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SUPPLEMENTARY INFORMATION

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Circadian oscillations of cytosolic free calcium regulate the *Arabidopsis* circadian clock

María Carmen Martí Ruiz[1,11], Katharine E. Hubbard[1,2,11], Michael J. Gardner[1,11], Hyun Ju Jung[1], Sylvain Aubry[1,3], Carlos T. Hotta[1,4], Nur Izzati Mohd-Noh[1,5], Fiona C. Robertson[1,6], Timothy J. Hearn[1], Yu-Chang Tsai[7], Antony N. Dodd[1,8], Matthew Hannah[9], Isabelle A. Carré[10], Julia M. Davies[1], Janet Braam[7] and Alex A. R. Webb[1]*

[1]Department of Plant Sciences, University of Cambridge, Cambridge, UK. [2]School of Biological, Biomedical and Environmental Sciences, University of Hull, Hull, UK. [3]Department for Plant and Microbial Biology, University of Zürich, Zürich, Switzerland. [4]Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo, São Paulo, SP, Brazil. [5]Department of Bioscience and Health Science, Faculty of Bioscience and Medical Engineering, Universiti Teknologi Malaysia, Skudai, Johor, Malaysia. [6]Department of Biochemistry, University of Zimbabwe, Harare, Zimbabwe. [7]Biochemistry and Cell Biology, Rice University, Houston, TX, USA. [8]School of Biological Sciences, Bristol Life Sciences Building, University of Bristol, Bristol, UK. [9]Bayer CropScience NV Innovation Center, Trait Discovery, Gent, Belgium. [10]School of Life Sciences, University of Warwick, Coventry, UK. [11]These authors contributed equally to this work: María Carmen Martí Ruiz, Katharine E. Hubbard, Michael J. Gardner. *e-mail: aarw2@cam.ac.uk

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.

	For artificial circadian $[Ca^{2+}]_{cyt}$ oscillations (Supplementary Figure 1b)				For Gene Expression (Figure 1 and Supplementary Figure 1c)
Time (h)	mM $CaCl_2$ added				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

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 ºC, 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.

Gene	Primers
CCA1	F: CCTCAAACTTCAGAGTCCAATGC
	R: GACCCTCGTCAGACACAGACTTC
LHY	F: ACGAAACAGGTAAGTGGCGACATT
	R: TGGGAACATCTTGAACCGCGTT
PRR9	F: CATCAAAAGCTTAGCCTCTCT
	R: CTGTGGACTGAACTTGGT
PRR7	F: GCACTTAAAGACCAGCCCCATTGA
	R: TCGTCGGAACATCCCTGTCATCAT
PRR5	F:AAGGTTCGTTACGAGAGCCGGAAG
	R: TTGGCCTTTGATTCGTGGTCGTTG
PRR3	F: TGACCCTTGGGTGCTTTCAGG
	R: AGAAGATGTCACAGCTCTAGCGGA
CHE	F: TAATGGGTGGTGGTGGTTCTG
	R: GCAAAGCTCCAGACTTGTCC
TOC1	F: TCACCATGAGCCAATGAAAA
	R: TTGAAACTTCTCCGCCAAAC
GI	F: GGTCGACGGTTTATCCAATCT
	R: CGGACTATTCATTCCGTTCTT
ZTL	F: TGACGAGGTTGTGTCTATGA
	R: AGCACCAGGAACAGTCTCTA
ELF3	F: GCACAGACTGATTAAGGTTCAAAAAC
	R: CTTCACTGGATAGCTTTTAGCAG
ELF4	F: TGTCGTTGACTTGTTGAATCAGTG

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

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= 0.016; *PRR5* ZT36 *t*=2.732, 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*=-0.410, p=0.703; *PRR9* ZT48 *t*=-3.902, p=0.018; *GI* ZT48 *t*=-0.346, p=0.746; *TOC1* ZT48 *t*=-0.132, p=0.901; *PRR3* ZT48 *t*=4.103, p= 0.015; *PRR7* ZT48 *t*= 4.613 p= 0.010; *PRR5* ZT48 *t*=-0.0336, p=0.975; *ZTL* ZT48 *t*=0.186, p=0.862; *ELF3* ZT48 *t*=1.748, p=0.155; *LUX* ZT48 *t*=-0.465, p= 0.666. The Mann-Whitney Rank Sum Tests for Fig. 1c provided with the following T, U and p values: *CCA1* ZT36 T=9 U=3 p=0.7; *PRR7* ZT36 T=12 U=3 p=0.7; *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.

