Circadian oscillations of cytosolic free calcium regulate the Arabidopsis circadian clock

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In the last decade, the view of circadian oscillators has expanded from transcriptional feedback to incorporate post-transcriptional, post-translational, metabolic processes and ionic signalling. In plants and animals, there are circadian oscillations in the concentration of cytosolic free Ca$^{2+}$ ($[\text{Ca}^{2+}]_{\text{cyt}}$), though their purpose has not been fully characterized. We investigated whether circadian oscillations of $[\text{Ca}^{2+}]_{\text{cyt}}$ regulate the circadian oscillator of Arabidopsis thaliana. We report that in Arabidopsis, $[\text{Ca}^{2+}]_{\text{cyt}}$ circadian oscillations can regulate circadian clock function through the Ca$^{2+}$-dependent action of CALMODULIN-LIKE24 (CML24). Genetic analyses demonstrate a linkage between CML24 and the circadian oscillator, through pathways involving the circadian oscillator gene TIMING OF CAB2 EXPRESSION1 (TOC1).

We show that circadian oscillations of $[\text{Ca}^{2+}]_{\text{cyt}}$ affect the abundance of CHE and affect circadian period through a Ca$^{2+}$-dependent regulatory protein of the plant specific CALMODULIN-LIKE (CML) family. We conclude that CML24 is part of the Arabidopsis circadian system, acting through a Ca$^{2+}$-dependent pathway to regulate TOC1.

Results

Circadian oscillator gene expression can be altered by $[\text{Ca}^{2+}]_{\text{cyt}}$ signals. We identified potential targets for $[\text{Ca}^{2+}]_{\text{cyt}}$ by examining the response of circadian oscillator transcripts to a single 24 h artificial oscillation of $[\text{Ca}^{2+}]_{\text{cyt}}$ in plants in which circadian oscillations of $[\text{Ca}^{2+}]_{\text{cyt}}$ were abolished, and later artificially induced (Fig. 1a and Supplementary Fig. 1). $[\text{Ca}^{2+}]_{\text{cyt}}$ signals did not restore high amplitude oscillations of clock transcripts such as those in light and dark cycles11. CCA1 HIKING EXPEDITION (CHE) was the only clock transcript whose abundance correlated with the $[\text{Ca}^{2+}]_{\text{cyt}}$ signal, having a dynamic opposite to the imposed $[\text{Ca}^{2+}]_{\text{cyt}}$ oscillation (maximum repression 5.2-fold at 12 h, 4.5-fold at 16 h and 3.1-fold at 8 h) (Fig. 1b). The CCA1 dynamic was modestly altered (1.9-fold activation at 20 h and 24 h) (Fig. 1b). This later increase in CCA1 transcript abundance might have been due to the earlier large repression of CHE. Artificial $[\text{Ca}^{2+}]_{\text{cyt}}$ oscillation had smaller effects on other circadian oscillator transcript abundance, the largest being a 2-fold reduction of PRR3 at 16 h and a 1.9-fold increase of LHY at 20 h (Fig. 1b).

To test further the effects of $[\text{Ca}^{2+}]_{\text{cyt}}$ on CHE transcript abundance, we screened a number of $[\text{Ca}^{2+}]_{\text{cyt}}$ agonists to identify those that had profound and persistent effects on $[\text{Ca}^{2+}]_{\text{cyt}}$ N-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride
Fig. 1 | Transcripts abundance of circadian clock genes is modulated by [Ca\^{2+}]_cyt. a, Imposing oscillations of external CaCl_2 restores circadian oscillations in [Ca\^{2+}]_cyt in unentrained seedlings. See also Supplementary Fig. 1c for calibrated data. Results represent the mean ± s.d. (n = 12 biological replicates) for one experiment. Experiments were repeated three times. b, Effect of the imposition of a 24 h oscillation of [Ca\^{2+}]_cyt on the transcript abundance (expressed as log2) of the circadian clock genes. Closed circles indicate unentrained water-treated samples, green circles the unentrained CaCl_2-treated plants. To generate the oscillation, CaCl_2 was applied as indicated by the shaded areas and as described in Supplementary Table 1. Results represent the mean (n = 2 biological replicates). c, 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). See also Supplementary Fig. 1. Single, double or triple asterisks indicate significance of *P ≤ 0.05, **P ≤ 0.01 and ***P ≤ 0.001, after two-tailed Student’s t-test analysis or two-sided Mann-Whitney rank sum test (ZT36 CCA1, PRR7 and ELF4; ZT48 ELF4 and LHY).
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Period, we wished to determine whether Ca2+

in regulating the clock

in planta.

We performed a screen of 75

vtc1

cml23

cml24

cml24

transcript did not alter the free-running period of the oscilla-
tor, because treating plants with the W7 solution at ZT0 and ZT12
did not alter the period of CCA1:LUC (ZT0 Control 24.4 ± 0.5h, W7 solution 24.2 ± 0.2h; ZT12 Control 24.4 ± 0.2h, W7 solution 24.4 ± 0.5h; P > 0.05). Thus, manipulation of [Ca2+]cyt demonstrated that both 24 h [Ca2+]cyt oscillations and shorter-term [Ca2+]cyt signals can regulate CHE transcript abundance (Fig. 1).

