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Circadian oscillations of cytosolic free calcium regulate the Arabidopsis circadian clock

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Abstract

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+} ($[Ca^{2+}]_{cyt}$), though their purpose has not been fully characterised. We investigated whether circadian oscillations of $[Ca^{2+}]_{cyt}$ regulate the circadian oscillator of *Arabidopsis thaliana*. We report that in Arabidopsis, $[Ca^{2+}]_{cyt}$ 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*).

Circadian oscillators confer competitive advantage by modulating physiology and development[1, 2]. In Eukaryotes, circadian oscillators are comprised of feedback loops of transcriptional regulators, however, the oscillator genes differ between the Kingdoms[2, 3]. In *Arabidopsis thaliana*, a morning loop is formed of *CIRCADIAN CLOCK ASSOCIATED1* (*CCA1*)[4] and *LATE ELONGATED HYPOCOTYL* (*LHY*)[5], *PSEUDO RESPONSE REGULATOR 7* (*PRR7*) and *PRR9*[6]. The evening feedback loop involves *TIMING OF CAB2 EXPRESSION1* (*TOC1*)[7] and *GIGANTEA* (*GI*)[8]. These loops are connected through *CCA1/LHY* mediated repression of *TOC1* expression[9] and *TOC1*-mediated repression of *CCA1* involving *CCA1 HIKING EXPEDITION* (*CHE*)[10].

Circadian oscillators also incorporate post-transcriptional, post-translational and metabolic processes[11-16]. In Arabidopsis, these include the F-box protein ZEITLUPE (ZTL)[17] regulated blue-light dependent degradation of TOC1[15, 18, 19] and photosynthetic sugars affect entrainment[11]. Several studies have revealed the importance of ionic signalling for circadian timekeeping[20-23]. In *Drosophila*, circadian oscillations of

intracellular Ca^{2+} ($[Ca^{2+}]$) regulate cellular oscillations *in vivo*[23] and temperature-induced increases in cytosolic $[Ca^{2+}]$ are involved in entrainment[21].

In plants, like in the mammalian suprachiasmatic nucleus, there are circadian oscillations of $[Ca^{2+}]_{cyt}$ [24]. They are driven by cyclic adenosine diphosphate ribose-mediated Ca^{2+}-release from internal stores[16, 25-28] to encode information about photoperiod, timing and light intensity[29, 30]. However, the functions regulated by circadian oscillations of $[Ca^{2+}]_{cyt}$ have not been identified. Therefore, it has been conjecture whether circadian oscillations of $[Ca^{2+}]_{cyt}$ represent an input to the oscillator or part of the timing mechanism, in addition to being an output[24].

We show that circadian oscillations of $[Ca^{2+}]_{cyt}$ 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 $[Ca^{2+}]_{cyt}$ signals

We identified potential targets for $[Ca^{2+}]_{cyt}$, by examining by microarray the response of circadian oscillator transcripts to a single 24 h artificial oscillation of $[Ca^{2+}]_{cyt}$ in plants in which circadian oscillations of $[Ca^{2+}]_{cyt}$ were abolished, and later artificially induced (Fig. 1a and Supplementary Fig. 1). $[Ca^{2+}]$ signals did not restore high amplitude oscillations of clock transcripts like those in light and dark cycles[31]. *CCA1 HIKING EXPEDITION* (*CHE*) was the only clock transcript whose abundance correlated with the $[Ca^{2+}]_{cyt}$ signal, having a dynamic opposite to the imposed $[Ca^{2+}]_{cyt}$ 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 $[Ca^{2+}]$ 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 $[Ca^{2+}]_{cyt}$ on *CHE* transcript abundance, we screened a number of $[Ca^{2+}]_{cyt}$ agonists to identify those that had profound and persistent effects on $[Ca^{2+}]_{cyt}$. N-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W7) in combination with $CaCl_2$ evoked sustained increases in $[Ca^{2+}]_{cyt}$[32]. This Ca^{2+} agonist caused a transient and large rise of $[Ca^{2+}]_{cyt}$ peaking at 848.5 ± 156.0 nM, followed by a sustained elevation for at least 2 h (Supplementary Fig. 1). Induction of large, sustained $[Ca^{2+}]_{cyt}$ increases at ZT36 and ZT48 by 2 h treatment with W7 and $CaCl_2$, confirmed that the major transcriptional response of the circadian oscillator was a repression of *CHE* abundance (Fig. 1c). Similar to the experiment with a single artificial 24 h $[Ca^{2+}]_{cyt}$ oscillation, the abundance of morning transcripts *CCA1* and *LHY* did not alter immediately after the $[Ca^{2+}]_{cyt}$ increase (Fig. 1c). This increase in $[Ca^{2+}]_{cyt}$ significantly altered the abundance of transcripts expressed later in the day, being an activator of *PRR9* and a repressor of *PRR7*, *PRR3* and *CHE* (fold changes: *PRR9* 3.2 (ZT48), *PRR7* 2.0 (ZT48), *PRR3* 1.7 (ZT36) and 2.1 (ZT48), *CHE* 3.4 (ZT36)) (Fig. 1c). Finally, the transcript levels of the evening genes were not affected (Fig. 1c). These Ca^{2+}-induced changes in *CHE* transcript did not alter the free running period of the oscillator, because treating plants with the W7 solution at ZT0 and ZT12 did not alter the period of *CCA1:LUC* (ZT0 Control 24.4 ± 0.5 h, W7 solution 24.2 ± 0.2 h; ZT12 Control 24.4 ± 0.2 h, W7 solution 24.4 ± 0.5 h; $p>0.05$). Thus, manipulation of $[Ca^{2+}]_{cyt}$ demonstrated that both 24 h $[Ca^{2+}]_{cyt}$ oscillations and shorter-term $[Ca^{2+}]_{cyt}$ 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 $[Ca^{2+}]_{cyt}$ affected circadian oscillator gene abundance but not period, we wished to determine whether Ca^{2+} signalling has a role in regulating the clock *in planta*. We performed a screen of 75 well characterised Ca^{2+} signalling mutants of transporters, transducers and sensors. Five lines had significantly increased circadian period of leaf movement compared to wildtype (Fig. 2 and Supplementary Table 2); *vitamin C* (*vtc) 1*-1[33] (Col-0 23.8 ± 0.1 h, *vtc1*-1 24.7 ± 0.2 h, p=0.001), *calmodulin-like (cml) 24*-1 and the double mutant *cml23*-2 *cml24*-1(Col-0 24.1 ± 0.1 h, *cml24*-1 24.6 ± 0.1 h, *cml23*-2 *cml24*-1 25.0 ± 0.1 h, p<0.001 for both) and also *cml24*-4 and the double mutant *cml23*-2 *cml24*-4[34, 35] (Col-0 23.9 ± 0.1 h, *cml24*-4 25.1 ± 0.1 h, *cml23*-2 *cml24*-4 25.6 ± 0.1 h, p<0.001 for both). *cml23*-2 only had a phenotype when in combination with alleles of CML24 (Fig. 2), suggesting that CML24 and CML23 could be redundant in the regulation of the clock. None of the overexpressing (*CML24-OX1* and *CML24-OX2*) or underexpressing lines (*CML24-U1* and *CML24-U1*) affected circadian period (Supplementary Table 2). As *CML24* (previously called *TCH2*)[36, 37] encodes a CALMODULIN-LIKE Ca^{2+}-sensor[34, 35] 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 $[Ca^{2+}]_{cyt}$ (Fig. 3a-3d) (Col-0 23.7 ± 0.1 h, *cml23*-2 *cml24*-4 26.1 ± 0.4 h for CCA1:LUC, p<0.001; Col-0 23.7 ± 0.2 h, *cml23*-2 *cml24*-4 25.1 ± 0.1 h for 35S:AEQ, p<0.001). To investigate the effect of *CML24* on the central oscillator in more detail, we analyzed *CCA1*, *PRR7* and *TOC1* transcript abundance in Col-0 and *cml23*-2 *cml24*-4. In the third day in LL, there was a substantial delay of 4 h in the phase of *CCA1*, *TOC1* and *PRR7* transcript abundance in the mutant plants (Fig. 3e), consistent with the lengthening of circadian period by ~1.5 h described in Fig. 3a-d. The transcript levels of the clock components were unaffected.

