Circadian gating of dark-induced increases in chloroplast- and cytosolic-free calcium in Arabidopsis

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SUMMARY

- Changes in the spatio-temporal concentration of free Ca^{2+} ($[Ca^{2+}]$) in different organelles of the cell contribute to responses of plants to physiological and environmental stimuli. One example are $[Ca^{2+}]$ increases in the stroma of chloroplasts during light to dark transitions, however the function and mechanisms responsible are not known, in part because there is a disagreement in the literature concerning whether corresponding dark-induced changes in cytosolic $[Ca^{2+}]$ ($[Ca^{2+}]_{cyt}$) can be detected.
- We have measured changes in $[Ca^{2+}]_{cyt}$ upon darkness in addition to the already known dark-induced increases in $[Ca^{2+}]_{stroma}$ in the aerial part of the *Arabidopsis thaliana* plant.
- These $[Ca^{2+}]_{cyt}$ transients depend on the photoperiod and time of the day, peaking at anticipated dusk and are superimposed on daily 24 h oscillations in $[Ca^{2+}]_{cyt}$. We also find that the magnitude of the dark-induced increases in Ca^{2+} in both the cytosol and chloroplasts are gated by the nuclear circadian oscillator.
- The modulation of the magnitude of dark-induced increases in $[Ca^{2+}]_{stroma}$ and $[Ca^{2+}]_{cyt}$ by transcriptional regulators in the nucleus that are part of the circadian oscillator demonstrate a new role for the circadian system in sub-cellular Ca^{2+} signalling, additional to its role in driving circadian oscillations $[Ca^{2+}]$ in the cytosol and chloroplasts.

KEY WORDS: calcium signalling, chloroplast, circadian clock, cytosol, light-dark transition.

INTRODUCTION

A wide range of plant cell responses to environmental stimuli are associated with specific changes in the spatio-temporal concentration of free Ca^{2+} ($[Ca^{2+}]$), known as "Ca^{2+} signatures", present in numerous cellular types and compartments (McAinsh et al., 1995; Kiegle et al., 2000; Marti et al., 2013; Sello et al., 2018). In the cytosol, Ca^{2+} signatures arise from fluxes of Ca^{2+} into the cytosol across the plasma membrane or by release from internal stores. Downstream pathways decode the Ca^{2+} signatures to allow the cell to respond appropriately (Dodd et al., 2010).

The time scale over which the Ca^{2+} signatures can occur varies greatly. Abiotic and biotic signals can cause rapid increases in $[Ca^{2+}]_{cyt}$ (Lynch et al., 1989; Price et al., 1994; Knight et al., 1997; Monshausen et al., 2009). On a slower scale, there are 24 h $[Ca^{2+}]_{cyt}$ daily rhythms, regulated by the circadian clock and light signalling (Dalchau et al., 2010). Diel oscillations of $[Ca^{2+}]_{cyt}$ in light and dark cycles, or constant light, rise to a peak of approximately 300 nM towards the middle and end of the photoperiod (Johnson et al. 1995, Love et al. 2004, Dalchau et al., 2010). Circadian oscillations of $[Ca^{2+}]_{cyt}$ occur predominantly in the spongy mesophyll cells (Marti et al., 2013), are driven by the rhythmic production of cyclic ADP ribose (Dodd et al., 2007) and are suppressed specifically by the circadian oscillator gene *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*) (Dodd et al., 2007). Daily and circadian oscillations of $[Ca^{2+}]_{cyt}$ form part of the circadian oscillator, regulating its function through sensing by CALMODULIN-LIKE 24, a Ca^{2+} sensor protein, which genetically interacts with the circadian oscillator protein TIMING OF CAB 1 (TOC1) (Marti et al., 2018).

