

1 **Supplementary Tables**

2 **Supplementary Table 1.** Related to Figure 1 and Supplementary Figure 1. CaCl₂

3 Treatments Used to Generate Artificial [Ca²⁺]_{cyt} Oscillations.

4 Dosing regime for generating artificial [Ca²⁺]_{cyt} oscillations in unentrained plants. Plants
5 were grown for 12 days in continuous light without stratification before being dosed with
6 the concentrations described.

7

Time (h)	For artificial circadian [Ca ²⁺] _{cyt} oscillations (Supplementary Figure 1b)				For Gene Expression (Figure 1 and Supplementary Figure 1c)
	mM CaCl ₂ added				
	Day 1	Day 2	Day 3	Day 4	Day 1
0	0.5	1	1	5	0
1	1	5	5	10	5
2	5	10	10	20	10
3	10	20	20	50	20
4	20	50	50	75	50
5	50	75	75	100	100
6	60	85	85	110	150
12	0	0	0	0	0

8

9 **Supplemental Methods**

10 **Effect of Ca²⁺ agonist solution on [Ca²⁺]_{cyt}**

11 Luminometry of changes in [Ca²⁺]_{cyt} in response to N-(6-Aminoethyl)-5-chloro-1-
12 naphthalenesulfonamide hydrochloride (W7) (Calbiochem) and CaCl₂ and subsequent
13 calibration of bioluminescence to estimate [Ca²⁺]_{cyt} were measured as follows.

14 Luminescence of at least 3 individual 12 day old 35S:AEQ Col-0 or *cml23-2 cml24-4*
15 seedlings was measured in opaque 96 imaging plates using FLUOstar (BMG Labtech,

16 Germany). Aequorin was reconstituted with 20 μM coelenterazine (20 °C, overnight) and
17 response to Ca²⁺ agonists (henceforth referred to as “W7 solution”) was determined by

18 injecting the W7 solution onto the plants to reach a final concentration of 660 μM W7 and
19 50 mM CaCl₂ (in 2.5 % (v/v) DMSO), respectively. The injection of room temperature

20 distilled water was used as a touch response control for all the treatments. To convert
21 luminescence into Ca²⁺ concentrations, 3 M CaCl₂ and 30% ethanol were added to

22 discharge the remaining aequorin. Measurements were made until the detected
 23 luminescence reached 10% of the first peak after discharge injection. $[Ca^{2+}]_{\text{cyt}}$ levels were
 24 determined according to [S1]. All experiments were repeated at least twice.

25

26 **The effect of NO agonists and antagonists on the circadian signalling network**

27 The response of *35S:AEQ* and *CAB2:LUC* to NO agonists and antagonists was measured
 28 in WS transformed seedlings as previously reported in [S2]. S-nitroso-N-
 29 acetylpenicillamine (SNAP, Calbiochem UK) was diluted in deionised water to the required
 30 concentration from a 600 mM stock in 100% (v/v) ethanol or methanol. 2-4-carboxyphenyl-
 31 4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (c-PTIO; Calbiochem UK) was diluted in
 32 deionised water from a 60 mM stock in 0.4 mg ml⁻¹ 4-(2-hydroxyethyl)-1-
 33 piperazineethanesulfonic acid (HEPES;Fisher, UK) buffer.

34

35 **Primers used for qPCR.**

36

Gene	Primers
<i>CCA1</i>	F: CCTCAAACCTTCAGAGTCCAATGC R: GACCCTCGTCAGACACAGACTTC
<i>LHY</i>	F: ACGAAACAGGTAAGTGGCGACATT R: TGGGAACATCTTGAACCGCGTT
<i>PRR9</i>	F: CATCAAAAAGCTTAGCCTCTCT R: CTGTGGACTGAACTTGGT
<i>PRR7</i>	F: GCACTTAAAGACCAGCCCCATTGA R: TCGTCGGAACATCCCTGTCATCAT
<i>PRR5</i>	F:AAGGTTTCGTTACGAGAGCCGGAAG R: TTGGCCTTTGATTTCGTGGTCGTTG
<i>PRR3</i>	F: TGACCCTTGGGTGCTTTCAGG R: AGAAGATGTCACAGCTCTAGCGGA
<i>CHE</i>	F: TAATGGGTGGTGGTGGTTCTG R: GCAAAGCTCCAGACTTGTCC
<i>TOC1</i>	F: TCACCATGAGCCAATGAAAA R: TTGAAACTTCTCCGCCAAAC
<i>GI</i>	F: GGTGACGGTTTATCCAATCT R: CGGACTATTCATTCCGTTCTT
<i>ZTL</i>	F: TGACGAGGTTGTGTCTATGA R: AGCACCAGGAACAGTCTCTA
<i>ELF3</i>	F: GCACAGACTGATTAAGGTTCAAAAAC R: CTTCACTGGATAGCTTTTAGCAG
<i>ELF4</i>	F: TGTCGTTGACTTGTGAATCAGTG

R: CGATGTGGGAGAATCTTGAC
LUX F: TAACGTGGAGGAGGAAGATCGA
 R: TCC ATCACCGTTTGATGTCTTT
UBQ10 F: GGCCTTGATAATCCCTGATGAATAAG
 R: AAAGAGATAACAGGAACGGAAACATAGT
PP2a F: TAACGTGGCCAAAATGATGC
 R: GTTCTCCACAACCGCTTGGT
IPP2 F: GTATGAGTTGCTTCTCCAGCAAAG
 R: GAGGATGGCTGCAACAAGTGT