CML24 regulates circadian period in a $[Ca^{2+}]_{cyt}$-dependent manner

Because circadian rhythms persist in conditions where the circadian oscillations of $[Ca^{2+}]_{cyt}$ are abolished, such as nicotinamide[16], sucrose[24] or monochromatic red light[29], circadian rhythms of $[Ca^{2+}]_{cyt}$ are not necessary for a rhythmic oscillator. However, we could test for necessity for oscillations in $[Ca^{2+}]_{cyt}$ in the correct regulation of circadian period by determining whether the action of CML24 in the circadian clock depends upon Ca^{2+}. If the effects of the CMLs are independent of Ca^{2+}, then the effects of mutation on circadian period should be additive to treatments that abolish circadian $[Ca^{2+}]_{cyt}$ oscillations. Whereas, if the action of the CMLs is dependent on Ca^{2+}, then mutation should have no further effect in the presence of Ca^{2+} antagonists. 50 mM nicotinamide increased the circadian period of leaf movement[16] but in its presence, *cml23*-2 *cml24*-4 was indistinguishable from the wild type (Col-0 27.7 ± 0.2 h, *cml23*-2 *cml24*-4 28.1 ± 0.2 h, p=0.490) (Fig. 4a). Sucrose abolishes $[Ca^{2+}]_{cyt}$ oscillations[24], however under high and low light conditions it has different effects on circadian period[11]. There was no significant difference between *cml23*-2 *cml24*-4 and wild type on 3% sucrose (high light Col-0 24.7 ± 0.1 h, *cml23*-2 *cml24*-4 25.0 ± 0.1 h, p = 0.051; low light Col-0 26.4 ± 0.2 h, *cml23*-2 *cml24*-4 26.9 ± 0.2 h, p=0.061) (Figs. 4b and 4c). Lastly, in monochromatic red light, the circadian period was not statistically different in the *cml23*-2 *cml24*-4 mutant compared to Col-0 (Col-0 26.3 ± 0.4 h, *cml23*-2 *cml24*-4 26.1 ± 0.2 h p=0.410) (Fig. 4d). The lack of an effect of *cml23*-2 *cml24*-4 in conditions that abolish circadian oscillations of $[Ca^{2+}]_{cyt}$ is consistent for loss-of-function mutations in putative Ca^{2+}-sensor proteins and CML24 acting downstream of $[Ca^{2+}]_{cyt}$.

Additionally, we found that *CML23* and *CML24* transcript abundance (Fig. 4e) was increased in response to the artificially imposed $[Ca^{2+}]_{cyt}$ 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 (ACGCGT)[32].

CML24 not only senses Ca^{2+}, it also regulates NO[35] [35] as shown by high levels of NO in *cml24* mutants[35]. 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 PROTEIN2: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)[38].

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 transcriptional 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 transcriptional 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 *CML23/24* and *CCA1, LHY, TOC1, ZTL, ELF3, ELF4* and *LUX.* Double *CML23/24* 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 ± 0.3 h, Mann-