Similar to the cytosol, there are chloroplastic circadian $[Ca^{2+}]$ oscillations, in addition to increases in chloroplast stromal $[Ca^{2+}]$ ($[Ca^{2+}]_{stroma}$) in response to biotic and abiotic signals (Johnson et al., 1995; Nomura et al., 2012; Sello et al., 2016). Changes in $[Ca^{2+}]$ in the chloroplasts regulate aspects of photosynthesis, organelle division and the import of nuclear-encoded proteins (Rocha and Vothknecht, 2012; Nomura and Shiina, 2014; Hochmal et al., 2015). Additionally, the environmental transition between light and darkness produces a prolonged and sustained increase in $[Ca^{2+}]_{stroma}$ (Sai & Johnson, 2002; Nomura et al., 2012; Sello et al., 2016; Loro et al., 2016) that depends on photoperiod. However, it was concluded that these dark induced increases in $[Ca^{2+}]_{stroma}$ are not modulated (or "gated") by the nuclear circadian oscillator (Sai & Johnson, 2002).

Many studies have tried to unravel the mechanisms and biological role that underlie the generation and dissipation of $[Ca^{2+}]$ transients in the chloroplasts upon darkness (Sai & Johnson, 2002; Loro et al.,2016; Sello et al., 2018; Frank et al., 2019). Recently, new Arabidopsis lines expressing Aequorin in different chloroplastic compartments have been developed (Sello et al., 2018) and two Arabidopsis chloroplast-targeted Ca^{2+} transporters, BIVALENT CATION TRANSPORTER 1 (BICAT1) and BICAT2, have been found to determine the amplitude of the dark-induced $[Ca^{2+}]_{stroma}$ increase (Frank et al., 2019). The later study suggested that the most straightforward explanation for the strong diminishment of the *bicat2* mutants $[Ca^{2+}]_{stroma}$ transient, is a dark-triggered influx of Ca^{2+} from the cytosol. However, the authors reported that this idea is currently not favoured because $[Ca^{2+}]_{cyt}$ recordings failed to detect a consistent decrease of $[Ca^{2+}]_{cyt}$ upon the onset of darkness (Sai & Johnson, 2002; Nomura et al., 2012; Sello et al., 2016; Sello et al., 2018) and therefore, it has led to the hypothesis that the generation of the dark-induced $[Ca^{2+}]_{stroma}$ signal is a result of Ca^{2+} being released from a hypothetical chloroplastic store. This hypothesis is also supported because buffering cytosolic Ca^{2+} with the chelator EGTA combined with digitonin, was without effect on Ca^{2+} transients in the chloroplasts (Loro et al., 2016). However, short stromal $[Ca^{2+}]$ spikes were strongly reduced, suggesting that cytosolic Ca^{2+} and/or cellular integrity are the source, or at least necessary for the spikes (Loro et al., 2016). Therefore, more studies are necessary to understand how and why the chloroplastic $[Ca^{2+}]$ transients upon darkness are generated.

The nuclear circadian oscillator can regulate photosynthetic activity in the chloroplast, at least in part because in Arabidopsis, the nuclear-encoded SIGMA FACTOR5 (SIG5) controls circadian rhythms of transcription of several chloroplast genes (Noordally et al., 2016). Because the nuclear circadian oscillator can regulate events in the plastids, we decided to revisit whether the generation of the dark-induced $[Ca^{2+}]_{stroma}$ signal was gated by the circadian clock, in order to understand how this signal is controlled. In parallel, we have investigated the regulation of Ca^{2+} signals in the cytosol by light to dark transitions to resolve a debate in the literature about the potential mechanisms for the regulation of plastid Ca^{2+} transients.

Here, we demonstrate that similar to the chloroplasts, there are reproducible and consistent increases in $[Ca^{2+}]_{cyt}$ at the onset of darkness in the aerial part of the plant, which are superimposed on the daily 24 h oscillations in $[Ca^{2+}]_{cyt}$. We report that dark-induced transient in $[Ca^{2+}]$ in both

the cytosol and stroma are gated by the circadian clock. This finding demonstrates an important new role for the circadian system in sub-cellular Ca^{2+} signalling, and also establishes a new link between eukaryotic circadian clocks and organelles of endosymbiotic origin.