37

38 **Supplementary Statistical Parameters**

39 The Student's *t*-test analyses for Fig.1c provided with a degree of freedom (d.f.) of 4 and
 40 the following *t*-values (*t*) and *p*-values (*p*): *CHE* ZT36 *t*=7.914, *p*=0.001; *PRR9* ZT36
 41 *t*=1.353, *p*=0.247; *GI* ZT36 *t*=0.468, *p*=0.664; *TOC1* ZT36 *t*=0.709, *p*=0.518; *PRR3* ZT36
 42 *t*=4.023, *p*= 0.016; *PRR5* ZT36 *t*=2.732, *p*=0.052; *ZTL* ZT36 *t*=1.172, *p*=0.306; *ELF3* ZT36
 43 *t*=1.458, *p*=0.219; *LUX* ZT36 *t*=-0.546, *p*= 0.614; *CHE* ZT48 *t*=1.547, *p*=0.197; *CCA1* ZT48
 44 *t*=-0.410, *p*=0.703; *PRR9* ZT48 *t*=-3.902, *p*=0.018; *GI* ZT48 *t*=-0.346, *p*=0.746; *TOC1* ZT48
 45 *t*=-0.132, *p*=0.901; *PRR3* ZT48 *t*=4.103, *p*= 0.015; *PRR7* ZT48 *t*= 4.613 *p*= 0.010; *PRR5*
 46 ZT48 *t*=-0.0336, *p*=0.975; *ZTL* ZT48 *t*=0.186, *p*=0.862; *ELF3* ZT48 *t*=1.748, *p*=0.155; *LUX*
 47 ZT48 *t*=-0.465, *p*= 0.666. The Mann-Whitney Rank Sum Tests for Fig. 1c provided with the
 48 following *T*, *U* and *p* values: *CCA1* ZT36 *T*=9 *U*=3 *p*=0.7; *PRR7* ZT36 *T*=12 *U*=3 *p*=0.7;
 49 *ELF4* ZT36 *T*=14 *U*=1 *p*=0.2; *ELF4* ZT48 *T*=12 *U*=3 *p*=0.7; *LHY* ZT48 *T*=13 *U*=2 *p*=0.4.

50 The Student's *t*-test analyses for Fig.2 provided with the following d.f, *t* and *p* values when
 51 mutants were compared to Col-0: Fig. 2a, *cml23-2* d.f=138, *t*=-0.687, *p*=0.493; *cml24-1*
 52 d.f=165, *t*=-4.078, *p*<0.001; *cml23-2 cml24-1* d.f=168, *t*=-6.716, *p*<0.001; Fig. 2b, *cml23-2*
 53 d.f=130, *t*=-2.016, *p*=0.046; *cml24-4* d.f=110, *t*=-7.905, *p*<0.001). The Mann-Whitney Rank
 54 Sum Tests for Fig. 2b for comparison of Col-0 vs. *cml23-2 cml24-4*, provided with the
 55 following values: *T*=2313, *U*=297 and *p*<0.001.

56 The Student's *t*-test for Fig.3c and the Mann-Whitney Rank Sum test for Fig. 3d, provided
 57 with the following values, d.f=14, *t*=-6.050 and *p*<0.001 and *T*=319.5, *U*=43.5 and *p*<0.001,

58 respectively. The Student's *t*-test analyses for Fig.3e provided with a d.f. of 4 and the
59 following t and p values: *CCA1* ZT48 $t=0.35$ $p=0.744$, ZT50 $t=-0.68$ $p=0.534$, ZT52 $t=-2.8$
60 $p=0.049$, ZT54 $t=-3.641$ $p=0.022$, ZT56 $t=-3.037$ $p=0.039$, ZT58 $t=-2.427$ $p=0.072$, ZT62
61 $t=0.826$ $p=0.455$, ZT64 $t=2.476$ $p=0.069$, ZT66 $t=-4.907$ $p=0.008$, ZT68 $t=3.802$ $p=0.019$,
62 ZT70 $t=-2.573$ $p=0.062$, ZT72 $t=1.762$ $p=0.153$; *TOC1* ZT48 $t=-3.865$ $p=0.018$, ZT50
63 $t=0.412$ $p=0.701$, ZT52 $t=1.491$ $p=0.21$, ZT54 $t=-7.576$ $p=0.002$, ZT56 $t=8.392$ $p=0.001$,
64 ZT58 $t=-2.452$ $p=0.07$, ZT60 $t=1.046$ $p=0.355$, ZT62 $t=-0.668$ $p=0.541$, ZT64 $t=1.289$
65 $p=0.267$, ZT66 $t=-0.38$ $p=0.723$, ZT68 $t=-0.441$ $p=0.682$, ZT70 $t=2.321$ $p=0.081$, ZT72 $t=-$
66 1.278 $p=0.27$; *PRR7* ZT50 $t=0.973$ $p=0.386$, ZT52 $t=-2.357$ $p=0.078$, ZT54 $t=4.948$
67 $p=0.008$, ZT56 $t=-0.525$ $p=0.627$, ZT58 $t=-4.613$ $p=0.010$, ZT60 $t=-0.0276$ $p=0.031$, ZT62
68 $t=-1.973$ $p=0.12$, ZT64 $t=0.477$ $p=0.658$, ZT66 $t=-2.308$ $p=0.082$, ZT68 $t=-0.728$ $p=0.507$,
69 ZT70 $t=15.107$ $p<0.001$, ZT72 $t=-6.133$ $p=0.004$. The Mann-Whitney Rank Sum Tests for
70 Fig. 3e, provided with the following values: *CCA1* ZT60 $T=15$, $U=0$ and $p=0.1$; *PRR7*
71 ZT48 $T=15$, $U=0$ and $p=0.1$.

