*[32], *abscisic acid insensitive 1 (abi1*-1)[33], *constitutive triple response 1 (ctr1-12/gin4*)[34], *hookless 1* (*hls1*-1)[35], *phytochrome A (phyA-201 phyB-5*)[36], *cryptochrome 1* (*cry1-1*)[37], *long hypocotyl 5* (*hy5*-215)[38], *constitutive photomorphogenic 1* (*cop1*-4)[39], *far-red elongated hypocotyl 3* (*fhy3*-1)[40], *phytochrome interacting factor 3* (*pif3*-3)[41], *spindly* (*spy*-3)[42] by crossing. Ler/*CCA1:LUC* and *gin2*-1/*CCA1:LUC* were backcrossed to Ler or *CCA1:LUC*, respectively, two times. The *gin2*-1(Col-0) line was used for sugar-insensitivity experiments to allow direct comparison to *prr* mutants and Col-0. Surface-sterilised seeds were sown on half-strength Murashige & 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 d, then grown in 12 h light-12 h dark cycles under 50 μmol m^{-2} s^{-1} cool fluorescent white light at constant 19°C.

Photon counting experiments

Clusters of 5-10 seedlings were grown in 1/2 MS agar media and entrained in light-dark cycles (50 μmol m^{-2} s^{-1} light). For LUC measurement, seedlings were dosed twice with 1-2 mM D-luciferin between 12 and 48 h before commencing photon counting. Seedlings were released into continuous light after 7-11 d in light-dark cycles. Luminescence was detected for 800 s at each time point with an HRPCS4 (Photek) or a LB985 Nightshade (Berthold) camera. Delayed fluorescence[17] was measured for 5 s in a LB985 Nightshade camera. During photon counting, light was supplied from red (660 nm) and blue (470 nm) LEDs at 50 μmol m^{-2} s^{-1} during light-dark cycles and either 50 μmol m^{-2} s^{-1} (continuous light) or 10 μmol m^{-2} s^{-1} (continuous low light). Where indicated, data was normalised to average counts across the experiment for each replicate. All period and relative amplitude error estimates where performed on rhythms between 24-120 h in continuous conditions on non-normalised data using Fast-Fourier Transformed Non-Linear Least Squares (FFT-NLLS) analysis, implemented in Biological Rhythms Analysis Software Suite (BRASS) (http://millar.bio.ed.ac.uk/PEBrown/BRASS/BrassPage.htm). All *n* values represent biological replicates, and all data are representative of independently repeated experiments. Tests were justified by determining minimum difference of means with a power of 0.9. Two-sided statistical tests, including assessment of normal distribution and equal variance, were performed in Excel.

Gating of short-term responses of LUC reporters to sugars at 1.5 h intervals for 24 h was performed in 8 d old seedlings from ZT0 in continuous low light. Signal was normalised to average signal across a time-course of several days. The change in normalised LUC reporter activity in seedlings before and after 3 h exposure to 90 mM sugars was subtracted from the change in normalised LUC reporter activity in untreated seedlings.

PRC experiments were performed in 8 d old seedlings from ZT0 in continuous low light. Seedlings growing on 1 μm nylon mesh on 1/2 MS were transferred to 1/2 MS containing 90 mM sugars for 3 h at 1.5 h intervals. Phase was determined based on the time of the circadian peaks following sugar pulses and the PRC was determined relative to phase in control seedlings after accounting for period differences as described[14].

Treatments

Sugars were added to media for a final concentration of 90 mM (3% w/v sucrose) unless indicated otherwise. Chemical treatments were added to media at the following concentrations: 20 μM DCMU, 5 μM norflurazon, 220 μg ml^{-1} lincomycin. Seedlings were transferred to treatments 48-60 h before release into continuous conditions. CO_2-free air was produced by pumping ambient air through self-indicating soda lime, a 0.45 μm filter and autoclaved deionized water into a sealed growth plate with an outlet. CO_2 concentration of the air from the outlet was confirmed at < 1ppm using an infra-red gas analyser (ADC 255-MK3). For experiments with dark-adapted seedlings, sugars were added with a micropipette as ~ 0.1 vol of the growth media to give ~30 mM final concentration. For gating and PRC experiments, seedlings were transferred to media containing 90 mM sugars. Treatments in the dark were performed under dim green light.