MATERIAL AND METHODS

Plant materials and growth conditions

Arabidopsis thaliana ecotypes Columbia-0 (Col-0), Wassilewskija-2 (Ws-2), Landsberg erecta (Ler) and *cry1*, *cry2*, *phyA* and *phyB* mutants carrying *CaMV 35S:AEQUORIN (35S:AEQ)* were described previously in Xu et al., 2007 and Sai & Johnson, 2002. *prr7*-11 *prr5*-10 *prr9*-11 (Nakamichi et al., 2005) plants carrying *35S:AEQ* targeted to the cytosol, were obtained as described in Xu et al., 2007.

Arabidopsis seeds were surface sterilised with 10% (v/v) NaClO and 0.1% (v/v) Triton X-100 for 3 min and rinsed 3 times with sterile dH_2O. Surface-sterilized seeds were sown onto 0.8% (w/v) bactoagar plates containing 0.5 strength Murashige and Skoog (pH 5.7 with 0.5 M KOH) without sucroseand stratified in the dark for 48 h at 4 ºC. Seeds were germinated and entrained in growth cabinets (Sanyo, UK) with constant temperature of 19 ºC and 100 µmol m^{-2} s^{-1} cool white light from fluorescent tubes under 12 h light/12 h dark cycles (LD 12:12), unless otherwise stated.

Aequorin imaging for dark-induced [Ca^{2+}] transient using ICCD225 photon-counting camera system

Photon counting was performed in a light-tight box using a Photek (Hastings, UK) ICCD225 photon-counting camera system mounted above the seedlings. The camera chambers supplied equal amounts of red (630 nm)/blue (470 nm) LED light in a mixed array (100 µmol m^{-2} s^{-1}) at the desired photoperiod and was cooled to 19-20 ºC. When just red or blue light was used the intensity was (50 µmol m^{-2} s^{-1}). Luminescence was recorded from clusters of 7 to 12 seedlings grown as described, and the data for one experiment was obtained as the sum of the signal of all the cluster together. Image analysis was done with Photek IFS32 software.

For measurements lasting > 1 day or for one-time point measurements, photon-counting images were captured every 2 h for 1500 s following a wait of 200 s post-illumination to allow light from delayed fluorescence to scatter or at the end of the photoperiod for 7000 s or a different time point when stated, respectively. In both, seedlings were incubated with 50 µl of 20 µM coelenterazine (Nanolight, UK) for 20 min in the dark the night before going into the camera box when they were 11 - 12 days old.

Estimation of daily and circadian oscillations of $[Ca^{2+}]_{cyt}$

Estimation of daily and circadian oscillations of $[Ca^{2+}]_{cyt}$ was performed as described by Love et al., 2004. Sixteen-bit images of the photon density generated from the 1500 s or the last 700 s of each integration were obtained and processed using the Photek IFS32 software. Circadian parameters were analysed using the BRASS plug-in for MS excel (http://www.amillar.org) to carry out Fast Fourier Transform Non-Linear Least Squares (FFT-NLLS) analysis (Plautz et al., 1997) with period limits between 18 and 35 hours at 95% confidence level. Rhythms were analysed for at least three cycles in constant conditions after the first 24 h. Traces with a relative amplitude error > 0.5 were considered arrhythmic.

Aequorin imaging for dark-induced $[Ca^{2+}]$ transient using a luminometer

Measurement of bioluminescence from the Ca^{2+} reporter aequorin and calibration to estimate $[Ca^{2+}]$ were determined as follows. Arabidopsis seedlings were grown as described for the camera system. When plants were 11 - 13 days old, 3 plants were placed within a luminometer tube (51 mm long × 12 mm diameter, Sarstedt, Leicester, UK) containing 1 ml of 0.8% (w/v) bactoagar media containing 0.5 strength Murashige and Skoog and incubated with 100 µl of 20 µM coelenterazine (Nanolight, UK) for 20 min in the dark. At the end of the photoperiod, bioluminescence was measured using a photon-counting luminometer (photomultiplier tube 9899A) cooled to -20ºC with a FACT50 housing (Electron Tubes, UK) (Marti et al., 2013). Aequorin bioluminescence was captured every second for at least 2 h and finally discharged by 1 ml of discharge solution (2 M $CaCl_2$ dissolved in 20% (v/v) ethanol). Measurements were made until the detected luminescence reached 10% of the first peak after discharge injection. $[Ca^{2+}]$ levels were determined according to Fricker et al. (1999).