72 The Student's *t*-test analyses for Fig. 4 provided with the following d.f., t and p values:
73 nicotinamide d.f=46 $t=-0.689$ $p=0.495$; high light water d.f=13 $t=-2.827$ $p=0.014$, high light
74 sucrose d.f=14 $t=-2.131$ $p=0.051$; low light water d.f=30 $t=-6.229$ $p<0.001$, low light
75 sucrose d.f=30 $t=1.944$ $p=0.061$; white light d.f=6 $t=-4.654$ $p=0.00349$, red light d.f=19
76 $t=0.585$ $p=0.5655$, blue light d.f=12 $t=-4.570$ $p=0.000644$. The Mann-Whitney Rank Sum
77 Tests for Fig. 4, provided with the following values: water (Fig. 4a) $T=216.5$ $U=80.5$
78 $p<0.001$; mannitol $T=81.5$ $U=2.5$ $p=0.001$; water (Fig. 4f) $T=31$ $U=3$ $p=0.002$, cPTIO $T=28$
79 $U=0$ $p<0.001$.

80 Kruskal-Wallis One Way ANOVA analyses for Fig. 5 and Fig.6 a and b provided with a d.f
81 of 2 and the following H and p values: *toc1-2* $H=41.741$ $p<0.001$, *cca1-11* $H=58.291$
82 $p<0.001$, *lhy-21* $H=47.679$ $p<0.001$, *ztl-3* $H=41.768$ $p<0.001$, *che-2* $H=37.030$ $p<0.001$,

83 *che-1* H=49.271 p<0.001. These analyses were followed by Dunn's method, see
84 Supplementary Table 3 for comparison between lines and precise p values. Kruskal-Wallis
85 One Way ANOVA analyses for Fig.6 c and d provided with a d.f of 2 and the following H
86 and p values: LD *che-2* H=33.924 p<0.001, *che-1* H=36.191 p<0.001; SD *che-2* H=31.352
87 p<0.001, *che-1* H=28.263 p<0.001. These analyses were followed by Tukey test (LD) or
88 Dunn's method (SD).

89 The Student's *t*-test analyses for Supplementary Fig. 3c provided with a d.f. of 4 and the
90 following t and p values: *CHE* ZT36 *t*=2.744, p=0.052; *PRR9* ZT36 *t*=0.0558, p=0.958;
91 *PRR3* ZT36 *t*=-4.008, p= 0.016; *PRR5* ZT36 *t*=-0.449, p=0.677; *PRR7* ZT36 *t*=1.033,
92 p=0.36; *CHE* ZT48 *t*=3.120, p=0.036; *PRR9* ZT48 *t*=-4.857, p=0.008; *PRR3* ZT48 *t*=2.131,
93 p= 0.1; *PRR7* ZT48 *t*= 2.631 p= 0.058; *PRR5* ZT48 *t*=-0.0366, p=0.973.

94 One Way ANOVA and Kruskal-Wallis One Way ANOVA analyses for Supplementary Fig.
95 4 provided with a d.f of 2 and the following H or F and p values: exp2 *toc1-2* H=37.517
96 p<0.001, exp3 *toc1-2* F=115.307 p<0.001, exp2 *che-2* H=27.507 p<0.001, exp3 *che-2*
97 F=3.878 p=0.026, exp2 *che-1* H=37.883 p<0.001. These analyses were followed by Holm-
98 Sidak method or Dunn's method, see Supplementary Table 3 for comparison between
99 lines and precise p values.

100 The Student's *t*-test analyses for Supplementary Fig. 5 provided with the following d.f, t
101 and p values when mutants were compared to the correspondent wild type: LD *cmI23-2*
102 *cmI24-4* d.f=29, *t*=-4.787, p<0.001; *che-1* d.f=29, *t*=3.270, p=0.003; *gi-11* d.f=30, *t*=35.570,
103 p<0.001; SD, *che-2* d.f=30, *t*=-8.268, p<0.001; *cca1-11* d.f=30, *t*=4.384, p<0.001, *gi-11*
104 d.f=27, *t*=2.333, p=0.027). The Mann-Whitney Rank Sum Tests for Supplementary Fig. 5
105 for comparison of the single mutants vs. their wild type, provided with the following values:
106 LD *che-2* T=360.000, U=0.000 p<0.001, *cca1-11* T=257.5, U=121.5 p=0.809, *elf3-4*

107 T=381.000, U=11.000 p<0.001; SD *cml23-2 cml24-4* T=351.000, U=8.500 p<0.001, *che-1*
108 T=288.500, U=103.500 p=0.365, *elf3-4* T=120.000, U=0.000 p<0.001.