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=0.267, ZT66 t=-0.38 p=0.723, ZT68 t=-0.441 p=0.682, ZT70 t=2.321 p=0.081, ZT72 t=-1.278 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.

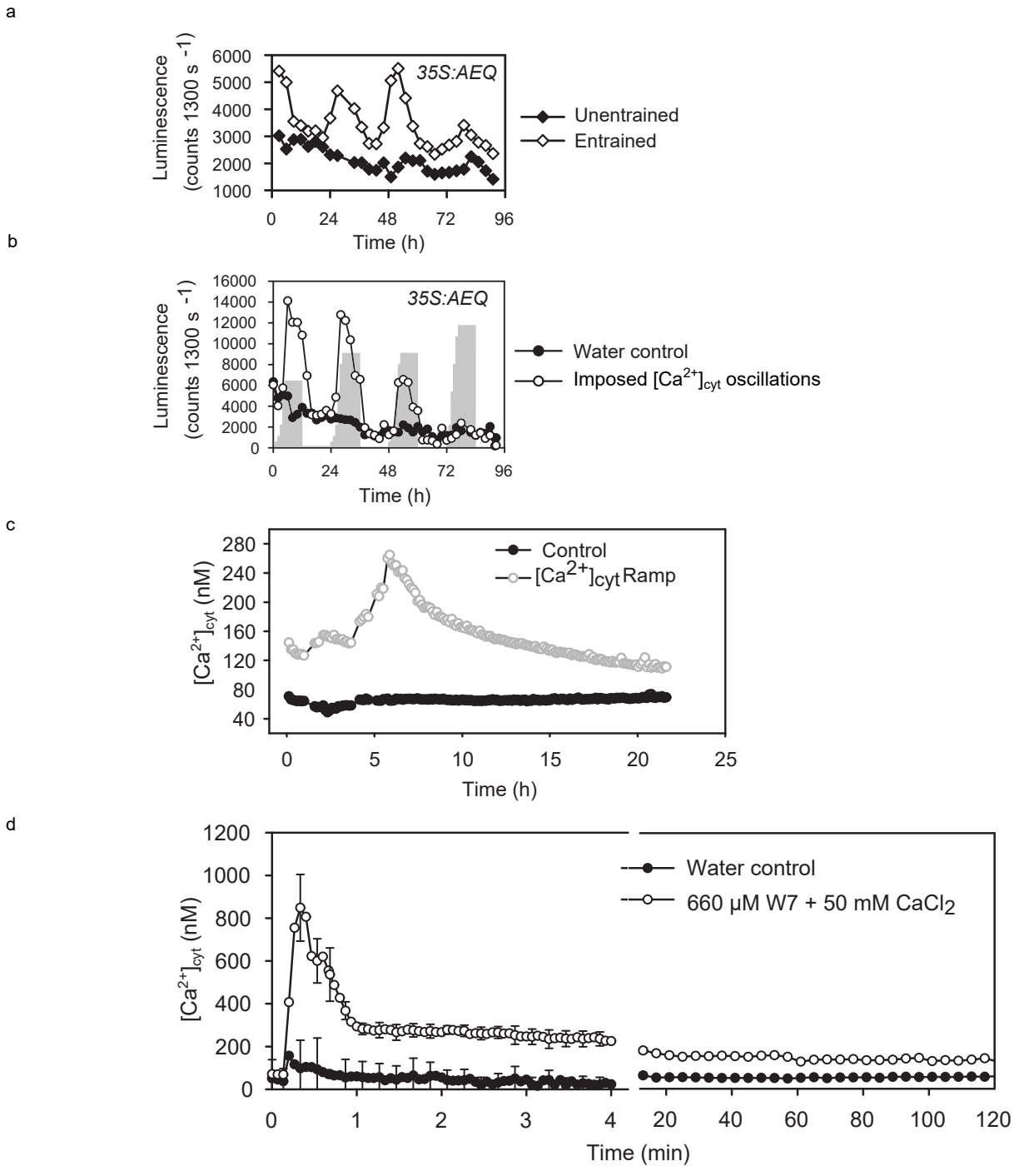
The Student´s *t*-test analyses for Supplementary Fig. 5 provided with the following d.f, t and p values when mutants were compared to the correspondent wild type: LD *cml23*-2 *cml24*-4 d.f=29, *t*=-4.787, p<0.001; *che*-1 d.f=29, *t*=3.270, p=0.003; *gi*-11 d.f=30, *t*=35.570, p<0.001; SD, *che*-2 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

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 T=288.500, U=103.500 p=0.365, *elf3*-4 T=120.000, U=0.000 p<0.001.