Quantitative real-time PCR

Ten d old seedlings growing in light-dark cycles (50 μmol m^{-2} s^{-1}) were transferred to treatments at dusk and leaf tissue was collected at 3 h intervals between 37 and 58 h later. Total RNA was extracted from three biological replicates of frozen leaf tissue using RNeasy Plant Mini Kit (Qiagen) and RNase-free DNase on-column treatment (Qiagen). cDNA was synthesised from 1 μg RNA with RevertAid First Strand cDNA Synthesis Kit (Fermentas) using oligo-dT primer. Technical replicates of gene specific products were amplified in 10

μL reactions using Rotor-Gene SYBR Green PCR Kit (Qiagen) on a Rotor-Gene 6000 real-time PCR machine fitted with a Rotor-Disc 100 (Qiagen). Primers were *UBQ10-F* 5′ GGCCTTGTATAATCCCTGATGAATAAG 3′ *UBQ10-R* 5′ AAAGAGATAACAGGAACGGAAACATAGT 3′ *CCA1-F* 5′ GATGATGTTGAGGCGGATG 3′ *CCA1-R* 5′ TGGTGTTAACTGAGCTGTGAAG 3′ *LHY-F* 5′ ACGAAACAGGTAAGTGGCGACATT 3′ *LHY-R* 5′ TGGGAACATCTTGAACCGCGTT 3′ *PRR9-F* 5′ CCACAGTAACGAATCAGAAGCAA 3′ *PRR9-R* 5′ TTGTCCAGCAATCCCCTCA 3′ *PRR7-F* 5′ GGAAACTTGGCGGATGAAAA 3′ *PRR7-R* 5′ CGAGGGCGTTGTTCTGCT 3′ *PRR5-F* 5′ CCGAATGAAGCGAAAGGACA 3′ *PRR5-R* 5′ GGATTGGACTTGACGAACG 3′. Relative transcript levels were determined by incorporating PCR efficiencies as described[43].

Sugar 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 a Sucrose/Fructose/D-Glucose Assay Kit (Megazyme). For chorophyll measurements, fresh tissue was extracted in methanol and concentrations determined as described[44].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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

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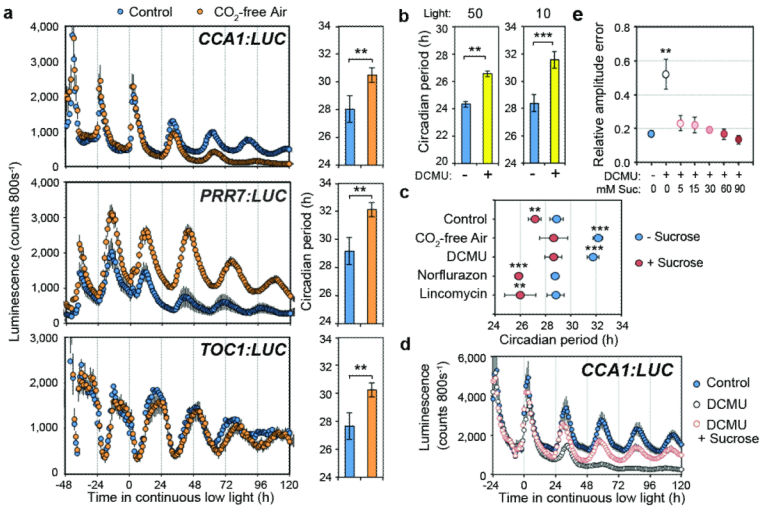