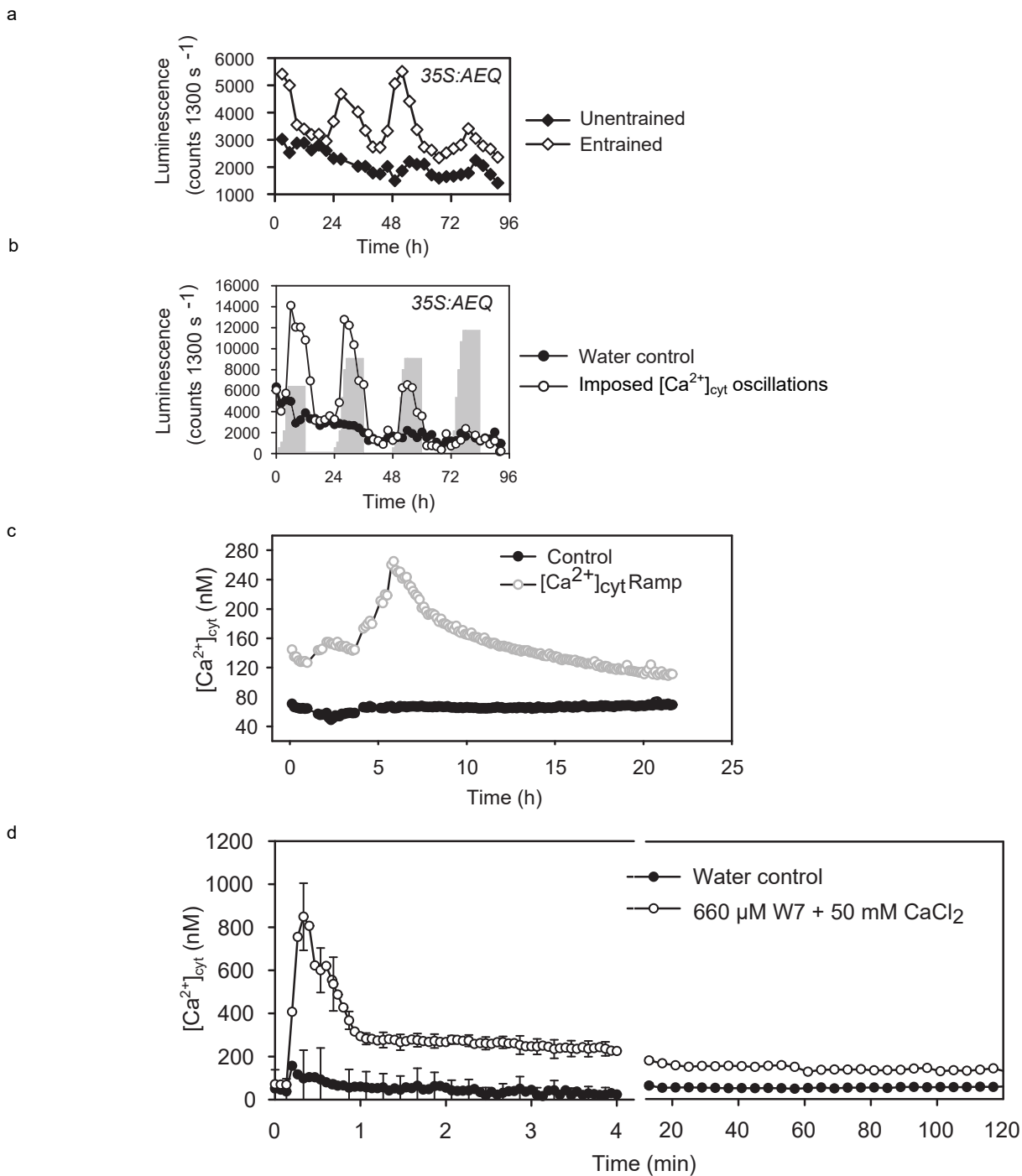
109

110 **Supplementary References**

- 111 1. Fricker, M.D., Plieth, C., Knight, H., Blancaflor, E., Knight, M.R., White, N.S., and
112 Gilroy, S. (1999). Fluorescence and luminescence techniques to probe ion activities in
113 living plant cells. In *Fluorescent and Luminescent Probes for Biological Activity*, W.T.
114 Mason, ed. (San Diego: Academic Press), pp. 569-596.
- 115 2. Xu, X., Hotta, C.T., Dodd, A.N., Love, J., Sharrock, R., Lee, Y.W., Zie, Q., Johnson,
116 C.H., and Webb, A.A.R. (2007). Distinct light and clock modulation of cytosolic free
117 Ca²⁺ oscillations and rhythmic *CHLOROPHYLL A/B BINDING PROTEIN2* promoters
118 activity in *Arabidopsis*. *Plant Cell* 19, 3474-3490.

119

120 **Supplementary Figures**

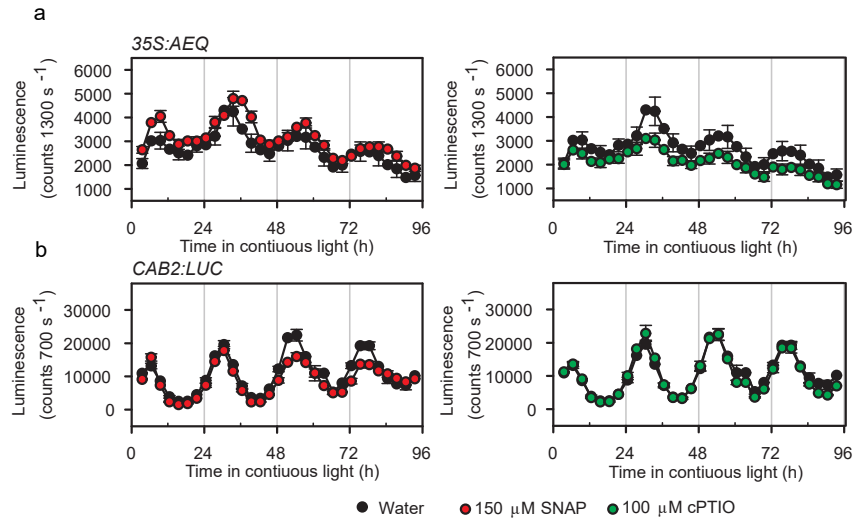


Supplementary Fig. 1. Related to Fig. 1. [Ca²⁺]_{cyt} Manipulation to Study the Effect of [Ca²⁺]_{cyt} Signals on Circadian Clock Genes Expression.