A reverse genetic screen identified calmodulin-like 24 (CML24) as a regulator of circadian period. Because transient increases in [Ca2+]cyt, affected circadian oscillator gene abundance but not period, we wished to determine whether Ca2+ signalling has a role in regulating the clock in planta. We performed a screen of 75 well characterized Ca2+ signalling mutants of transporters, transducers and sensors. Five lines had significantly increased circadian period of leaf movement compared to wild type (Fig. 2 and Supplementary Table 2). As previously called CML24-U1 (Col-0 23.8 ± 0.1h, P < 0.001), calmodulin-like (cml) 24 on the central oscillator in more detail, we analysed because treating plants with the W7 solution at ZT0 and ZT12
did not alter the period of CCA1:LUC (ZT0 Control 24.4 ± 0.5h, W7 solution 24.2 ± 0.2h; ZT12 Control 24.4 ± 0.2h, W7 solution 24.4 ± 0.5h; P > 0.05). Thus, manipulation of [Ca2+]cyt demonstrated that both 24 h [Ca2+]cyt oscillations and shorter-term [Ca2+]cyt signals can regulate CHE transcript abundance (Fig. 1).

CML24 regulates circadian period in a [Ca2+]cyt-dependent manner. Because circadian rhythms persist in conditions where the circadian oscillations of [Ca2+]cyt are abolished, such as nicotineamide48, sucrose50 or monochromatic red light59, circadian rhythms of [Ca2+]cyt are not necessary for a rhythmic oscillator. However, we could test for necessity for oscillations in [Ca2+]cyt in the correct regulation of circadian period by determining whether the action of CML24 in the circadian clock depends on Ca2+. If the effects of the CMLs are independent of Ca2+, then the effects of mutation on circadian period should be additive to treatments that abolish circadian [Ca2+]cyt. Additionally, CML24 could be redundant in the regulation of the clock. None of the overexpressing (CML24-OX1 and CML24-OX2) or under-expressing lines (CML24-U1 and CML24-U1) affected circadian period (Supplementary Table 2). As CML24 (previously called TCH2)60 encodes a CALMODULIN-LIKE Ca2+-sensor61,62 and two different alleles, cml24-1 and cml24-4 had a significantly longer period than Col-0 for leaf movements (Fig. 2), we decided to further characterize whether CML24 is involved in the regulation of the circadian oscillator. The long circadian period of cml23-2 cml24-4 double mutants was confirmed by measuring promoter activity of CCA1 fused to luciferase (CCA1:LUC) and circadian oscillations of [Ca2+]cyt, (Fig. 3a-d) (Col-0 23.7 ± 0.1h, cml23-2 cml24-4 26.1 ± 0.4h for CCA1:LUC, P < 0.001; Col-0 23.7 ± 0.2h, cml23-2 cml24-4 25.1 ± 0.1h for 35S:AEQ, P < 0.001). To investigate the effect of CML24 on the central oscillator in more detail, we analysed CCA1, PRR7 and TOC1 transcript abundance in Col-0 and cml23-2 cml24-4. In the third day in white light, there was a substantial delay of 4h in the phase of CCA1, TOC1 and PRR7 transcript abundance in the mutant plants (Fig. 3c), consistent with the lengthening of circadian period by ~1.5h described in Fig. 3a-d. The transcript levels of the clock components were unaffected.

Fig. 2 | CML24 regulates circadian period in Arabidopsis. Average normalized traces of leaf positions (left panels). Fast Fourier transform-nonlinear least squares (FFT-NLLS) analysis of the circadian period for leaf movement experiments: dots indicate individual samples and black bars mean period ± s.e.m. (right panels). a, The results of cml23-2 and cml24-1 single and double mutants. Col-0 n = 70, cml23-2 n = 70, cml24-1 n = 97, double mutant n = 94; b, The results of cml23-2 and cml24-4 single and double mutants. Col-0 n = 63, cml23-2 n = 69, cml24-4 n = 49, double mutant n = 110, rhythmic leaves. Red lines indicate Col-0, grey lines indicate cml23cml24 double mutants, and light blue cml24 single mutants. cml24-2 traces for leaf position were removed for clarity. All plants were grown under 12h light: 12h dark cycles before the experiments. Data represent three (a) or two (b) independent experiments. Single or triple asterisks indicate significance of *P ≤ 0.05 and ***P ≤ 0.001, after two-tailed Student’s-t-test or two-sided Mann-Whitney rank sum test. n.s., not significant.
oscillation (Fig. 1a), with their peaks in phase with the imposed [Ca\(^{2+}\)]\(_{cyt}\) rhythm, suggesting [Ca\(^{2+}\)]\(_{cyt}\) positively regulates CML abundance. This could be explained by the presence in their promoters of the Ca\(^{2+}\)-responsive cis element CAM box (AGCGGT\(^n\))\(^1\).