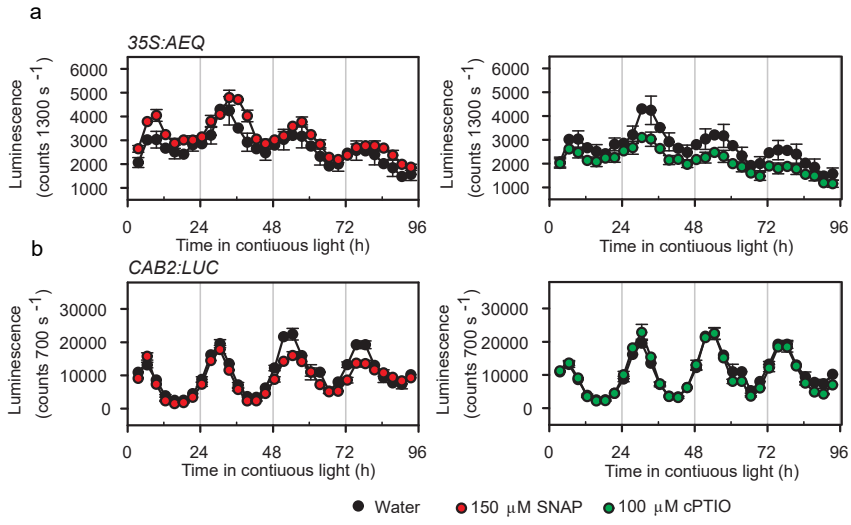
Supplementary References

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

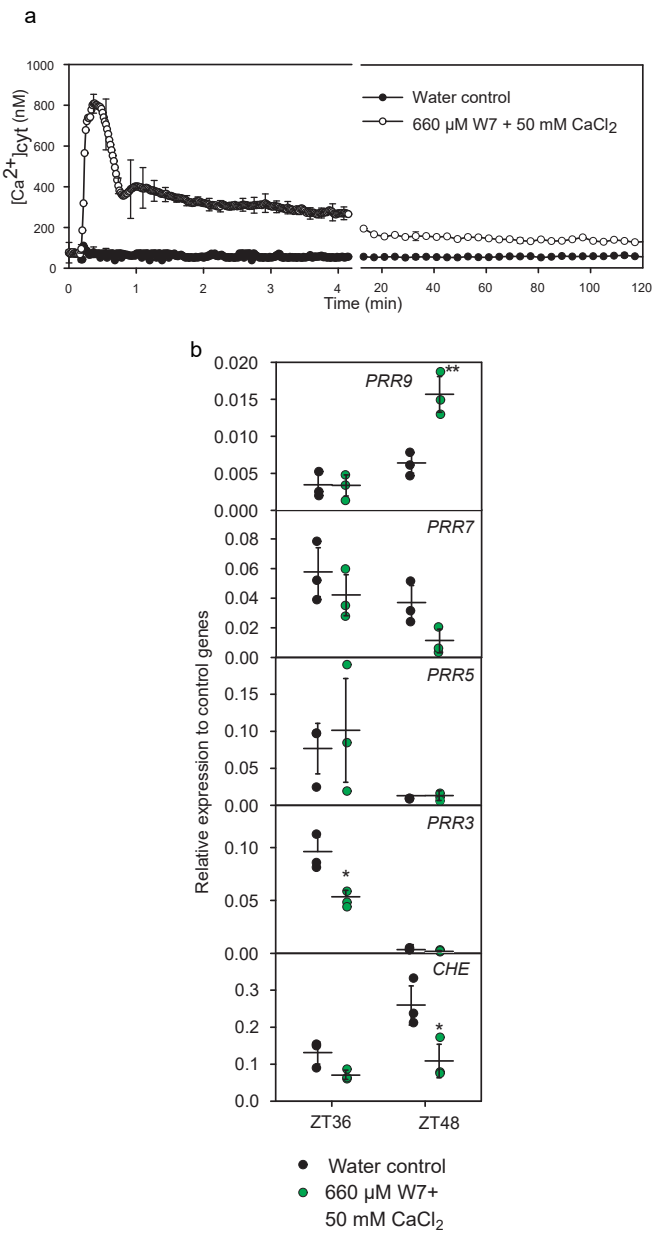
Supplementary Figures



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 $CaCl_2$ to unentrained seedlings, restores circadian oscillations in $[Ca^{2+}]_{cyt}$ (open circles) when compared to unentrained water-treated samples (closed circles). $CaCl_2$ 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 $CaCl_2$ 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 $CaCl_2$ (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).