195 Whitney Rank Sum Test p<0.001; Fig. 5a)[29]. In the triple mutant *toc1*-2 *cml23*-2 *cml24*-
196 4, the long period arising from mutations in *CML23*/*CML24* was absent, having a period
197 that was indistinguishable from the single mutant *toc1*-2, and significantly shorter than
198 *cml23*-2 *cml24*-4 mutant (One-way ANOVA p=1 and p<0.001, respectively) (Fig. 5a,
199 Supplementary Fig. 4 and Supplementary Table 3). This indicates that *toc1*-2 is
200 epistatic to *cml23*-2 *cml24*-4. We did not find epistatic interactions between mutations
201 in *CML23*/*CML24* and *CCA1, LHY* or *ZTL. cml23*-2 *cml24*-4 increased period in the
202 *cca1*-11, *lhy*-21 and *ztl*-3 backgrounds, resulting in additive phenotypes (Fig. 5b-5d and
203 Supplementary Table 3). Analysis of genetic interactions between *CML23/CML24* and
204 *ELF3, ELF4* and *LUX* is complicated by the arrhythmic phenotypes caused by loss-of-
205 function of these evening complex genes. Triple mutants of *cml23-2 cml24*-4 and
206 members of the evening complex where therefore all arrhythmic in LL (Supplementary
207 Fig. 4). Crossing the wild type genetic backgrounds used in this study was without
208 effect (Supplementary Table 3).
209
210 *TOC1*-mediated repression of *CCA1* involves *CHE* [10]. Therefore, the regulation of *CHE*
211 by $[Ca^{2+}]_{cyt}$ and interaction between mutations in *CML23*/*CML24* and *TOC1*, prompted
212 investigation of whether *CHE* is also part of the genetic pathway by which *CML24*
213 regulates circadian period. We identified epistatic interaction between mutations in
214 *CML23*/*CML24* and *CHE* in the regulation of circadian leaf movements. As previously
215 reported for *CCA1:LUC*$^+$ rhythms, the circadian period of leaf movement in *che*-1 and
216 *che*-2 single mutants was indistinguishable from the background (two-tailed Student´s
217 t-test p=0.461 and p=0.681, respectively; Fig. 6a and 6b)[10]. In the triple mutant *che*-2
218 *cml23*-2 *cml24*-4, the long period arising from mutations in *CML23*/*CML24* was absent,
219 being indistinguishable from the single mutant *che*-2 and significantly shorter than the
220 *cml23*-2 *cml24*-4 mutant (One-way ANOVA p>0.05 and p<0.05, respectively) (Fig. 6a,
221 Supplementary Fig. 4 and Supplementary Table 3). However, we found no evidence
222 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[10], 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[35], 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 $[Ca^{2+}]_{cyt}$-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 $[Ca^{2+}]_{cyt}$ can feed back into the circadian oscillator. We demonstrate that $[Ca^{2+}]_{cyt}$ 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 $[Ca^{2+}]_{cyt}$ rhythms are abolished. We conclude that correct circadian period is dependent on CML24 and circadian rhythms of $[Ca^{2+}]_{cyt}$. Epistatic analysis suggests that *TOC1* and likely *CHE* genetically interact with *CML24* (Supplementary Fig. 6). Additionally, we show that $[Ca^{2+}]_{cyt}$ 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[35]. Our new data demonstrates 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)[35], 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*-11, *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)[39], *che*-1 and *che*-2 (no effect on period)[10], *prr3-1* and *prr5-3* (□1 h)[40], *lnk1*-1 (no effect on period), *lnk2*-2 (1 h), *lnk1*-1 *lnk2*-2 (2 h)[41], *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 $[Ca^{2+}]_{cyt}$ 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[35]. 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 $[Ca^{2+}]_{cyt}$ as shown by the absence of an effect of the *cml23*-2 *cml24*-4 mutation when circadian oscillations of $[Ca^{2+}]_{cyt}$ are suppressed[16, 24, 29] (Fig. 4a-4d). This also demonstrates that sucrose regulates circadian period through a pathway independent of CML23 and CML24 because in low light, added sugar shortened circadian period[11] which was unaffected by *cm23*-2 *cml24*-4 (Fig. 4c). In the presence of nicotinamide, the circadian 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[42]. The Arabidopsis genome encodes over 50 CaM and CMLs[42,43] 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[44] 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 *cml* 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 CML23/24. Additionally, circadian period was not affected by a transient increase in $[Ca^{2+}]_{cyt}$ following W7 treatment. This is not surprising, because *che* mutants, in which *CHE* transcript abundance is constitutively reduced, period is unaffected[10].

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 IIb[45], being similar to the $[Ca^{2+}]_{cyt}$-regulated Site II promoter element (AGGCCCAT)[32]. $[Ca^{2+}]_{cyt}$-regulation of Site II is most likely through the TCP family of transcription factors[32], of which *CHE* is a member. CHE binds to the class I TCP-binding site (TBS) (GGTCCCAC) in the *CCA1* promoter and represses its expression[10]. 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

Plant material and growth

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[10] and the point mutation single mutants *toc1*-2[9] and *lux*-4[46] were provided by Steve Kay (The Scripps Research Institute, USA); the T-DNA insertion mutants *cca1*-11, *lhy*-21, *elf3*-4 and *elf4*-1[48] were donated by Seth Davis (University of York, UK); the T-DNA single mutant *ztl*-3 (SALK_035701)[17] was obtained from Nottingham Arabidopsis Seed Centre (NASC), UK. To obtain the triple mutants of the circadian oscillator genes 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.

Growth of *Arabidopsis thaliana*, photon-counting imaging of aequorin and luciferase luminescence and transformation techniques were as described in [29] unless otherwise stated.

$[Ca^{2+}]_{cyt}$ manipulation

To obtain plants with undetectable circadian $[Ca^{2+}]_{cyt}$ rhythms (unentrained), meaning that $[Ca^{2+}]_{cyt}$ remained at basal levels, 35S:AEQ WS seeds[29] were grown in opaque 7 mm x 9 mm plastic rings sealed at the base with 0.5 µm nylon mesh (Normesh, UK) on sucrose-free 0.5 MS agar seeds, germinated without stratification and grown in continuous white light (LL) for at least 12 days. Artificial $[Ca^{2+}]_{cyt}$ rhythms were induced in these plants by step-wise addition of external $CaCl_2$ 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 $[Ca^{2+}]_{cyt}$ 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 $CaCl_2$ (Supplementary Table 1) was injected into wells of a 96 well plate containing individual 12 day old *35S: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 luminesce was measured[48]. 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 $[Ca^{2+}]_{cyt}$ measurements after treatment with N-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W7) (Calbiochem) see Supplementary Methods.

Microarray analysis

Plants treated with $CaCl_2$ as described above to generate 24 h $[Ca^{2+}]_{cyt}$ oscillations were harvested every 4 h for 24 h. RNA was isolated as described in [16]. RNA was hybridised to GeneChips *Arabidopsis* ATH1 Genome Array using NASC's 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 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 $CaCl_2$, 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_2.

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 ºC 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 of three biological replicates of at least four pooled plants each, using the RNeasy Plant Mini Kit (QIAGEN) and RNase-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 gene-specific products were amplified using the Rotor-Gene SYBR Green PCR Kit on a Rotor-Gene 6000 Real-Time PCR machine (QIAGEN). Primers used are detailed in Supplementary Methods. Relative transcript levels were determined by incorporating PCR efficiencies[49].

Leaf movement analysis

Analysis of circadian rhythms of the first true leaves was performed as described in [16] without experimenter knowledge of seed lines. In those experiments where nicotinamide was applied, 50 mM Nicotinamide (Sigma) or deionized water was applied once a day for 2 days before the start of imaging; 50 µl of solution was applied to the aerial parts of each seedling. The experiments were repeated twice but for the triple mutants, *cml23*-2 *cml24*-4 *che*-2 and *cml23*-2 *cml24*-4 *toc1*-2 were repeated three times. For *cml23*-2 *cml24*-4 *toc1*-2 two independent lines were used.