CML24 not only senses Ca\(^{2+}\), it also regulates NO\(^\dagger\) as shown by high levels of NO in cml24 mutants\(^\circ\). We therefore, tested whether the high NO in the mutants could be the cause of the long period by two experiments. We investigated the effect of NO on circadian regulation of [Ca\(^{2+}\)]\(_{cyt}\) and CHLOROPHYLL A/B BINDING PROTEIN12:LUC (CAB2:LUC) in wild type plants and found no evidence for a role for NO, because the NO donor, SNAP, and the scavenger, cPTIO, were without effect on circadian rhythms (Supplementary Fig. 2). The high NO levels found in cml23-2 cml24-4, were not responsible for the long period phenotype, because the mutant long period phenotype persisted in the presence of cPTIO (Fig. 4f)\(^\circ\).

We conclude that the effect of the cml23-2 cml24-4 mutations on the circadian clock requires [Ca\(^{2+}\)]\(_{cyt}\) and is independent of the effects of CML24 on NO generation.

**CML24 regulates circadian period by a pathway involving TOC1 and possibly CHE.** To investigate how CML24 affects circadian period, we tested whether it is involved in the [Ca\(^{2+}\)]\(_{cyt}\)-mediated transcriprional regulation of circadian clock genes. The clock transcripts regulated by [Ca\(^{2+}\)]\(_{cyt}\) in Col-0 (Fig. 1c) were also regulated by [Ca\(^{2+}\)]\(_{cyt}\) in cml23-2 cml24-4 (Supplementary Fig. 3), suggesting that CML24 is not involved in the transcriprional regulation by [Ca\(^{2+}\)]\(_{cyt}\).

We then investigated the genetic linkage between CML24 and components of the oscillator. We studied epistatic interactions in the control of circadian rhythms of leaf movement between mutations in CML23/CML24 and CCA1, LHY, TOC1, ZTL, ELF3, ELF4 and LUX. Double CML23/CML24 mutants were used for epistasis due to having a larger measurable effect on period compared to the single CML24 mutants. We identified epistasis between mutations in CML23/CML24 and TOC1 in the regulation of the period of leaf movement (Fig. 5a, Supplementary Fig. 4 and Supplementary Table 3). toc1-2 single mutant had a short period (C24 24.3 ± 0.1 h, toc1-2 21.4 ± 0.3h, Mann–Whitney rank sum test P < 0.001; Fig. 5a)\(^\circ\). In the triple mutant toc1-2 cml23-2 cml24-4, the long period arising from mutations in CML23/CML24 was absent, having a period that was indistinguishable from the single mutant toc1-2, and significantly shorter than cml23-2 cml24-4 mutant (one-way ANOVA P = 1 and P < 0.001, respectively) (Fig. 5a, Supplementary Fig. 4 and Supplementary Table 3). This indicates that toc1-2 is epistatic to cml23-2 cml24-4. We did not find epistatic interactions between mutations in CML23/CML24 and CCA1, LHY or ZTL. CML23-2 cml24-4 increased period in the cca1-11, lhy-21 and ztl-3 backgrounds, resulting in additive phenotypes (Fig. 5b–d and Supplementary Table 3). Analysis of genetic interactions between CML23/CML24 and ELF3, ELF4 and LUX is complicated by the rhythmic phenotypes caused by loss-of-function of these evening complex genes. Triple mutants of cml23-2 cml24-4 and members of the evening complex where therefore all arrhythmic in white light (Supplementary Fig. 4). Crossing the wild type genetic backgrounds used in this study was without effect (Supplementary Table 3).

TOC1-mediated repression of CCA1 involves CHE\(^\circ\). Therefore, the regulation of CCA1 by [Ca\(^{2+}\)]\(_{cyt}\) and interaction between mutations in CML23/CML24 and TOC1, prompted investigation of whether CHE is also part of the genetic pathway by which CML24 regulates circadian period. We identified epistatic interaction between mutations in CML23/CML24 and CHE in the regulation of circadian leaf movements. As previously reported for CCA1:LUC\(^+\) rhythms, the circadian period of leaf movement in che-1 and che-2 single mutants was indistinguishable from the background (two-tailed Student’s t-test P = 0.461 and P = 0.681, respectively; Fig. 6a,b)\(^\circ\).