Figure 1. Photosynthetic sugars influence the circadian clock in Arabidopsis

a, LUC reporter rhythms (mean ± s.e.m.) and period estimates in seedlings grown in ambient or CO_2-free air in 10 μmol m^{-2} s^{-1} light, n =4. **b**, Period estimates of PRR7:LUC in continuous light in the presence or absence of DCMU (mean ± s.d.) n = 4. **c**, Period estimates of PRR7:LUC rhythms in continuous low light treated with inhibitors in the presence or absence of exogenous sucrose, (mean ± s.d.) n = 4. **d**, CCA1:LUC rhythms (mean ± s.e.m.) and **e**, relative amplitude error of CCA1:LUC rhythms in seedlings treated with DCMU in the presence or absence of exogenous sucrose, (mean ± s.d.) n = 4. ** P < 0.01 *** P < 0.001 by t-test.

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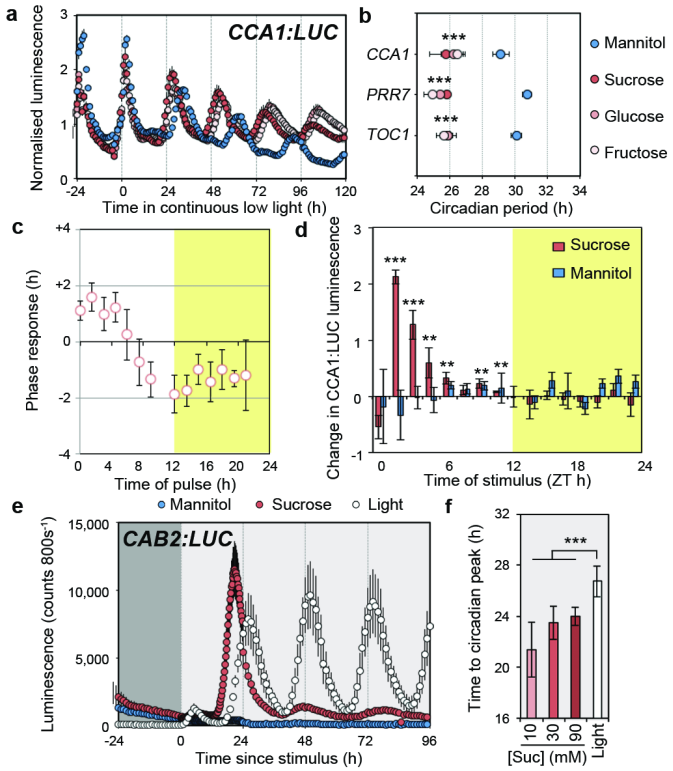

Figure 2. Metabolically active sugar is a *zeitgeber* which acts differently to light

a, CCA1:LUC rhythms (mean ± s.e.m) and **b**, period estimates in seedlings grown in continuous low light with the indicated sugar n =4. **c**, Phase response of CCA1:LUC rhythms to pulses of sucrose in continuous low light, (mean ± s.d.) n = 8. **d**, Change in normalised CCA1:LUC activity 3 h after sucrose or mannitol treatment in continuous low light, (mean ± s.d.) n = 4. **e**, CAB2:LUC rhythms in dark-adapted seedlings treated with sucrose or mannitol in continuous dark or transferred to continuous light, (mean ± s.e.m.) n = 4. **f**, Time to first circadian peak of CAB2:LUC after treatment with sucrose or light as in e, (mean ± s.d.) n = 4. ** $P < 0.01$ *** $P < 0.001$ by t-test.