Photoperiodic flowering time screening

Plants were sown directly onto soil and grown in 20 □C and 100 µmol m^{-2} s^{-1} in either long day (LD) conditions (16 h L:8 h D) or short day (SD) conditions (8 h L: 16 h D). Flowering time was defined as when the emerging bolt reached a height of approximately 5 mm. Screening experiments typically had 6 to 8 plants of each line per

414 growth condition, and confirmation experiments had 15 to16 plants of each line per
415 growth condition.
416
417 **Statistical Analysis**
418 All statistical tests, n number, the measure of the centre and the error bars are
419 described in figure legends when appropriate. Other statistical parameters are listed in
420 Supplementary Statistical Parameters section. For comparison between two groups,
421 two-tailed Student´s *t*-test or two-sided Mann-Whitney Rank Sum Test were used. Both
422 test analyses were considered significant if $p<0.05$, $p<0.01$ and $p<0.001$. For
423 comparison between more than two groups, one-way ANOVA followed by Holm-Sidak
424 method or Kruskal-Wallis one-way ANOVA followed by Dunn´s method or Tukey test
425 were used. ANOVA tests were performed with an alpha level of 0.05 or 0.001.
426
427 **Data availability**
428 The authors declare that the data supporting the findings of this study are available
429 within the paper (Figures 1 to 6) and its supplementary files (Supplementary Figure 1 to
430 5 and Supplementary Tables 2 and 3). Additionally, microarray data are available at
431 NASC Arrays (http://arabidopsis.info/affy), experiment reference NASCARRAYS-529.
432
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559

560 **Figure legends**

561 **Fig. 1.** Transcripts Abundance of Circadian Clock Genes is Modulated by $[Ca^{2+}]_{cyt}$.

562 **a**, Imposing oscillations of external $CaCl_2$ restores circadian oscillations in $[Ca^{2+}]_{cyt}$ in
563 unentrained seedlings. See also Supplementary Fig. 1c for calibrated data. Results
564 represent the mean ± S.D. (n=12 biological replicates) for one experiment. Experiments
565 were repeated three times.

566 **b**, Effect of the imposition of a 24 h oscillation of $[Ca^{2+}]_{cyt}$ on the transcript abundance
567 (expressed as log2) of the circadian clock genes. Closed circles indicate unentrained
568 water-treated samples, green circles the unentrained $CaCl_2$-treated plants. To generate
569 the oscillation, $CaCl_2$ was applied as described by the shaded areas and in
570 Supplementary Table 1. Results represent the mean (n=2 biological replicates).

571 **c,** Plants treated at ZT36 and ZT48 with a solution containing 660 µM W7 and 50 mM
572 $CaCl_2$ for 2 h, were assayed for changes in the abundance of circadian clock
573 transcripts by qPCR. Dots represent each measurement and the black bars the mean ±
574 S.D. (n= 3 biological replicates). See also Supplementary Fig. 1. Single, double or triple

asterisk indicate significance of ≤ 0.05, ≤ 0.01 and ≤ 0.001, respectively 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*).

Fig. 2. CML24 Regulates Circadian Period in Arabidopsis.

Average normalized traces of leaf positions (left panels). 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**, Col-0 n=70, *cml23*-2 n=70, *cml24*-1 n=97, double mutant n=94; **b**, Col-0 n=63, *cml23*-2 n=69, *cml24*-4 n=49, double mutant n=110, rhythmic leaves. **a** shows the results of *cml23*-2 and *cml24*-1 single and double mutants, **b** shows the results of *cml23*-2 and *cml24*-4 single and double mutants. Red lines indicate Col-0, grey lines indicate *cml23cml24* double mutants, and light blue *cml24* single mutants. *cml23*-2 traces for leaf position were removed for clarity. All plants were grown under 12 h L: 12 h D cycles before the experiments. Data represent three (**a**) or two (**b**) independent experiments. Single or triple asterisk indicate significance of ≤ 0.05 and ≤ 0.001, respectively, after two-tailed Student's t test or two-sided Mann-Whitney Rank Sum test (**b**, Col-0 *vs*. double mutant).

Fig. 3. CML24 has profound effect on the regulation of the Arabidopsis circadian clock.

The *cml23*-2 *cml24*-4 mutant has a long circadian period of 35S:AEQ and CCA1:LUC luminescence. Mean normalized luminescence ± S.D. of 35S:AEQ (**a**) and CCA1:LUC (**b**) for wildtype (red circles) and *cml23*-2 *cml24*-4 (grey circles) from two independent experiments (**a**, n=8 biological replicates; **b**, Col-0 n=23 and *cml23*-2 *cml24*-4 n=24, biological replicates). **c** and **d** show the FFT-NLLS analysis of the samples used in **a** and **b**, respectively. Triple asterisk indicate significance of ≤ 0.001, after two-tailed Student´s t test analysis (**c**) and two-sided Mann-Whitney Rank Sum Test (**d**). **e,** *CCA1*, *TOC1* and *PRR7* transcripts abundance were analysed at the time point indicated in plants grown for 12 days in 12h:12h light:dark cycles and transferred to

601 continuous light at ZT0. qPCR results represent the mean ± S.D. (n=3 biological
602 replicates). Single, double or triple asterisk indicate significance of ≤ 0.05, ≤ 0.01 and ≤
603 0.001, respectively after two-tailed Student´s t test analysis or Mann-Whitney Rank
604 Sum Test (*CCA1* ZT60 and *PRR7* ZT48).

605 **Fig. 4.** Circadian oscillations of $[Ca^{2+}]_{cyt}$ are necessary for the correct function of the
606 Circadian Oscillator.