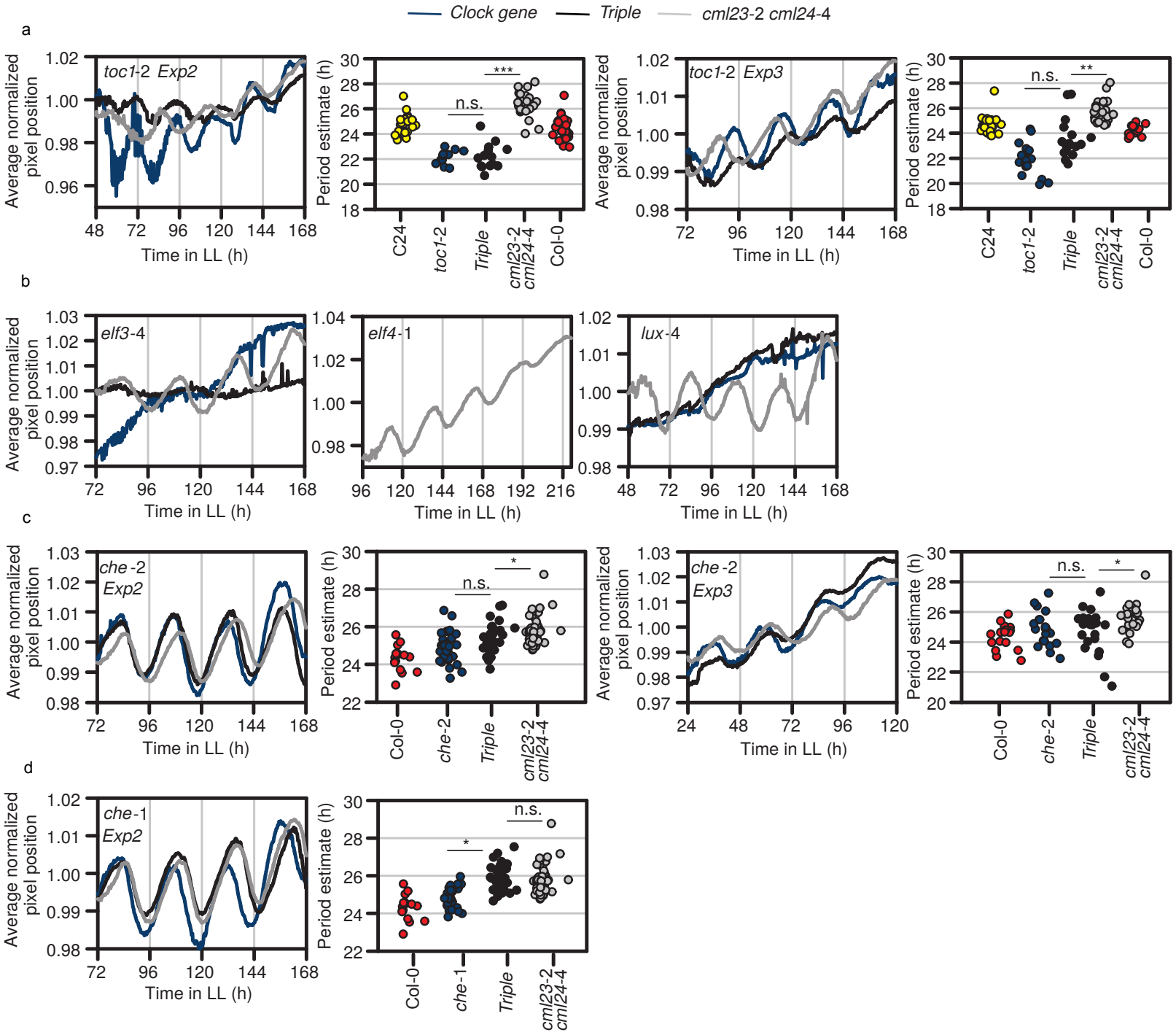
Supplementary Fig. 2. NO levels do not affect circadian period.
The NO donor SNAP (red) and the NO scavenger cPTIO (green) do not affect the circadian oscillations of $[Ca^{2+}]_{cyt}$ (**a**) or CAB2:LUC activity (**b**). Seedlings were imaged in LL, and SNAP or cPTIO were applied every 3 h. Results 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.