In the triple mutant che-2 cml23-2 cml24-4, the long period arising from mutations in CML23/CML24 was absent, being indistinguishable from the single mutant che-2 and significantly shorter than the cml23-2 cml24-4 mutant (one-way ANOVA P > 0.05 and P < 0.05, respectively) (Fig. 6a, Supplementary Fig. 4 and Supplementary Table 3). However, we found no evidence that che-1 was epistatic to cml23-2 cml24-4. che-1 cml23-2 cml24-4 triple mutants had a significantly longer period relative to che-1 and similar to cml23-2 cml24-4 (one-way ANOVA, P < 0.05 and P > 0.05, respectively) (Fig. 6b, Supplementary Fig. 4 and Supplementary Table 3). This is consistent with che-2 being a stronger allele than che-1 in the lhy background\(^\circ\), explaining why there is an epistatic interaction with cml23-2 cml24-4 and che-2 but not with che-1.
Because the circadian oscillator contributes to the photoperiodic regulation of flowering and cml23-2 cml24-4 mutants are late-flowering\(^3\), we tested epistasis between CML23/CML24 and CHE by measurement of flowering time. che-2 is an early flowering mutant and, as suggested above, possibly the stronger allele (Supplementary Fig. 5). As in the circadian experiments, there is an epistatic relationship between mutations in CML23/CML24 and CHE in the regulation of flowering time (Fig. 6c and Supplementary Fig. 5) when che-2 was used. che-2 cml23-2 cml24-4 mutant flowered at the same time as che-2, and significantly earlier than cml23-2 cml24-4 (one-way ANOVA, \(P > 0.05\) and \(P < 0.05\), respectively) (Fig. 6c and Supplementary Fig. 5). However, and similarly to leaf movement, flowering time provided no evidence of epistasis between che-1 and cml23-2 cml24-4 (Fig. 6d and Supplementary Fig. 5).

The data suggest that the \([Ca^{2+}]_\text{cyt}\)-dependent regulation of circadian period by CML24 is not directly mediated by CCA1, LHY and ZTL and that CML24 might regulate TOC1 and CHE, because functional copies of these two clock genes are required to express the cml23-2 cml24-4 phenotype.

**Discussion**

The Ca\(^{2+}\)-sensor CML24 is a regulator of Arabidopsis circadian period. We tested the hypothesis that circadian oscillations of \([Ca^{2+}]_\text{cyt}\) can feed back into the circadian oscillator. We demonstrate that \([Ca^{2+}]_\text{cyt}\) signals can regulate the expression of the Ca\(^{2+}\)-binding CALMODULIN-LIKE24 (CML24) and that CML24 also regulates circadian period, with the loss-of-function phenotype being absent when \([Ca^{2+}]_\text{cyt}\) rhythms are abolished. We conclude that correct circadian period is dependent on CML24 and circadian rhythms of \([Ca^{2+}]_\text{cyt}\). Epistatic analysis suggests that TOC1 and probably CHE genetically interact with CML24 (Supplementary Fig. 6). Additionally, we show that \([Ca^{2+}]_\text{cyt}\) signals can regulate the expression of CHE in a CML24-independent manner and transcriptional regulation of CHE is unlikely to regulate circadian period.

It was previously reported that CML24 regulates flowering time\(^3\). Our new data demonstrate that CML24 also regulates circadian period in Arabidopsis because two different alleles (cml24-1 and cml24-4) alone or in combination with the null allele of CML23 (cml23-2)\(^3\), had a long circadian period (Fig. 2). We found that CML24 has robust and profound effects on period (Fig. 3). The period lengthening persisted in different clock mutant backgrounds such as cca1-1, lhy-21 and ztl-3 (Fig. 5). The magnitude of the period lengthening of the CML24 mutants (from 0.6 to 2 h) is larger or similar to previously reported mutations in important circadian genes: \(prr7-11\) and \(prr9-1\) (0-2 h), che-1 and che-2 (no effect on period)\(^3\), \(prr3-1\) and \(prr5-3\) (\(\sim 1\) h\(^3\)), \(lnk1\) (no effect on period), \(lnk2\) (1 h), \(lnk1\) and \(lnk2\) (2 h)\(^3\), che-1/lhy and che-2/lhy double mutants have a significantly shorter circadian period (\(0.5\) or 1 h, respectively) compared to the lhy mutant\(^10\). CML24-OX1 and CML24-OX2 were without phenotype, which might be expected for a sensor protein whose activity depends on and might be limited by Ca\(^{2+}\) concentration, rather than abundance of the sensor protein. Meaning that the presence of 24 h \([Ca^{2+}]_\text{cyt}\) oscillations might be critical for the production of the physiological response as observed in Fig. 4a-d and that in the over-expressor lines, the Ca\(^{2+}\) signature might be still decoded. Nevertheless, the limitation of other protein targets of CML24 and activators cannot be ruled out.

CML24 binds Ca\(^{2+}\) at EF hands to cause a conformational change but has no other identified functional domains\(^3\). Our data are consistent with CML23 and CML24 acting as Ca\(^{2+}\)-sensors because we demonstrate that the effect of CML23 and CML24 mutations on circadian period depends on \([Ca^{2+}]_\text{cyt}\) as shown by the absence of an effect of the cml23-2 cml24-4 mutation when circadian oscillations of \([Ca^{2+}]_\text{cyt}\) are suppressed\(^6,20\) (Figs. 4a-d). This also demonstrates that sucrose regulates circadian period through a pathway.