(a) Growth of seedlings in constant light without stratification (unentrained seedlings) results in the absence of [Ca²⁺]_{cyt} rhythms (closed diamonds) compared to entrained seedlings (open diamonds). (b) Imposing ramps of external CaCl₂ to unentrained seedlings, restores circadian oscillations in [Ca²⁺]_{cyt} (open circles) when compared to unentrained water-treated samples (closed circles). CaCl₂ was applied as shown by the shaded areas and as described in Supplementary Table 1. Results represent the mean for luminescence values from a minimum of three experiments consisting of 6 replicates each. Relative Amplitude Error (R.A.E) was used for rhythmicity analysis. Rhythms were considered robust if R.A.E.<0.5 and with poor robustness if R.A.E.>0.5.

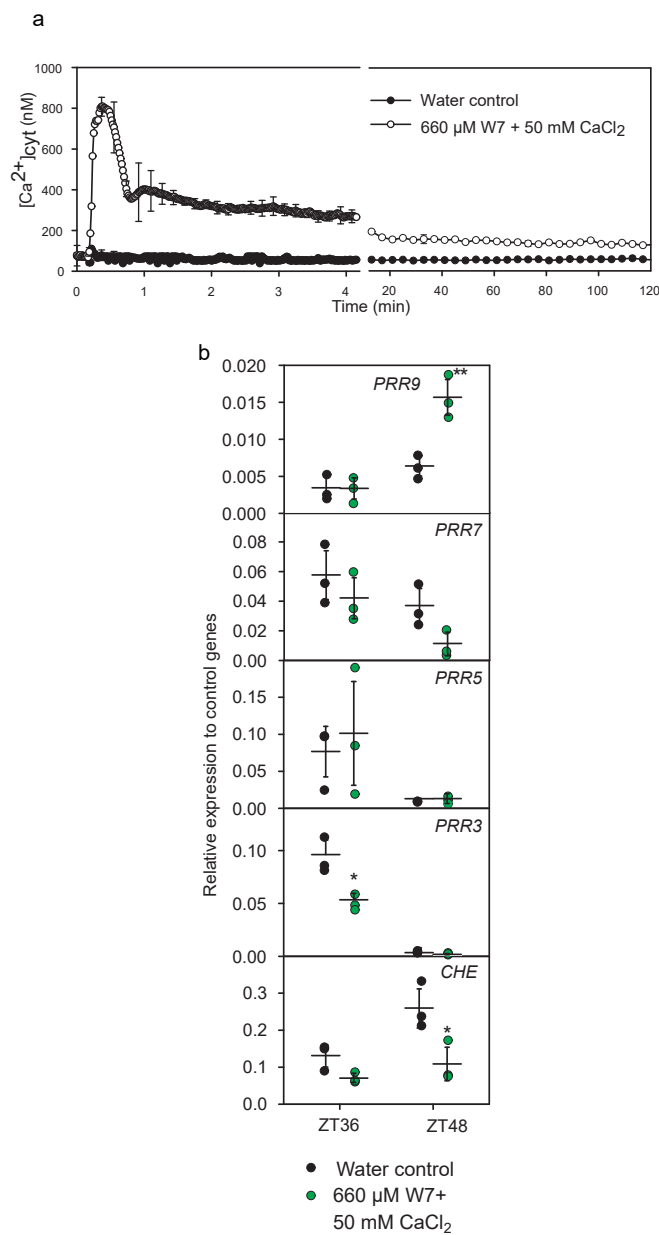
(c) A peak of [Ca²⁺]_{cyt} with a similar phase to that in entrained seedlings was generated using a 'ramp' of external CaCl₂ concentrations in unentrained seedlings. 35S:AEQ seedlings were treated as indicated in Supplementary Table 1. Photons were counted for 5 s every 8 min for 24 h. Results represent the mean from 3 independent experiments (n=12, biological replicates each).

(d) 35S:AEQ seedlings were grown for 12 days in light:dark cycles (12h:12h) and then placed in 96-well plates containing 20 mM coelenterazine. The effect of 660 μM W7 and 50 mM CaCl₂ (open circles) on [Ca²⁺]_{cyt} was measured using photon counting luminometry and compared to plants treated with distilled water (closed circles). Results represent the mean ± S.D. from one of two independent experiments (n=3 biological replicates).



Supplementary Fig. 2. NO levels do not affect circadian period.