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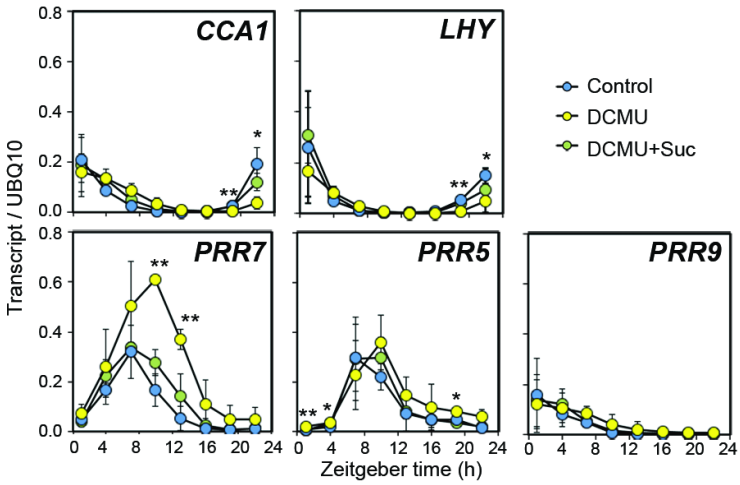

Figure 3. Photosynthetic sugar represses *PRR7* late in the photoperiod

Leaf transcript levels relative in seedlings treated with DCMU in the presence or absence of exogenous sucrose in light-dark conditions, (mean ± s.d.) $n = 3$. * $P < 0.05$ ** $P < 0.01$ by *t*-test.

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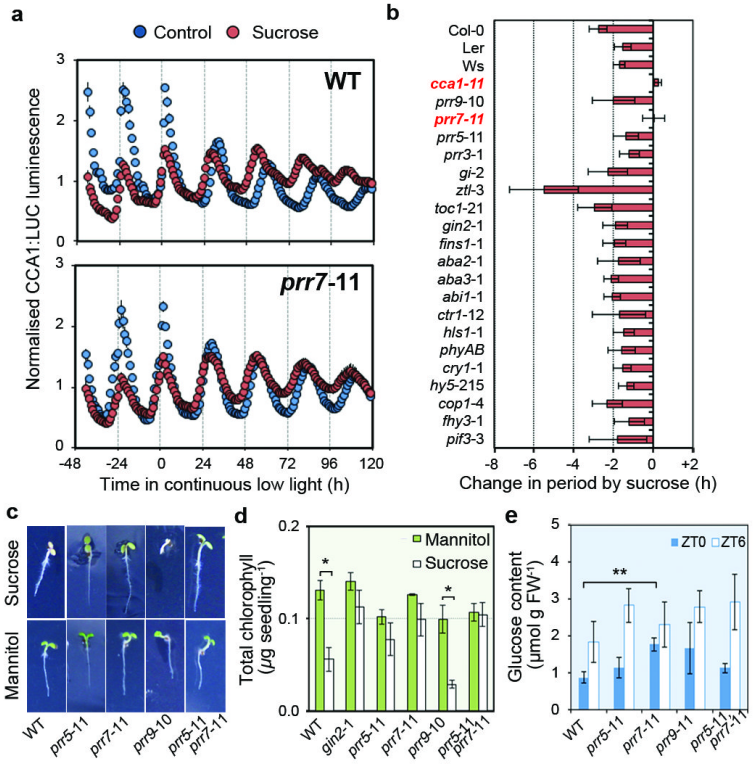

Figure 4. PRR7 contributes to circadian sugar signalling

a, CCA1:LUC rhythms in wild-type and *prr7*-11 seedlings in continuous low light in the presence or absence of exogenous sucrose, (mean ± s.e.m.) $n = 4$. **b**, Change in period of CCA1:LUC rhythms in circadian, sugar and light signalling mutants, CAB2:LUC (Ws and *cca1*-11) or CCR2:LUC (*toc1*-21) grown in the presence of sucrose compared to control media in continuous low light (mean ± s.d.) $n = 8$. **c**, Seedlings germinated on 180 mM sucrose or mannitol. **d**, Total chlorophyll content of seedlings germinated on sucrose or mannitol, (mean ± s.d.) $n = 3$. **d**, Glucose content of seedlings, (mean ± s.d.) $n = 3$. * $P < 0.05$ ** $P < 0.01$ by *t*-test.

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