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**Fig. 4 | Circadian oscillations of \([Ca^{2+}]_\text{cyt}\) are necessary for the correct function of the circadian oscillator.** a. Circadian period estimates of leaf movement in continuous high light (80 \(\mu\)mol m\(^{-2}\) s\(^{-1}\)) of Col-0 and cml23-2 cml24-4 plants treated with either 50 mM nicotinamide (nic, Col-0 n = 15, cml23-2 cml24-4 n = 33, biological replicates) or water (Col-0 n = 16, cml23-2 cml24-4 n = 29, biological replicates). b, c. Circadian period estimates of CCA1:LUC rhythms in continuous high light (80 \(\mu\)mol m\(^{-2}\) s\(^{-1}\)) (b) or continuous low light (10 \(\mu\)mol m\(^{-2}\) s\(^{-1}\)) (c) of Col-0 and cml23-2 cml24-4 plants grown in the presence of either high light (Col-0 n = 7, cml23-2 cml24-4 n = 8, low light n = 16, biological replicates), 90 mM sucrose (suc, high light n = 8, low light n = 16, biological replicates) or 90 mM mannitol (man, Col-0 n = 8, cml23-2 cml24-4 n = 7, biological replicates). d. Circadian period estimates of CCA1:LUC rhythms in Col-0 and cml23-2 cml24-4 plants under continuous high mixed red (660 nm) and blue (470 nm) light (80 \(\mu\)mol m\(^{-2}\) s\(^{-1}\)) (n = 4, biological replicates) and continuous monochromatic blue or red light (40 \(\mu\)mol m\(^{-2}\) s\(^{-1}\)) (blue n = 7, red Col-0 n = 11 and cml23-2 cml24-4 n = 10, biological replicates). e. Effect of the imposition of a ramp of external Ca\(_{\text{Cl}}\)\(_2\) (Fig. 1a) on the expression (log2) of CML24 and CML23. Ca\(_{\text{Cl}}\)\(_2\) was applied as described by the shaded areas and in Supplementary Fig. 1c. Plant material was harvested from the onset of treatment every 4 h for 24 h to extract RNA for probing with microarray. Results represent the mean (n = 2 biological replicates). f. Circadian period estimates of CCA1:LUC rhythms in continuous high light (80 \(\mu\)mol m\(^{-2}\) s\(^{-1}\)) of Col-0 (n = 7 biological replicates) and cml23-2 cml24-4 (n = 8 biological replicates) plants treated from the day before going into continuous high light either with water or 200 \(\mu\)M cPTIO. Period estimates were obtained by BRASS and are shown as mean ± s.e.m. Data were obtained from one independent experiment. Experiments were repeated at least twice. Single, double or triple asterisks indicate significance of \(P \leq 0.05\), ** \(P \leq 0.01\) and *** \(P \leq 0.001\), after two-tailed Student’s t-test analysis or two-sided Mann–Whitney rank sum test (a (water), b (mannitol) and f).
period in wild type and *cml23-2 cml24-4* was the same. However, the period of the double mutant was increased by nicotinamide (Fig. 4a), suggesting that nicotinamide might target both \([Ca^{2+}]_{cyt}\) dependent and -independent pathways, or additional Ca\(^{2+}\)-sensors might be involved\(^{11}\). The *Arabidopsis* genome encodes over 50 CaM and CMLs\(^{31,42}\) and other Ca\(^{2+}\) sensors, which could also contribute to circadian regulation.

**CML24 genetically interacts with TOC1 and possibly with CHE to regulate circadian period.** The absence of the *cml23-2 cml24-4* circadian phenotype in *toc1-2 cml23-2 cml24-4* indicates that the CMLs proteins are unable to exert their regulator function if TOC1 is absent (Fig. 5a and Supplementary Fig. 4). **CML24 and TOC1** are expressed in diverse tissues and organs\(^{11}\), which is consistent with our genetic studies, but more studies are necessary to conclude how cytosolic CML24 regulates TOC1 function. CHE might have a role because there was a genetic interaction between *CML23/CML24* and CHE in the regulation of circadian period when the *che-2* allele was used (Fig. 6).

Because we found that Ca\(^{2+}\) alone or in combination with W7 was able to suppress CHE transcript abundance in Col-0 plants (Fig. 1) and in the *cmI* double mutant (Supplementary Fig. 3), we suggest that the genetic interaction between *CML23/CML24* and CHE is not dependent on transcriptional regulation and that the effect of W7 is not through an effect on *CML23/CML24*. Additionally, circadian period was not affected by a transient increase in \([Ca^{2+}]_{cyt}\) following W7 treatment. This is not surprising, because in *che* mutants, in which CHE transcript abundance is constitutively reduced, period is unaffected\(^{11}\).