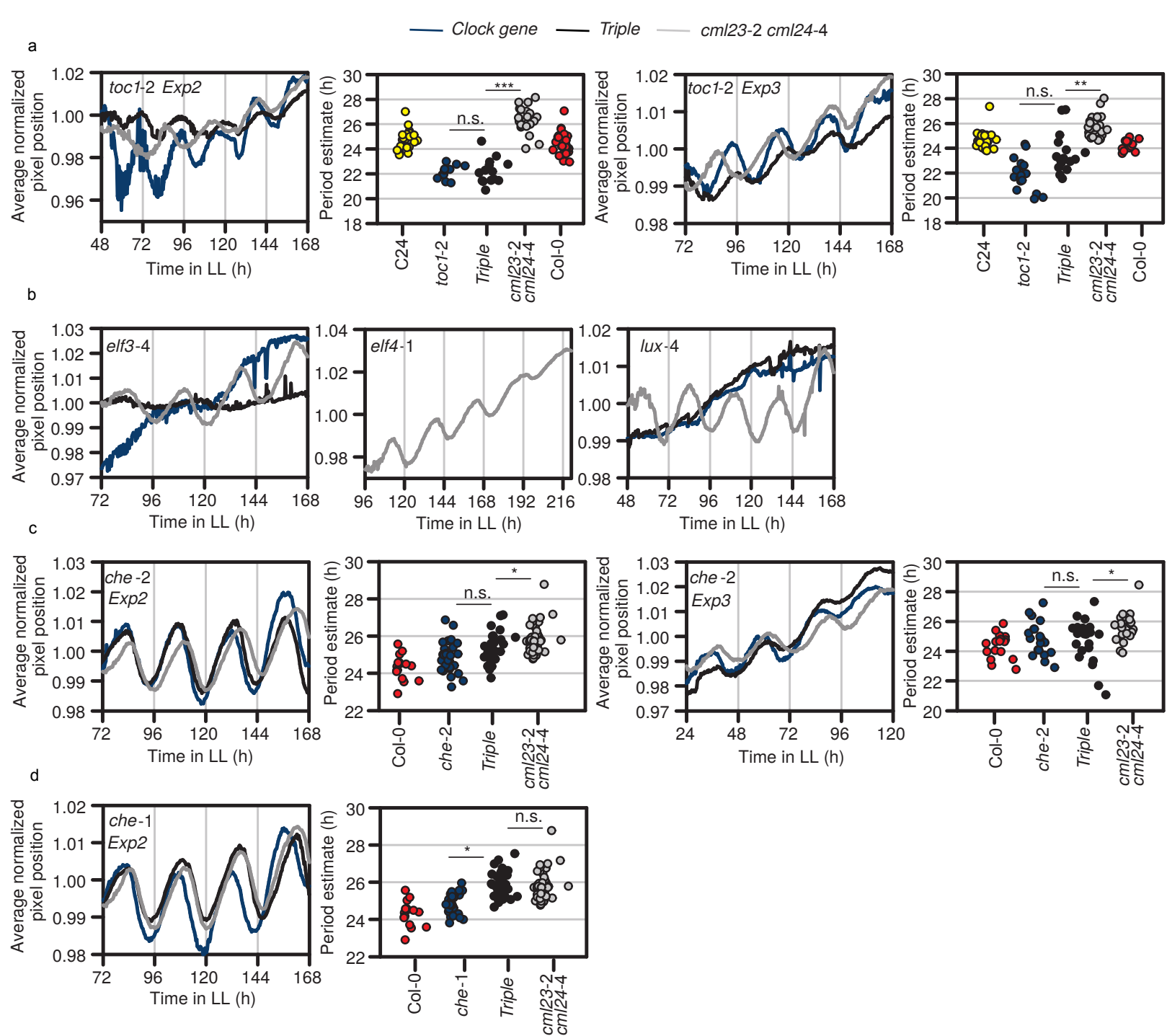
The NO donor SNAP (red) and the NO scavenger cPTIO (green) do not affect the circadian oscillations of $[Ca^{2+}]_{cyt}$ (**a**) or CAB2:LUC activity (**b**). Seedlings were imaged in LL, and SNAP or cPTIO were applied every 3 h. Results show mean \pm S.D. from a representative experiment ((**a**) water n=20, SNAP n=16, cPTIO n=12; (**b**) water n=11, SNAP n=12, cPTIO n=11).



Supplementary Fig. 3. The $[Ca^{2+}]_{cyt}$ Transcriptional Regulation of the Clock is not Dependent on CML24.

