**Supplementary Tables**

**Supplementary Table 1.** Related to Figure 1 and Supplementary Figure 1. CaCl$_2$ Treatments Used to Generate Artificial [Ca$^{2+}$]$_{cyt}$ Oscillations.

Dosing regime for generating artificial [Ca$^{2+}$]$_{cyt}$ oscillations in unentrained plants. Plants were grown for 12 days in continuous light without stratification before being dosed with the concentrations described.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>For artificial circadian [Ca$^{2+}$]$_{cyt}$ oscillations (Supplementary Figure 1b)</th>
<th>For Gene Expression (Figure 1 and Supplementary Figure 1c)</th>
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<tr>
<td></td>
<td>mM CaCl$_2$ added</td>
<td>mM CaCl$_2$ added</td>
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<tr>
<td>Day 1</td>
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<tr>
<td>12</td>
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**Supplemental Methods**

**Effect of Ca$^{2+}$ agonist solution on [Ca$^{2+}$]$_{cyt}$**

Luminometry of changes in [Ca$^{2+}$]$_{cyt}$ in response to N-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W7) (Calbiochem) and CaCl$_2$ and subsequent calibration of bioluminescence to estimate [Ca$^{2+}$]$_{cyt}$ were measured as follows.

Luminescence of at least 3 individual 12 day old 35S:AEQ Col-0 or cml23-2 cml24-4 seedlings was measured in opaque 96 imaging plates using FLUOstar (BMG Labtech, Germany). Aequorin was reconstituted with 20 μM coelenterazine (20 °C, overnight) and response to Ca$^{2+}$ agonists (henceforth referred to as “W7 solution”) was determined by injecting the W7 solution onto the plants to reach a final concentration of 660 μM W7 and 50 mM CaCl$_2$ (in 2.5 % (v/v) DMSO), respectively. The injection of room temperature distilled water was used as a touch response control for all the treatments. To convert luminescence into Ca$^{2+}$ concentrations, 3 M CaCl$_2$ and 30% ethanol were added to
discharge the remaining aequorin. Measurements were made until the detected
luminescence reached 10% of the first peak after discharge injection. [Ca^{2+}]_{cyt} levels were
determined according to [S1]. All experiments were repeated at least twice.

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

Primers used for qPCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
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| CCA1 | F: CCTCAAAACTTCAGAGTCCAATGC  
     R: GACCCTCGTCAGACACACACCTTC |
| LHY  | F: ACGAAACAGGTAAGTGGCGACATT  
     R: TGGGAAACATCTTGAACCGCGTT |
| PRR9 | F: CATCAAAAGCTTAGCCTCTCT   
     R: CTGTGGACTGAACTTGGT |
| PRR7 | F: GCACCTAAAGACCAGCCCATTGA  
     R: TCGTGGAACATCCCTTGTACATCAT |
| PRR5 | F: AAGGTTCGGTTACGAGAGCCGGAAG  
     R: TTGGGCTTTCTGATCGTGCTGG |
| PRR3 | F: TGACCCCTGGTCTTTCCAGG  
     R: AGAAAGATGTCACAGCTTAGCGGA |
| CHE  | F: TAATGGGTGGTGTTGTTCTTG  
     R: GCAAAAGCTCCAGACTTGT |
| TOC1 | F: TCACCATGAGCCCAATGAAAA  
     R: TTGAAACTTCTCCGCCAAAC |
| GI   | F: GGTGCAAGGTTTATCCAATCT |
| ZTL  | F: TGACGGAGGTGCTATGA  
     R: AGCACCAGGAAAGCTCAGT |
| ELF3 | F: GCACAGACTGATTAAGGTTCAAAAAC  
     R: CTTCACTGGAGATTTAGTG |
| ELF4 | F: TGTCGGACTGTTGGAATCAGT |
Supplementary Statistical Parameters

The Student’s t-test analyses for Fig.1c provided with a degree of freedom (d.f.) of 4 and the following t-values (t) and p-values (p): CHE ZT36 t=7.914, p=0.001; PRR9 ZT36 t=1.353, p=0.247; GI ZT36 t=0.468, p=0.664; TOC1 ZT36 t=0.709, p=0.518; PRR3 ZT36 t=4.023, p=0.052; ZTL ZT36 t=1.172, p=0.306; ELF3 ZT36 t=1.458, p=0.219; LUX ZT36 t=0.546, p=0.614; CHE ZT48 t=1.547, p=0.197; CCA1 ZT48 t=4.103, p=0.015; PRR7 ZT48 t=4.613, p=0.010; PRR5 ZT48 t=-0.0336, p=0.975; ELF4 ZT36 T=9 U=3 p=0.7; PRR7 ZT36 T=12 U=3 p=0.7; ELF4 ZT48 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.