Whilst we do not consider that the Ca\(^{2+}\)-induced transcriptional changes in CHE affect circadian period, it might be of functional significance because it is consistent with the CHE binding site, also known as Site Ib\(^{30}\), being similar to the \([Ca^{2+}]_{cyt}\)-regulated Site II promoter element (AGGGCCAT\(^3\)). [Ca\(^{2+}\)]\(_{cyt}\)-regulation of Site II is most likely through the TCP family of transcription factors\(^{3}\), of which CHE is a member. CHE binds to the class I TCP-binding site (TBS) (GGTCCCACT) in the *CCA1* promoter and represses its expression\(^{3}\). In addition to CHE, CCA1 transcript was the only clock gene that was modestly activated around 8 h after the last pulse of CaCl\(_2\) and CHE repression (Fig. 1b). CHE oscillates 9 h out of phase with *CCA1* transcript\(^{10}\) and at a similar phase with \([Ca^{2+}]_{cyt}\) oscillations. Whilst we conclude that transcriptional changes in CHE are unlikely to mediate changes in circadian period, it raises the possibility that CHE transmits information about Ca\(^{2+}\) signals to the *CCA1* promoter.

Our data identify roles for Ca\(^{2+}\) and CML24 in the circadian clock. These findings unveil for first time in plants a function for circadian oscillations of \([Ca^{2+}]_{cyt}\) and expand the architecture of the plant circadian oscillator.

**Methods**

*Arabidopsis* mutant lines used in the reverse genetic screen were supplied generously from laboratories working in the area of Ca\(^{2+}\) signalling in plants and are listed in Supplementary Table 2. The T-DNA insertion mutants *che-1* and *che-2* and the point mutation single mutants *toc1-2* and *lhy-4* were provided by Steve Kay (The Scripps Research Institute, USA); the T-DNA insertion mutants *cca1-11, lhy-21*, *elf3-4* and *elf4-1* were donated by Seth Davis (University of York, UK); the T-DNA single mutant *ztl-3* (SALK_035701\(^1\)) was obtained from Nottingham *Arabidopsis* Seed Centre (NASC), UK. To obtain the triple mutants of the circadian oscillator gene with the *cml23-2 cml24-4* double mutant (*Col-0*), the single mutants *che-1* (*Col-0*), *che-2* (*Col-0*), *cca1-11* (*WS*), *lhy-21* (*WS*), *toc1-2* (*C24*), *ztl-3* (*Col-0*), *elf3-4* (*WS*), *elf4-1* (*WS*) and *lux-4* (*C24*), were crossed independently to *cml23-2 cml24-4* (*Col-0*) double mutants to generate triple mutants. The F2 progeny was then self-fertilized to obtain an F3 generation. The F3 and F4 generations were then genotyped to ensure all the mutant alleles were homozygous. F4 or subsequent generations were used for the epistatic study. Similarly, to the circadian clock mutants, different Arabidopsis ecotypes (WS and C24) were also crossed to Col-0.

Fig. 5 | Epistatic analysis of leaf movements rhythms shows that TOC1 is functionally linked to CML24 to regulate circadian period. Average normalized traces of leaf positions and FFT-NLLS analysis of the circadian period for leaf movement experiments. a–d, Results of *cml23-2 cml24-4* with *toc1-2* (*Col-0 n = 33, C24 n = 31*), double mutant (*22, clock gene mutant = 24, triple mutant = 25*) (a), with *cca1-11* (*Col-0 n = 7, WS-2 n = 6*, double mutant = 29, clock gene mutant = 21, triple mutant = 19) (b), with *lhy-21* (*Col-0 n = 24, WS-2 n = 24*, double mutant = 22, clock gene mutant = 24, triple mutant = 14) (c) and with *ztl-3* (*Col-0 n = 22*, double mutant = 25, clock gene mutant = 26, triple mutant = 15) (d). Grey lines indicate *cml23-2 cml24-4*, blue clock gene single mutants and black the triple mutants, respectively. 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 are presented from one experiment representative of two (*cca1-11, lhy-21, ztl-3*) or three (toc1-2) independent experiments. See also Supplementary Fig. 4 and Supplementary Table 3. Single or triple asterisks indicate significance of \(*P ≤ 0.05\) and \(* *P ≤ 0.001\), after Kruskal–Wallis one-way ANOVA on ranks followed by Dunn’s method was used to compare the triple mutant to the single and *cml23-2 cml24-4* double mutant.

The presence of nicotinamide, the circadian independent of CML23 and CML24 because in low light, added sugar shortened circadian period\(^{11}\) that was unaffected by *cml23-2 cml24-4* (Fig. 4c).
Growth of Arabidopsis thaliana, photon-counting imaging of aequorin and luciferase luminescence and transformation techniques were as described in[29] unless otherwise stated.