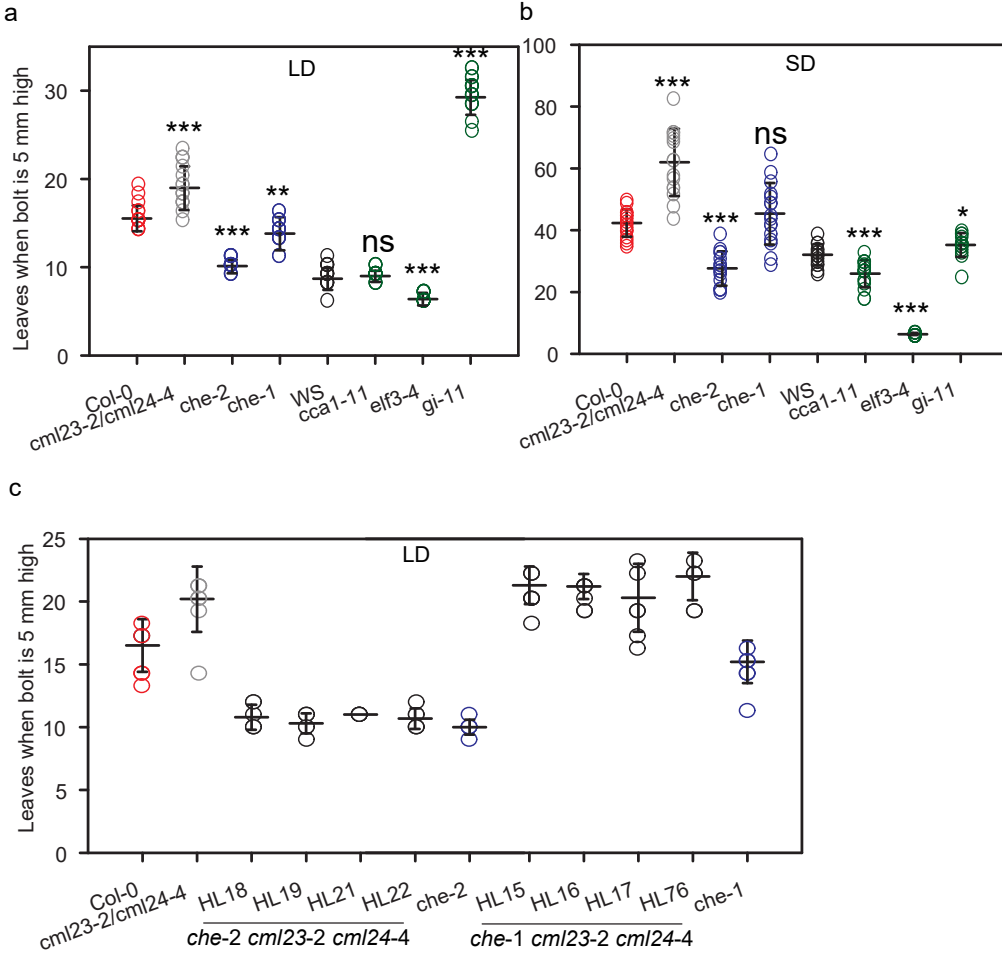
(a) Effect of external Ca^{2+} signals on $[Ca^{2+}]_{cyt}$ signaling in *cml23-2 cml24-4* plants. *cml23-2 cml24-4* Arabidopsis seedlings expressing 35S:AEQ were grown for 12 days on $\frac{1}{2}$ MS agar in LD and then placed in 96 well plates containing 20 mM coelenterazine. The effect of a solution containing 660 μ M W7 and 50 mM $CaCl_2$ (open circles) on $[Ca^{2+}]_{cyt}$ was measured using photon counting luminometry and compared to plants treated with distilled water (closed circles). Results represent the mean \pm S.D. from one of two independent experiments (n=3 biological replicates).

(b) *cml23-2 cml24-4* plants treated at ZT36 and ZT48 with a solution containing 660 μ M W7 and 50 mM $CaCl_2$ for 2 h, were assayed for changes in the abundance of circadian clock transcripts by qPCR. Dots represent each measurement and the black bars the mean \pm S.D. (n= 3 biological replicates). Single or double asterisk indicate significance of ≤ 0.05 and ≤ 0.01 , respectively, after two-tailed Student's t test analysis.



Supplementary Fig. 4. Related to Fig. 5 and 6. Epistatic Analysis of Leaf Movements Rhythms for *CML23/CML24* with *ELF3*, *ELF4*, *LUX*, *TOC1* and *CHE*.

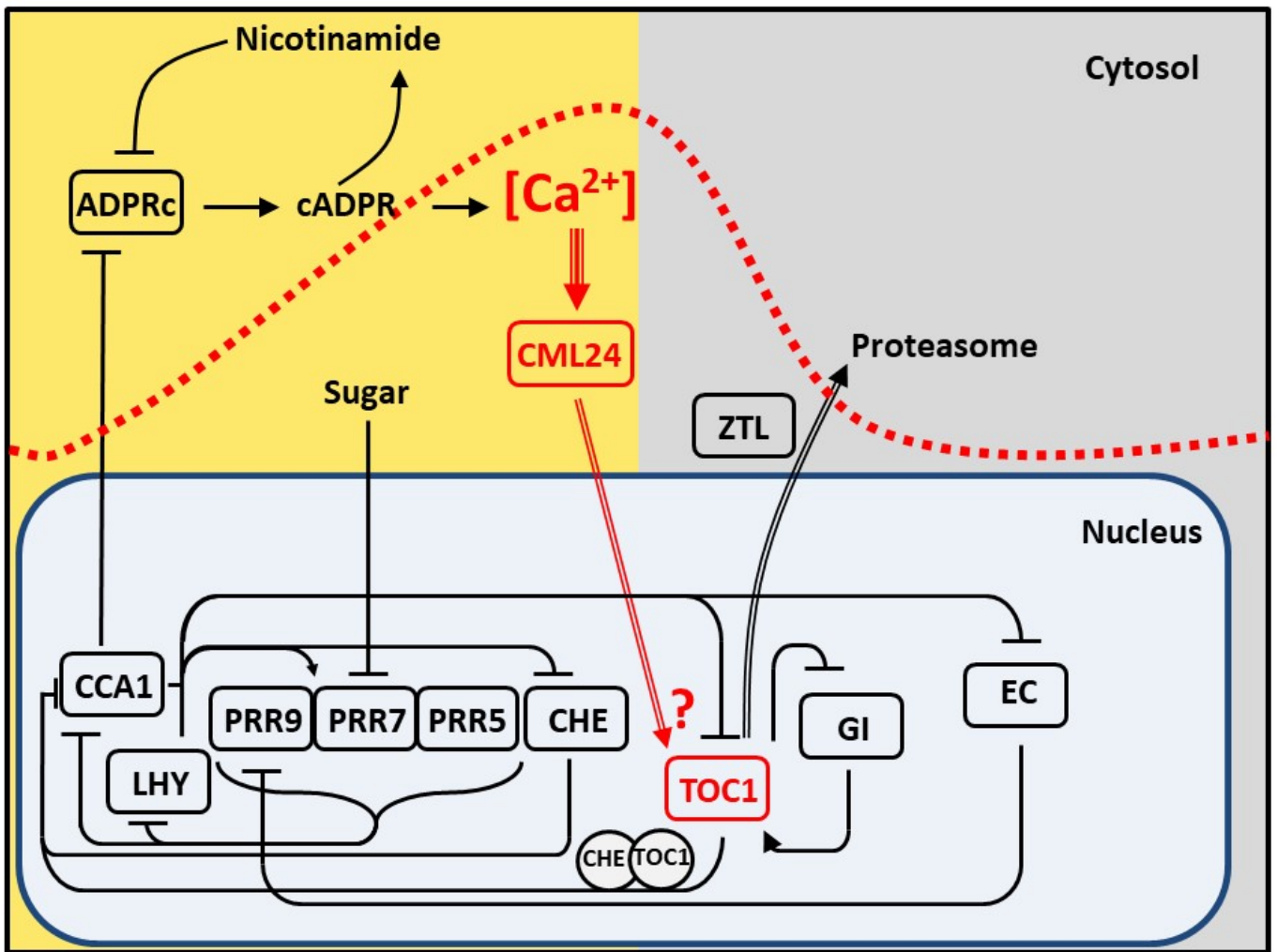
Average normalized traces of leaf positions and FFT-NLLS analysis of the circadian period for leaf movement experiments. **a** shows the results of *cml23-2 cml24-4* with *toc1-2* (exp2/3 Col-0 n=26/13, C24=21/29, *cml23-2 cml24-4*=22/25, *toc1-2*=11/17, triple mutant=14/16), **b** with *elf3-4*, *elf4-1* and *lux-4*, **c** with *che-2* (exp2/3 Col-0 n=16/18, *cml23-2 cml24-4*=48/21, *che-2* =31/23, triple mutant=35/22) and **d** with *che-1* (exp2 Col-0 n=16, *cml23-2 cml24-4*=48, *che-1*=29, triple mutant=46). Because rhythms were not detected in **b** for these single and triple mutants, FFT-NLLS analyses are not shown. Wild-type traces for leaf position were removed for clarity. All plants were grown under 12 h L: 12 h D cycles before the experiments. Data in **a**, **c** and **d** show the independent replicates of the experiments for *toc1-2*, *che-2* and *che-1* presented in Fig. 5 and 6. Data in **b** show one independent experiment representative of two. Data in **a** were obtained using a different triple mutant line than the one used in Fig. 5. Single or double asterisk indicate significance of ≤ 0.05 and ≤ 0.01 , respectively, after One-way ANOVA followed by Holm-Sidak method or Kruskal-Wallis One Way Analysis of Variance on Ranks followed by Dunn's method, when the triple mutant was compared to the single and *cml23-2 cml24-4* double mutant. See also Supplementary Table 3.