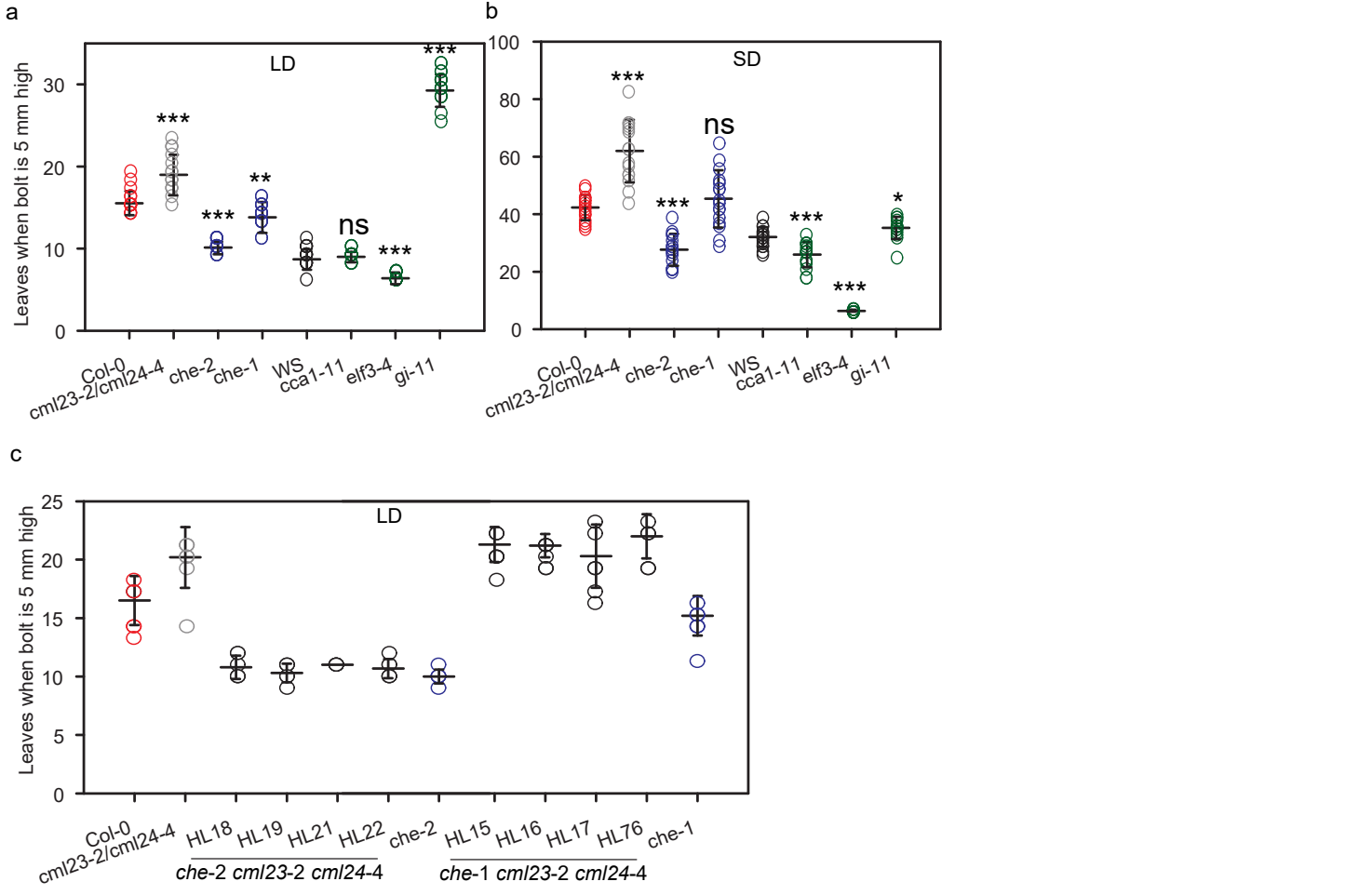
(**a**) Effect of external Ca^{2+} signals on $[Ca^{2+}]_{cyt}$ signaling in *cml23*-2 *cml24*-4 plants. *cml23*-2 *cml24*-4 Arabidopsis seedlings expressing 35S:AEQ were grown for 12 days on ½ MS agar in LD and then placed in 96 well plates containing 20 mM coelenterazine. The effect of a solution containing 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*.