[Ca\textsuperscript{2+}]\textsubscript{i}\textsuperscript{cyt}, manipulation. To obtain plants with undetectable circadian [Ca\textsuperscript{2+}]\textsubscript{i}\textsuperscript{cyt} rhythms (unentrained), meaning that [Ca\textsuperscript{2+}]\textsubscript{i}\textsuperscript{cyt} remained at basal levels, 35 S:AEQ WS seeds\textsuperscript{a} were grown in opaque 7 mm × 9 mm plastic rings sealed at the base with 0.5 mm nylon mesh (Normesh, UK) on sucrose-free 0.5 MS agar, germinated without stratification and grown in continuous white light for at least 12 days. Artificial [Ca\textsuperscript{2+}]\textsubscript{i}\textsuperscript{cyt} rhythms were induced in these plants by step-wise addition of external CaCl\textsubscript{2} during the subjective day, followed by removal (Supplementary Table 1). During treatment, seedlings were floating on the mesh rafts on temperature-adjusted solutions. All experiments were repeated at least twice.

Induction of a single 24 h [Ca\textsuperscript{2+}]\textsubscript{i}\textsuperscript{cyt} peak was carried essentially the same except using a FLUOstar plate reader (BMG Labtech, Germany). 200 µl of treatment solution to provide final concentrations of 0 mM to 150 mM CaCl\textsubscript{2} (Supplementary Table 1) was injected into wells of a 96 well plate containing individual 12-day-old 35 S:AEQ transformed seedlings that had previously been reconstituted with 20 µM coelenterazine (20 °C, overnight). Seedlings were washed with temperature-adjusted deionized water before measurement and luminescence was measured\textsuperscript{11}.

Solutions were replaced with the successive treatment every hour. Results were assessed from three independent experiments each consisting of a minimum of 12 replicates per treatment.

For [Ca\textsuperscript{2+}]\textsubscript{i}\textsuperscript{cyt} measurements after treatment with N-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W7) (Calbiochem) see Supplementary Methods.

Microarray analysis. Plants treated with CaCl\textsubscript{2} as described above to generate 24 h [Ca\textsuperscript{2+}]\textsubscript{i}\textsuperscript{cyt} oscillations were harvested every 4 h for 24 h. RNA was isolated as described in\textsuperscript{48}. RNA was hybridised to GeneChips Arabidopsis ATH1 Genome Array using NASCA International Affymetrix service. Raw intensity data were normalized across chips using RMA (http://www.bioconductor.org) automatically log transforming the expression data. Array 1-22_Calcium_12h_Rep2_ATH1 was normalized across chips using RMA (http://www.bioconductor.org) automatically log transforming the expression data. Array 1-22_Calcium_12h_Rep2_ATH1 was removed from the analysis after failing hybridization quality control.

qPCR analysis. Experiment to determine the effect of W7: Col-0 and cml23-2 cml24-4 plants were treated with W7 solution (660 µM W7 and 50 mM CaCl\textsubscript{2}, containing a final concentration of 2.5 % (v/v) DMSO) or deionized water at ZT36 and ZT48 in constant light for 2 h and then frozen in liquid N\textsubscript{2}.

Fig. 6 | Epistatic analyses of leaf movements rhythms and flowering time shows that CHE is functionally linked to CML24. Average normalized traces of leaf positions and FFT-NLLS analysis of the circadian period for leaf movement experiments. a,b, Results of cml23-2 cml24-4 with che-2 (Col-0 n = 29, cml23-2 cml24-4 = 48, che-2 = 47, triple mutant = 40) (a) and with che-1 (Col-0 n = 29, cml23-2 cml24-4 = 48, che-1 = 39, triple mutant = 46) (b). Wild-type traces for leaf position were removed for clarity. Data are presented from one experiment representative of two (che-1) or three (che-2) independent experiments (Supplementary Fig. 4 and Supplementary Table 3). c,d, Flowering time responses under long (16 h:8 h) or short day conditions (8 h:16 h) (c) and che-1 cml23-2 cml24-4 (d) mutants. Number of leaves were recorded when the emerging bolt was 5 mm high. Dots represent the individual plants and the black bars the mean ± s.d. (n = 16; in LD Col-0 n = 15; in SD cml23-2 cml24-4 n = 15 and che-2 triple mutant n = 13). Single or triple asterisks indicate significance of *P ≤0.05 or ***P ≤0.001, after Kruskal–Wallis one-way ANOVA analysis followed by Tukey test (LD) or Dunn’s method (a, b and SD), when the triple mutant was compared to the che and cml23-2 cml24-4 mutants. Flowering rate was calculated using the number of days since germination when the number of leaves was recorded. che-2 cml23-2 cml24-4 mutant used was HLI8 and che-1 cml23-2 cml24-4 was HL15. An independent experiment in LD was done using three different mutants lines (Supplementary Fig. 5).
Experiment to determine the effect of cml23-2 cml24-4 mutation on the circadian clock transcript abundance: Col-0 and cml23-2 cml24-4 mutant were grown at 20 °C under 12 light/12 dark and then transferred into constant light conditions. After 2 days under constant light, plants were collected every 2 h from ZT48 to ZT72. Total RNA was extracted from three biological replicates of at least four pooled plants each, using the RNeasy Plant Mini Kit (QIAGEN) and RNeasy-Free DNase on-column treatment (QIAGEN). CDNA was synthesized from 500 ng RNA with the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) using oligo(dT) primers. The Pseudo-Response Homeolog probes 1 participate in the phase entrainment of circadian clocks to feeding. Cell 142, 943–953 (2010).