The Student’s t-test analyses for Fig.2 provided with the following d.f, t and p values when mutants were compared to Col-0: Fig. 2a, cml23-2 d.f.=138, t=−0.687, p=0.493; cml24-1 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 d.f=130, t=−2.016, p=0.046; cml24-4 d.f.=110, t=−7.905, p<0.001. The Mann-Whitney Rank Sum Tests for Fig. 2b for comparison of Col-0 vs. cml23-2 cml24-4, provided with the following values: T=2313, U=297 and p<0.001.

The Student’s t-test for Fig.3c and the Mann-Whitney Rank Sum test for Fig. 3d, provided 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,
respectively. The Student’s t-test analyses for Fig.3e provided with a d.f. of 4 and the following t and p values: CCA1 ZT48 t=0.35 p=0.744, ZT50 t=-0.68 p=0.534, ZT52 t=-2.8 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 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, ZT70 t=-2.573 p=0.062, ZT72 t=1.762 p=0.153; TOC1 ZT48 t=-3.865 p=0.018, ZT50 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, 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 p=0.27; PRR7 ZT50 t=0.973 p=0.386, ZT52 t=-2.357 p=0.078, ZT54 t=4.948 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 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, ZT70 t=15.107 p<0.001, ZT72 t=-6.133 p=0.004. The Mann-Whitney Rank Sum Tests for Fig. 3e, provided with the following values: CCA1 ZT60 T=15, U=0 and p=0.1; PRR7 ZT48 T=15, U=0 and p=0.1.

The Student’s t-test analyses for Fig. 4 provided with the following d.f., t and p values: nicotinamide d.f=46 t=-0.689 p=0.495; high light water d.f=13 t=-2.827 p=0.014, high light sucrose d.f=14 t=-2.131 p=0.051; low light water d.f=30 t=-6.229 p<0.001, low light 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 t=0.585 p=0.5655, blue light d.f=12 t=-4.570 p=0.000644. The Mann-Whitney Rank Sum Tests for Fig. 4, provided with the following values: water (Fig. 4a) T=216.5 U=80.5 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 U=0 p<0.001.

Kruskal-Wallis One Way ANOVA analyses for Fig. 5 and Fig.6 a and b provided with a d.f of 2 and the following H and p values: toc1-2 H=41.741 p<0.001, cca1-11 H=58.291 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,
che-1 $H=49.271 \ p<0.001$. These analyses were followed by Dunn´s method, see Supplementary Table 3 for comparison between lines and precise p values. Kruskal-Wallis One Way ANOVA analyses for Fig.6 c and d provided with a d.f of 2 and the following H and p values: LD che-2 $H=33.924 \ p<0.001$, che-1 $H=36.191 \ p<0.001$; SD che-2 $H=31.352 \ p<0.001$, che-1 $H=28.263 \ p<0.001$. These analyses were followed by Tukey test (LD) or Dunn´s method (SD).

The Student´s t-test analyses for Supplementary Fig. 3c provided with a d.f. of 4 and the following t and p values: CHE ZT36 $t=2.744$, p=0.052; PRR9 ZT36 $t=0.0558$, p=0.958; PRR3 ZT36 $t=-4.008$, p=0.016; PRR5 ZT36 $t=0.449$, p=0.677; PRR7 ZT36 $t=1.033$, p=0.36; CHE ZT48 $t=3.120$, p=0.036; PRR9 ZT48 $t=4.857$, p=0.008; PRR3 ZT48 $t=2.131$, p=0.1; PRR7 ZT48 $t=2.631$, p=0.058; PRR5 ZT48 $t=-0.0366$, p=0.973.

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

The Student´s t-test analyses for Supplementary Fig. 5 provided with the following d.f, t and p values when mutants were compared to the correspondent wild type: LD cml23-2 $t=-4.787$, p=0.001; che-1 d.f=29, $t=3.270$, p=0.003; gi-11 d.f=30, $t=-35.570$, 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 d.f=27, $t=2.333$, p=0.027). The Mann-Whitney Rank Sum Tests for Supplementary Fig. 5 for comparison of the single mutants vs. their wild type, provided with the following values: 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.....
Supplementary References


Supplementary Figures
Supplementary Fig. 1. Related to Fig. 1. [Ca^{2+}]_{cyt} Manipulation to Study the Effect of [Ca^{2+}]_{cyt} Signals on Circadian Clock Genes Expression. 

(a) Growth of seedlings in constant light without stratification (unentrained seedlings) results in the absence of [Ca^{2+}]_{cyt} rhythms (closed diamonds) compared to entrained seedlings (open diamonds). 

(b) Imposing ramps of external CaCl₂ to unentrained seedlings, restores circadian oscillations in [Ca^{2+}]_{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 [Ca^{2+}]_{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 [Ca^{2+}]_{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 ± 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 ½ MS agar in LD and then placed in 96 well plates containing 20 mM coelenterazine. The effect of a solution containing 660 μM 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 ± 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 μM 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 ± 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 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 mutants 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 ± 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 ± 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.