607 **a**, Circadian period estimates of leaf movement in continuous high light (80 µmol m^{-2} s^{-1})
608 of Col-0 and *cml23*-2 *cml24*-4 plants treated with either 50 mM nicotinamide (Col-0
609 n=15, *cml23*-2 *cml24*-4 n=33, biological replicates) or water (Col-0 n=16, *cml23*-2
610 *cml24*-4 n=29, biological replicates). **b**, Circadian period estimates of CCA1:LUC
611 rhythms in continuous high light (80 µmol m^{-2} s^{-1}) or **c**, continuous low light (10 µmol m^{-2} s^{-1}) of Col-0 and *cml23*-2 *cml24*-4 plants grown in the presence of either water (high
613 light Col-0 n=7, *cml23*-2 *cml24*-4 n=8, low light n=16, biological replicates), 90 mM
614 sucrose (high light n=8, low light n=16, biological replicates) or 90 mM mannitol (Col-0
615 n=8, *cml23*-2 *cml24*-4 n=7, biological replicates). **d**, Circadian period estimates of
616 CCA1:LUC rhythms in Col-0 and *cml23*-2 *cml24*-4 plants under continuous high mixed
617 red (660 nm) and blue (470 nm) light (80 µmol m^{-2} s^{-1}) (n=4, biological replicates) and
618 continuous monochromatic blue or red light (40 µmol m^{-2} s^{-1}) (blue n=7, red Col-0 n=11
619 and *cml23*-2 *cml24*-4 n=10, biological replicates). **e**, Effect of the imposition of a ramp
620 of external $CaCl_2$ (Fig. 1a) on the expression (log2) of *CML24* and *CML23*. $CaCl_2$ was
621 applied as described by the shaded areas and in Supplementary Fig. 1c. Plant material
622 was harvested from the onset of treatment every 4 h for 24 h to extract RNA for probing
623 with microarray. Results represent the mean (n=2 biological replicates). **f**, Circadian
624 period estimates of CCA1:LUC rhythms in continuous high light (80 µmol m^{-2} s^{-1}) of
625 Col-0 (n=7 biological replicates) and *cml23*-2 *cml24*-4 (n=8 biological replicates) plants
626 treated from the day before going into continuous high light either with water or 200 µM
627 cPTIO. Period estimates were obtained by BRASS and are shown as mean ± S.E.M.

628 Data were obtained from 1 independent experiment. Experiments were repeated at
629 least twice. Single, double or triple asterisk indicate significance of ≤ 0.05, ≤ 0.01 and ≤
630 0.001, respectively after two-tailed Student´s t test analysis or two-sided Mann-Whitney
631 Rank Sum test (**a** (water), **b** (mannitol) and **f**).

632 **Fig. 5.** Epistatic Analysis of Leaf Movements Rhythms Shows that *TOC1* is
633 Functionally Linked to *CML24* to Regulate Circadian Period.

634 Average normalized traces of leaf positions and FFT-NLLS analysis of the circadian
635 period for leaf movement experiments. **a** shows the results of *cml23*-2 *cml24*-4 with
636 *toc1*-2 (Col-0 n=33, C24=31, double mutant=22, clock gene mutant=24, triple
637 mutant=25), **b** with *cca1*-11 (Col-0 n=7, Ws-2=6, double mutant=29, clock gene
638 mutant=21, triple mutant=19), **c** with *lhy*-21 (Col-0 n=24, Ws-2=24, double mutant=22,
639 clock gene mutant=24, triple mutant=14) and **d** with *ztl*-3 (Col-0 n=22, double
640 mutant=25, clock gene mutant=26, triple mutant=15). Grey lines indicate *cml23*-2
641 *cml24*-4, blue clock gene single mutants and black the triple mutants, respectively.
642 Wild-type traces for leaf position were removed for clarity. All plants were grown under
643 12 h L: 12 h D cycles before the experiments. Data are presented from one experiment
644 representative of two (*cca1*-11, *lhy*-21, *ztl*-3) or three (*toc1*-2) independent
645 experiments. See also Supplementary Fig. 4 and Supplementary Table 3. Single or
646 triple asterisk indicate significance of ≤ 0.05 and ≤ 0.001, respectively, after Kruskal-
647 Wallis One Way Analysis of Variance on Ranks followed by Dunn´s method was used
648 to compare the triple mutant to the single and *cml23*-2 *cml24*-4 double mutant.

649 **Fig. 6.** Epistatic Analyses of Leaf Movements Rhythms and Flowering Time Shows that
650 *CHE* is Functionally Linked to *CML24*.

651 Average normalized traces of leaf positions and FFT-NLLS analysis of the circadian
652 period for leaf movement experiments. **a** shows the results of *cml23*-2 *cml24*-4 with
653 *che*-2 (Col-0 n=29, *cml23*-2 *cml24*-4=48, *che*-2=47, triple mutant=40) and **b** with *che*-1

(Col-0 n=29, *cml23*-2 *cml24*-4=48, *che*-1 =39, triple mutant=46). 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). Flowering time responses under long (16 h:8 h) or short days conditions (8 h:16 h) for *che*-2 *cml23*-2 *cml24*-4 (**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 asterisk indicate significance of ≤ 0.05 or ≤ 0.001, respectively 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 HL18 and *che*-1 *cml23*-2 *cml24*-4 was HL15. An independent experiment in LD was done using three different mutants lines (Supplementary Fig. 5).

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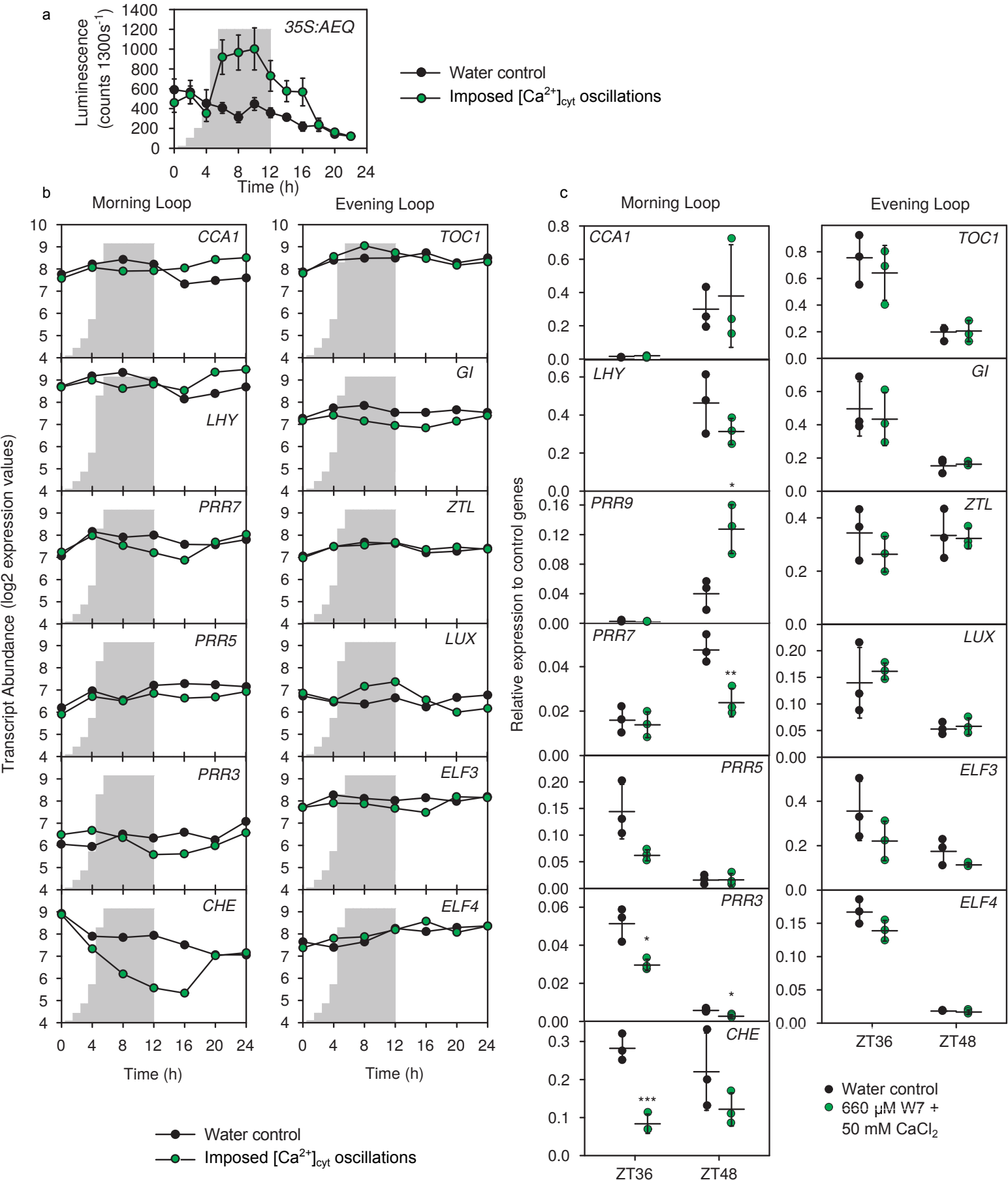
Author contributions