Statistical analysis

F-Test Two-Sample for Variances followed by Two-tailed Student´s t test, were performed to compare the changes in area under the aequorin luminescence curves from plants transfer from light to darkness or plants that were in the dark during 6 h.

RESULTS

Changes in cytosolic free calcium in Arabidopsis upon darkness

Using an ICCD 225 photon-counting camera to detect luminescence of AEQUORIN in the cytoplasm we detected a very prolonged increase in luminescence 3.5 min after the plants were transferred from white light to darkness at the end of the photoperiod (12 h after the onset of light) (Fig. 1a, Supporting Information Fig. S1). The dark-induced $[Ca^{2+}]_{cyt}$ transient at dusk could be divided in two phases. Firstly, we observed a "spike" that peaks around 10 min after the onset of darkness and secondly, a sustained increase that peaks around 30 min after the start of darkness and decays over the next 2 h. During the first 3.5 min aequorin luminescence is contaminated by photons originating from delayed chlorophyll fluorescence and as a consequence, aequorin luminescence and therefore changes in $[Ca^{2+}]_{cyt}$ immediately following the light to dark transition could not be detected. Plants that were recorded 6 h into the dark period did not have any change in luminescence (Fig. 1a, Supporting Information Fig. S2). Statistical analysis of the area under the curve for the luminescence traces in plants that were transferred from light to darkness and plants that were already in the dark during 6 h, resulted in significant differences between the two conditions studied (L to D 10453 ± 1359, D to D 7321 ± 203; Student t-test for equal variances, P=0.04, df=8, t=2.26) suggesting it is the transfer from light to dark that results in the change of aequorin luminescence. No signal was detected from non-transgenic Col-0 plants (Fig. 1b, Supporting Information Fig. S2) grown and assayed using the same method as the transgenic plants (Fig. 1a), including treatment with coelenterazine, allowing us to conclude that the increase in luminescence that occurs from 3.5 min after darkness is due to changes in aequorin luminescence and therefore $[Ca^{2+}]_{cyt}$.

The dark-induced $[Ca^{2+}]_{cyt}$ signal measured using the cytosolic aequorin in plants that were grown in 12h : 12h light : dark cycles, differs to that from a dark-induced signal from aequorin targeted to the stroma (Sai & Johnson, 2002). Comparison of the cytosolic and stromal $[Ca^{2+}]$ signals in Fig. 1c shows that the prolonged $[Ca^{2+}]_{cyt}$ signature does not mimic the $[Ca^{2+}]_{stroma}$ signature, mostly in the sustained increase of the cytosolic signal that peaks around 30 min after the start of darkness and that decays over the next 2 h, whereas the stromal $[Ca^{2+}]$ signal consists of two peaks at approximately 8 and 25 min lasting 40 min, followed by a prolonged increase that last about 2 h after dark (Fig.1c, Supporting Information Fig. S3). The difference in dynamics between the

luminescence reported by the stromal- and cytosolic-targeted aequorin suggests that the two signals are distinct, emanating from different compartments and that there are specific dark-induced increases in $[Ca^{2+}]_{cyt}$. Interestingly, the dark-induced $[Ca^{2+}]_{stroma}$ spike measured by the photon-counting camera was slightly different to those previously measured by others (Sai and Johnson 2002; Nomura et al., 2012; Sello et al., 2016; Sello et al., 2018) and ourselves (Supporting Information Fig. S3) in a luminometer, consisting of a rapid increase in $[Ca^{2+}]_{stroma}$ that reaches a peak between 25 min after dusk and decays close to basal levels within the next 2 h. Thus, we conclude there is an increase in $[Ca^{2+}]_{cyt}$ in response to darkness, not detected previously in some of the previous studies (Nomura et al., 2012; Sello et al., 2016; Sello et al., 2018).