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Author contributions

M.C.M.R., K.E.H., M.J.G., S.A., C.T.H., N.I.M-N., F.C.R., T.J.H., H.J.J. and A.N.D. performed the experiments and analysed the data. The effects of Ca$^{2+}$ on circadian gene expression experiments were designed by M.J.G. and M.C.M.R. and performed by them with K.E.H., S.A., C.T.H., F.C.R. and A.N.D. Reverse genetic screening was performed by K.E.H. Analysis of cml23/cml24 mutants was performed by M.C.M.R., K.E.H., N.I.M-N., T.J.H. and H.J.J. Y.-C.T. provided lines before publication and advice. M.C.M.R., K.E.H. and A.A.R.W. wrote the manuscript. M.H., I.A.C., J.M.D., J.B. and A.A.R.W. managed the project, advised on interpretation and obtained the funding.

Competing interests

The authors declare no competing interests.

Additional information

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- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
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- For null hypothesis testing, the test statistic (e.g. $F$, $t$, $r$) with confidence intervals, effect sizes, degrees of freedom and $P$ value noted
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Software and code

Policy information about availability of computer code

Data collection

Aequorin or luciferase 24 h rhythms data were collected automatically using the softwares associated to the photon counting cameras, IndiGO software (version 2.0.5.0, Berthold technologies) or Image32 software (version 5.33, Photek). Ct values for qPCR experiments were collected using Rotor-Gene Q series software (version 1.7, QIAGEN). Aequorin luminescence values for determination of Ca2+ concentration were obtained using FLUORstar Optima software (BMG Labtech, Germany).

Data analysis

Luciferase and aequorin image analyses were done using IndiGO software (version 2.0.5.0, Berthold technologies) or Image32 software (version 5.33, Photek). Leaf movement images were analyzed using MetaMorph (version 7.6.0.0). Circadian rhythms data from luciferase imaging, aequorin imaging and leaf movement were analyzed using the BRASS (version 3.0) plug-in for MS excel (http://www.amillar.org) to carry out Fast Fourier Transform Non-Linear Least Squares (FFT-NLLS) analysis. Raw intensity data from microarray were normalized across chips using RMA (http://www.bioconductor.org). qPCR primers efficiencies were calculated using LinRegPCR (product version 1.0.0.0). Statistical analyses were performed in SigmaPlot (version 11.0 and 13.0).

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The authors declare that the data supporting the findings of this study are available within the paper (Figures 1 to 6) and its supplementary files (Supplementary Figure 1 to 5 and Supplementary Tables 2 and 3). Additionally, microarray data are available at NASC Arrays (http://arabidopsis.info/affy), experiment reference NASCARRAYS-529.

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | The sample size was chosen based in previous experiments and experience, but it is not reported in the manuscript. |
|-------------|---------------------------------------------------------------------------------------------------------------|
| Data exclusions | For microarray, the array 1-22_Calcium_12h_Rep2_ATH1 was removed from the analysis after failing hybridization quality control, this criteria was pre-establised.  
For the analysis of circadian period, only the plants/leaves considered rhythmic by BRASS were used to calculate period, this is a pre-established criteria.  
For Ct values, if one out of the three technical replicates had a difference greater than 0.5, the data was excluded. This is a pre-established criteria. |
| Replication | Microarray experiement was done once, but each data point/sample included two biological replicates (but the one mentioned in data exclusions section). To validate the microarray finding about CHE, we used a qPCR experiment. The qPCR experiments were done twice, including 3 biological replicates each and each one of these included three technical replicates; in both attemps the replication was successful. Leaves movements experiments for the screen showed in Table S2 were done once. Just those lines that were promising were analyzed further, including larger number of samples and then the experiments were repeated at least twice. Replication was successful.  
Flowering time experiments and camera experiments were done at least twice and replication was successful. |
| Randomization | Randomization of samples in camera, leaf movement and flowering time experiments is the normal way to do it and were assumed, so it is not specifically reported in the manuscript. |
| Blinding | Leaf movements experiments were performed and analyzed in blind without the experimenter knowledge of seeds lines. For these particular experiments this is the normal way to do it. This is to avoid bias during the data analysis, where rhythmic traces have to be selected by eye in an early step of the analysis. |

Reporting for specific materials, systems and methods

Materials & experimental systems

- n/a Involved in the study
- Unique biological materials
- Antibodies
- Eukaryotic cell lines
- Palaeontology
- Animals and other organisms
- Human research participants

Methods

- n/a Involved in the study
- ChIP-seq
- Flow cytometry
- MRI-based neuroimaging