Supplementary Fig. 5. Related to Fig. 6. Flowering Time Study of *che-2* and *che-1* single mutants and four *cml23-2 cml24-4 che-1* and *cml23-2 cml24-4 che-2* triple mutant lines.

Flowering time responses under long day (16 h:8 h L:D) (**a**) or short day conditions (8 h: 16 h L:D) (**b**) for Col-0, *cml23-2 cml24-4* (Col-0), *che-2* (Col-0) and *che-1* (Col-0). *cca1-11*(WS), *elf3-4* (WS) and *gi-11* (WS) were used as controls. Number of leaves were recorded when the emerging bolt was 5 mm high. Data represent the mean \pm S.D. (n=16; in LD Col-0 n=15 and *gi-11* n=11; in SD *cml23-2 cml24-4* and *elf3-4* n=15, *che-2* triple mutant and *gi-11* n=13). Single, double or triple asterisk indicate significance of ≤ 0.05 , ≤ 0.01 or ≤ 0.001 , respectively after two-tailed Student's t test (LD, *cml23-2 cml24-4*, *che-1*, *gi-11*; SD, *che-2*, *cca1-11*, *gi-11*) or two-sided Mann-Whitney Rank Sum test analysis (SD, *cml23-2 cml24-4*, *che-1*, *elf3-4*; LD, *che-2*, *cca1-11*, *elf3-4*) compared to their control (Col-0 or WS).

(c) Flowering time screen of *che-1*, *che-2* and different *cml23-2 cml24-4 che-1* and *cml23-2 cml24-4 che-2* mutant lines under LD conditions. Four lines of each triple mutant were assayed (HL lines). Number of leaves were recorded when the emerging bolt was 5 mm high. Data represent the mean \pm S.D (n=6, HL76 n=5).



Supplementary Fig. 6. Model for a proposed loop by which $[Ca^{2+}]_{cyt}$ affects the circadian clock period in Arabidopsis. During the day CCA1 represses ADPRc activity. Reduced CCA1 levels toward the middle and end of the day allow cADPR to rise, resulting in a $[Ca^{2+}]_{cyt}$ increase. At its peak, $[Ca^{2+}]_{cyt}$ activates *CML24* expression and during the evening, $[Ca^{2+}]_{cyt}$ controls TOC1 function through *CML24* in preparation for the morning events. Loops/pathways previously reported are shown in black. The proposed pathway by which $[Ca^{2+}]_{cyt}$ feeds-back into the clock is shown in red. Continuous single lines denote transcriptional regulation, continuous double lines denote post-transcriptional regulation and continuous triple line denotes both. A single $[Ca^{2+}]_{cyt}$ circadian oscillation is shown as a dotted red line. The relative timing of action for each component during a day–night cycle is shown from left to right. Yellow area indicates subjective day; grey area indicates subjective night. Question mark denotes the possible post-transcriptional regulation of TOC1 by *CML24* and that more studies are necessary to conclude how cytosolic *CML24* regulates TOC1 function.