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 analyzed 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 prior to 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 financial interests.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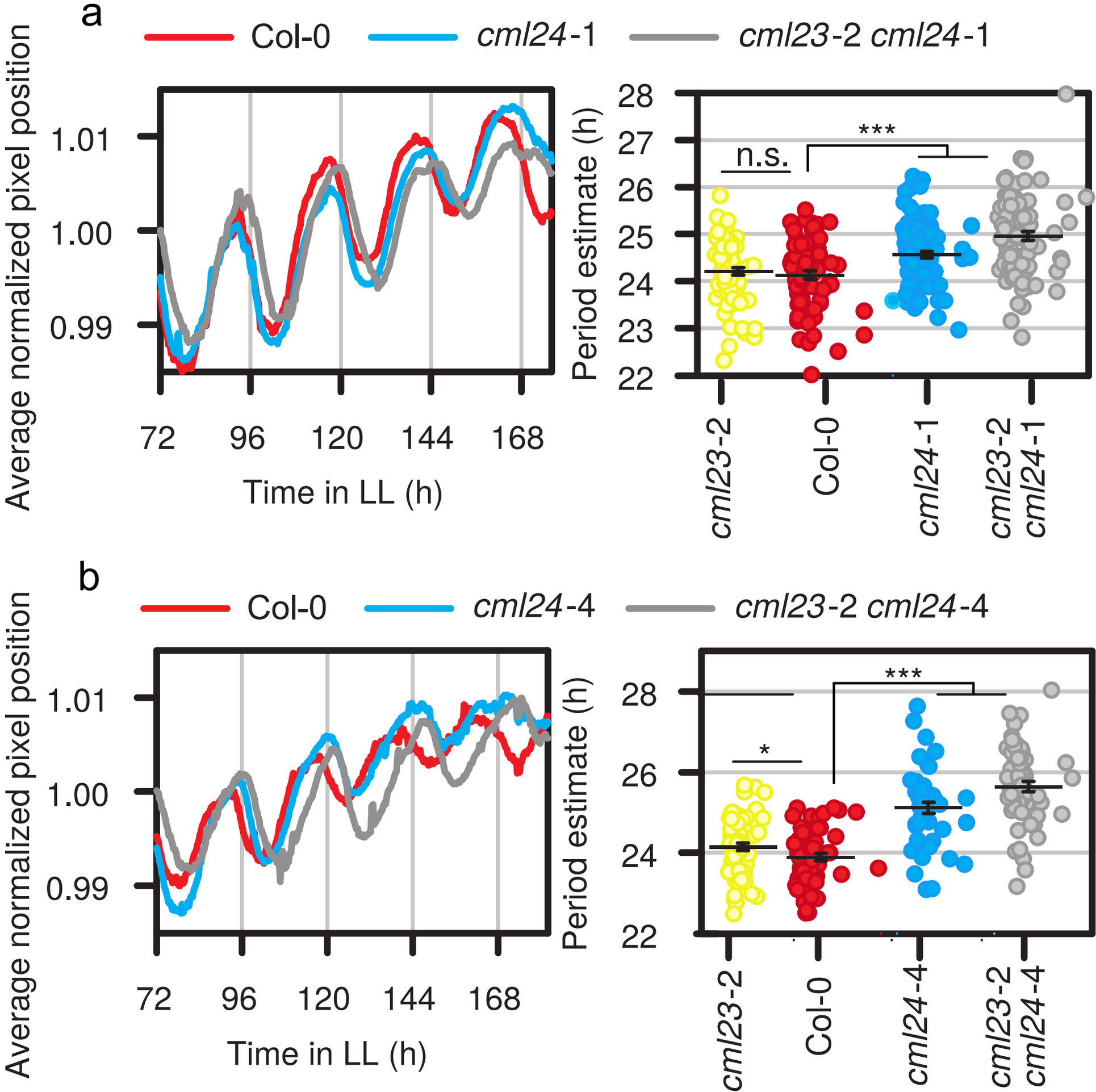