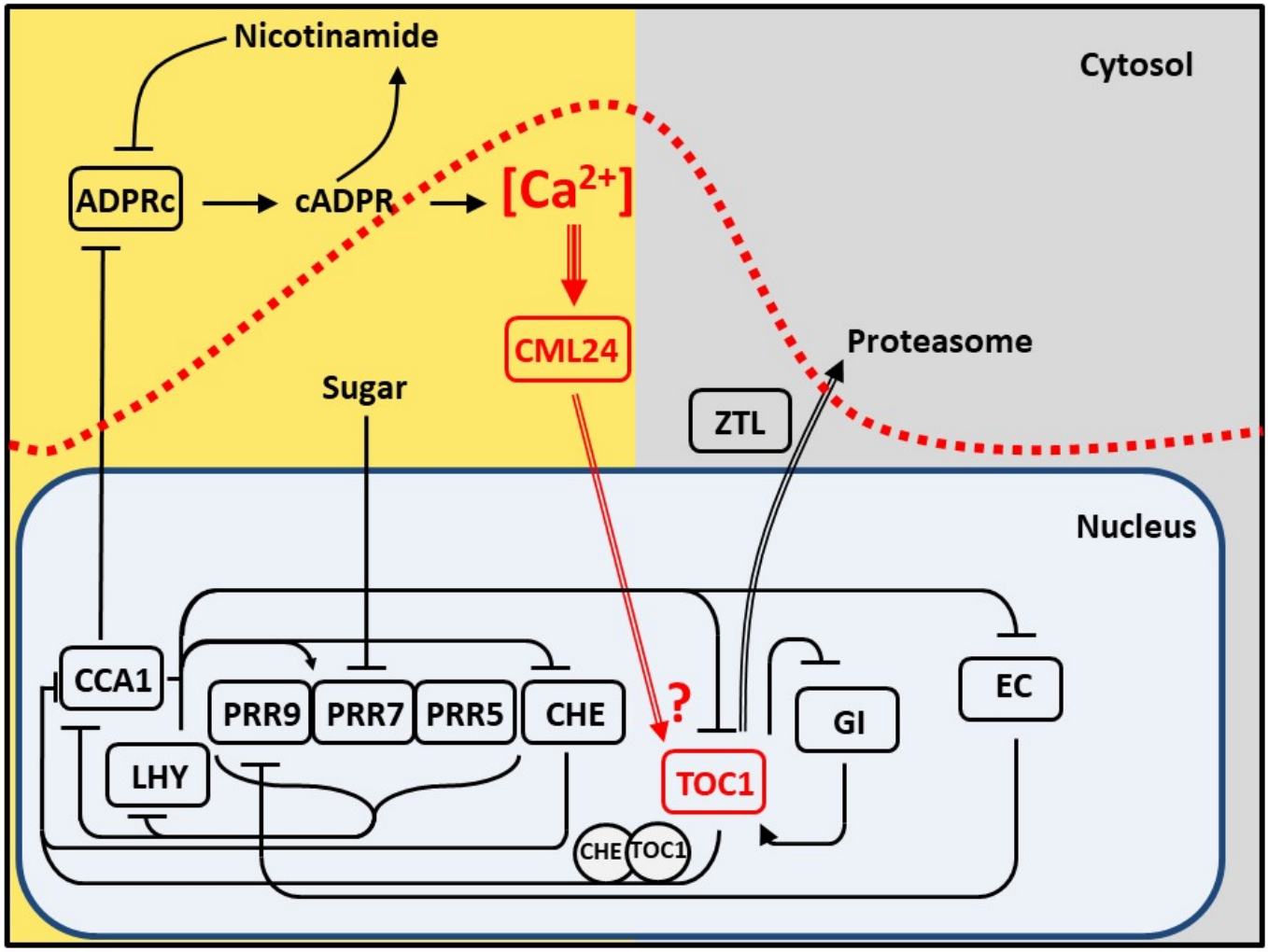
Average normalized traces of leaf positions and FFT-NLLS analysis of the circadian period for leaf movement experiments. **a** shows the results of *cml23*-2 *cml24*-4 with *toc1*-2 (exp2/3 Col-0 n=26/13, C24=21/29, *cml23*-2 *cml24*-4=22/25, *toc1*-2=11/17, triple mutant=14/16), **b** with *elf3*-4, *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 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 posible post-transcriptional regulation of TOC1 by CML24 and that more studies are necessary to conclude how cytosolic CML24 regulates TOC1 function.