We were unable to calibrate the magnitude of the increase in $[Ca^{2+}]_{cyt}$ measured using intensified CCD arrays because the discharge of available aequorin with excess Ca^{2+} to perform a normalization saturated pixels on the CCD detector. To obtain an estimate of the magnitude of the dark-induced increase in $[Ca^{2+}]_{cyt}$ that occur after 3.5 min after darkness, when chlorophyll fluorescence has dissipated, we revisited the use of luminometry in the same camera experiment conditions, with three plants per sample to avoid the leaves covering each other (Supporting Information Fig. S4). In 12 out of 14 experiments we detected changes in $[Ca^{2+}]_{cyt}$ after the transition into darkness. These changes peaked around 10 min after darkness and lasted 10 min with a variable estimated amplitude of 5 nM to 65 nM $[Ca^{2+}]_{cyt}$. This might be an underestimate because the normalization assumes all cells contribute equally to the response. The signature detected with the luminometer in the cytosol (Supporting Information Fig. S4) was broadly like the one detected in the camera but contained less detail (Fig. 1a, Supporting Information Fig. S1). For the cytosolic signal, the sustained increase peaking around 30 min and decayed over the next 2 h in darkness was not detected using luminometry.

The dark-induced cytosolic free calcium transient emanates from green tissue

To determine from where in the plant the dark-induced $[Ca^{2+}]_{cyt}$ transient arises, we performed a luminometry experiment using excised green tissue or roots, to separate the signal coming from the two tissues during the data acquisition. When the luminescence from the two different tissues was analyzed separately, the dark-induced $[Ca^{2+}]_{cyt}$ increase was detected in the green tissue and no increase was observed in the roots (Fig. 2, Supporting Information Fig. S5).

Identification of the signaling pathways leading to dark-induced cytosolic-free calcium increases

Since the dark-induced increase in $[Ca^{2+}]_{cyt}$ was dependent on prior illumination (Fig. 1a) we tested whether a particular photoreceptor system is involved in the perception of light prior to darkness. Growth of plants in either monochromatic red or blue light alone allowed the $[Ca^{2+}]_{cyt}$ changes upon darkness, suggesting that the $[Ca^{2+}]_{cyt}$ change that occurs when plants are transferred from light into darkness involves both red and blue photoreceptors systems (Fig. 3a). Monochromatic light resulted in a larger spike at 10 min than seen when plants were grown in white light, though we are uncertain why this might be.

To test further the involvement of the red and blue photoreceptors, we performed a light to dark transition using *cry1*, *cry2*, *phyA* and *phyB* Arabidopsis mutants. Statistical analysis of the changes in the area under the curve for the luminescence comparing *cry* mutants with wild type plants (Fig. 3b), resulted in no significant differences (Ler 8135 ± 906, *cry1* 6677 ± 60; Student t-test for unequal variances, P=0.32, df=2, t=4.3; Ler 8135 ± 906, *cry2* 4676 ± 132; Student t-test for unequal variances, P=0.09, df=2, t=4.3). These analyses indicated that the dark-induced $[Ca^{2+}]_{cyt}$ changes were still present in these mutant lines, suggesting that the remaining photoreceptor system in the mutants were able to respond to the transition into darkness. In Fig. 3c we tested the effect of the red photoreceptors mutants *phyA* and *phyB* on the dark-induced $[Ca^{2+}]_{cyt}$ changes. The absence of *PHYB* did not affect the response (Ler 8135 ± 906, *phyB* 14117 ± 1639; Student t-test for equal variances, P=0.06, df=4, t=2.77). However, we found that in the *phyA* mutant, the dark-induced $[Ca^{2+}]_{cyt}$ was absent (Ler 8135 ± 906, *phyA* 3837 ± 332; Student t-test for equal variances, P=0.02, df=4, t=2.77), suggesting that at least, *PHYA* is directly involved in the light signaling pathway that leads to the dark-induced cytosolic-free calcium increases.