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Time in LL (h)
Period estimate (h)
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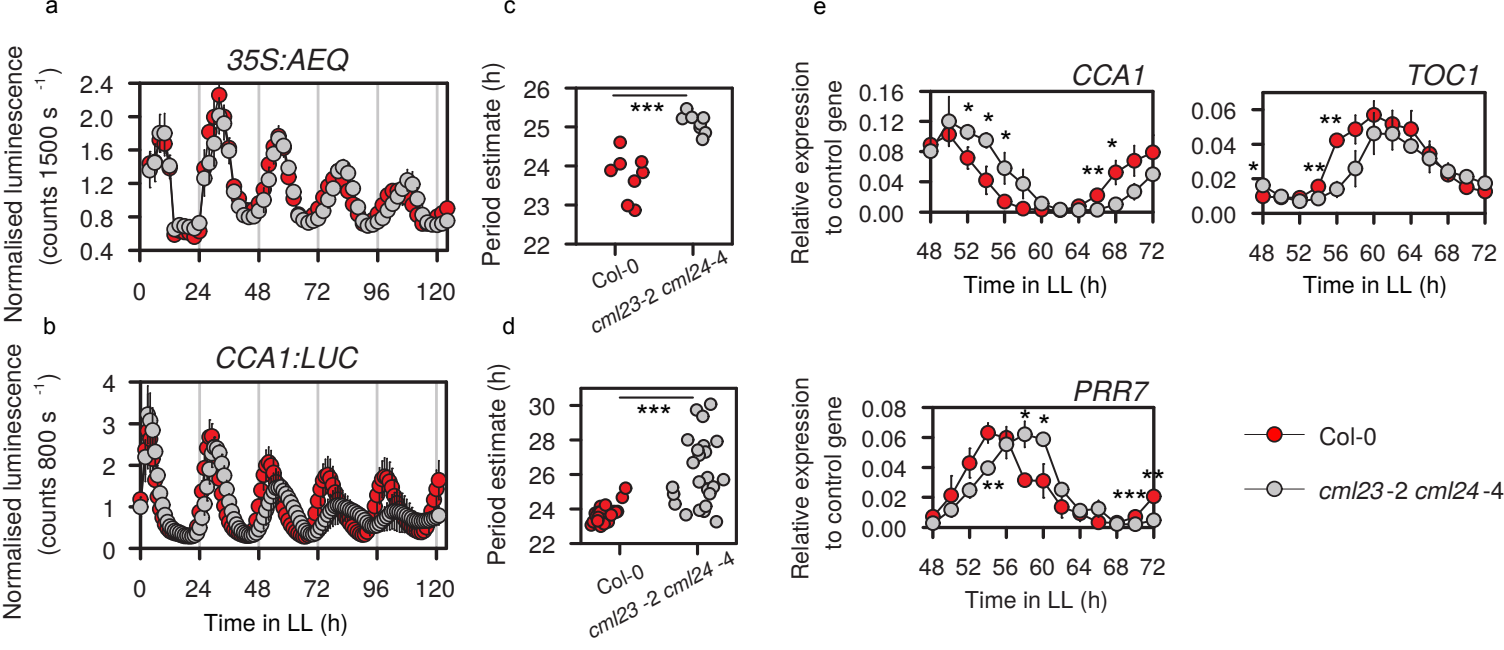
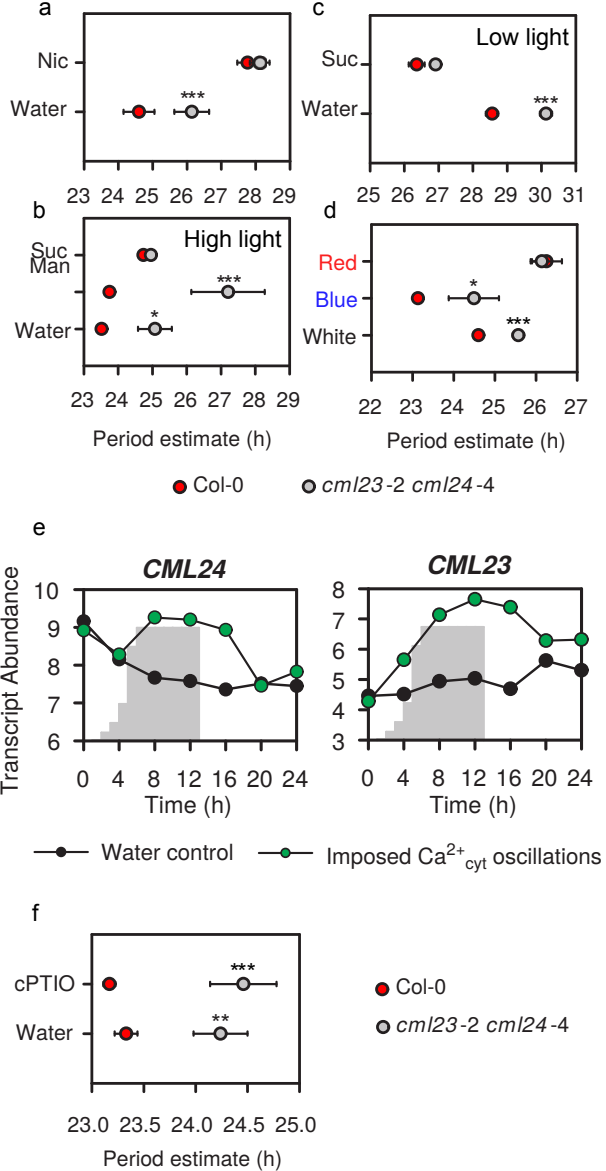
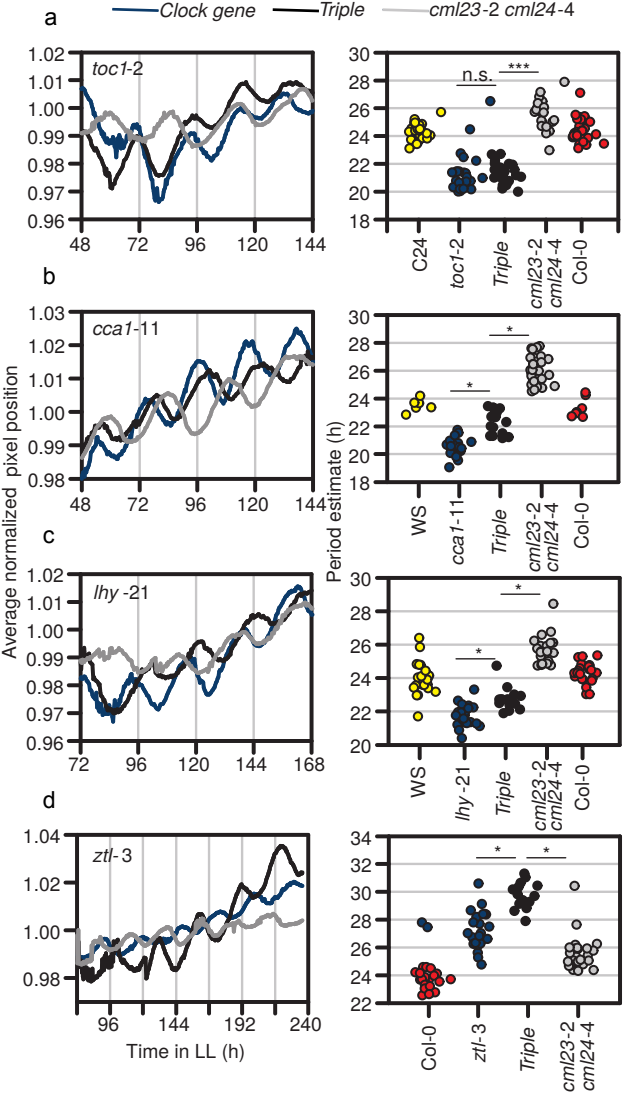
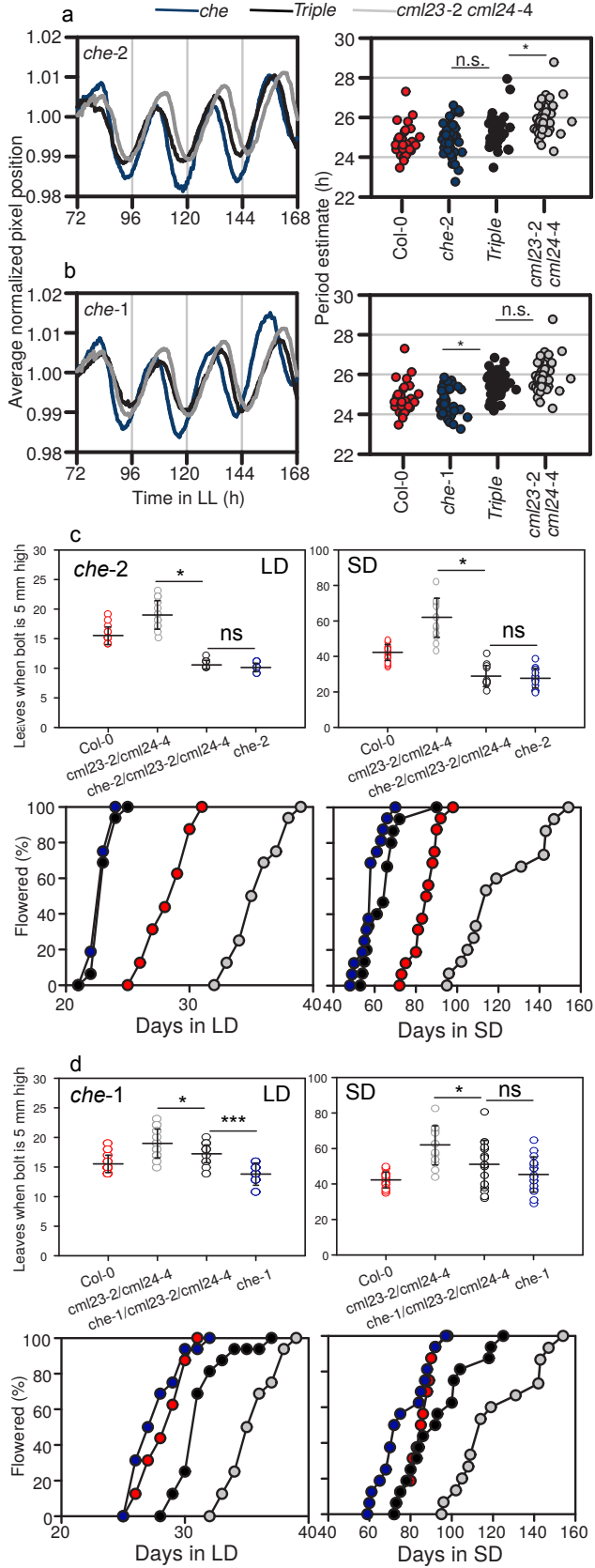
26
24
22
*
***
cml23-2
Col-0
cml24-4
cml23-2 cml24-4


a
35S:AEQ
Normalised luminescence (counts 1500 s -1)
2.4
2.0
1.6
1.2
0.8
0.4
0
24
48
72
96
120
c
Period estimate (h)
26
25
24
23
22
***
Col-0
cml23-2 cml24-4
e
CCA1
Relative expression to control gene
0.16
0.12
0.08
0.04
0.00
48 52 56 60 64 68 72
Time in LL (h)
TOC1
0.06
0.04
0.02
0.00
b
CCA1:LUC
Normalised luminescence (counts 800 s -1)
4
3
2
1
0
Time in LL (h)
d
Period estimate (h)
30
28
26
24
22
PRR7
0.08
0.06
0.04
0.02
0.00
Col-0
cml23-2 cml24-4


a
Nic
Water
***
23 24 25 26 27 28 29
c
Low light
Suc
Water
***
25 26 27 28 29 30 31
b
High light
Suc
Man
Water
***
*
23 24 25 26 27 28 29
Period estimate (h)
d
Red
Blue
White
*
***
22 23 24 25 26 27
Period estimate (h)
Col-0
cml23-2 cml24-4
e
CML24
CML23
Transcript Abundance
10 9 8 7 6
8 7 6 5 4 3
0 4 8 12 16 20 24
Time (h)
Water control
Imposed Ca2+cyt oscillations
f
cPTIO
Water
***
**
23.0 23.5 24.0 24.5 25.0
Period estimate (h)
Col-0
cml23-2 cml24-4


Clock gene
Triple
cml23-2 cml24-4
a
toc1-2
n.s.
***
C24
toc1-2
Triple
cml23-2 cml24-4
Col-0
b
cca1-11
*
*
WS
cca1-11
Triple
cml23-2 cml24-4
Col-0
Average normalized pixel position
Period estimate (h)
c
lhy-21
*
*
WS
lhy-21
Triple
cml23-2 cml24-4
Col-0
d
ztl-3
*
*
Col-0
ztl-3
Triple
cml23-2 cml24-4
Time in LL (h)


a
che
Triple
cml23-2 cml24-4
che-2
Average normalized pixel position
Period estimate (h)
n.s.
*
Col-0
che-2
Triple
cml23-2 cml24-4
b
che-1
Time in LL (h)
n.s.
*
Col-0
che-1
Triple
cml23-2 cml24-4
c
che-2
LD
SD
Leaves when bolt is 5 mm high
*
ns
Col-0
cml23-2/cml24-4
che-2/cml23-2/cml24-4
che-2
Flowered (%)
Days in LD
Days in SD
d
che-1
LD
SD
Leaves when bolt is 5 mm high
*
***
ns
Col-0
cml23-2/cml24-4
che-1/cml23-2/cml24-4
che-1
Flowered (%)
Days in LD
Days in SD