Additionally, we performed a set of experiments to investigate whether the alteration of $[Ca^{2+}]_{cyt}$ after darkness was derived from internal stores or from the extracellular space. Firstly, we decided to evaluate whether Ca^{2+} influx to the cytosol might be across the plasma membrane using two inhibitors of plasma membrane-mediated influx of Ca^{2+}, 1 mM $GdCl_3$ and 10 mM $LaCl_3$) (Véry & Davies, 2000) that were added 30 min before the measurement of $[Ca^{2+}]_{cyt}$ at the end of the photoperiod. The dark-induced $[Ca^{2+}]_{cyt}$ transient was insensitive to Gd^{3+} and La^{3+} when the area changes under the traces were determined (Control 12844 ± 642, Ga^{3+} 12406 ± 1657, Student t-

test for equal variances P=0.79, df=3, t=3.18; Control 12844 ± 642, La^{3+} 16816 ± 7296, Student t-test for unequal variances, P=0.68, df=2, t=12.71) (Fig. 3d), suggesting that the primary pathway by which dark increases $[Ca^{2+}]_{cyt}$ in Arabidopsis might be from an intracellular compartment. Cyclic ADP ribose (cADPR) is a Ca^{2+} signalling molecule synthesized by ADP-ribosyl cyclase (ADPRc) that can release Ca^{2+} into the cytosol from the ER and vacuole (Leckie et al., 1998; Navazio et al., 2000; Sánchez et al., 2004). ADPRc activity and cADPR can be detected, and the enzymatic protein been recently identified (Dodd et al., 2007; Awal et al., 2016; Wan et al., 2019). cADPR is thought to regulate circadian oscillations of $[Ca^{2+}]_{cyt}$ (Dodd et al., 2007). We tested for a potential involvement of cADPR using nicotinamide, an ADPRc activity inhibitor, and cPTIO, a nitric oxide (NO) scavenger, because NO increases ADPRc activity (Awal et al., 2016). When plants were incubated with 20 mM nicotinamide since plants were seven days old or 0.3 mM cPTIO which was added 30 min before the measurement, the dark-induced $[Ca^{2+}]_{cyt}$ transient was abolished or decreased (Control 12844 ± 642, nicotinamide 9583 ± 451, Student t-test for equal variances P=0.04, df=3, t=3.18; Control 12844 ± 642, cPTIO 5953 ± 49, Student t-test for unequal variances, P=0.009, df=2, t=4.30) (Fig. 3d), suggesting that ADPRc activity might be necessary for the dark-induced $[Ca^{2+}]_{cyt}$ signal. Additionally, we added 20 µM DCMU, an inhibitor of the photosynthetic electron transport chain, 24 h before measurement, to test whether photosynthesis was involved in the generation of the $[Ca^{2+}]_{cyt}$ signal upon darkness. The addition of DCMU negatively affected the dark-induced $[Ca^{2+}]_{cyt}$ increase (Control 12844 ± 642, DCMU 8991 ± 885, Student t-test for equal variances P=0.02, df=4, t=2.77), suggesting that photosynthesis may affect the $[Ca^{2+}]_{cyt}$ signal upon darkness (Fig. 3d).

Dark-induced transients in cytosolic free calcium might encode information about photoperiod and time of the day

Transition to darkness is the laboratory mimic of end of day at the onset of night. Therefore, we tested the hypothesis that changes in $[Ca^{2+}]_{cyt}$ upon darkness might encode information about day length. We grew plants under different photoperiods such as long day cycles (16 h:8 h) (LD 16:8) and short day cycles (8 h:16 h) (SD 8:16). The $[Ca^{2+}]$ response in the cytosol was affected by the length of day (Fig. 4). The dark-induced $[Ca^{2+}]_{cyt}$ changes were very similar in plants grown in SD 8:16 and 12 h:12 h light : dark cycles (Fig. 4, a and b). However, when plants were grown in LD 16:8, the $[Ca^{2+}]_{cyt}$ signature was different showing a larger peak at 7 min that lasted for 5 min before returning to the basal level (Fig. 4c). We also examined whether the dark-induced

304 $[Ca^{2+}]_{stroma}$ signal was sensitive to day-length. It has been reported that in LD 16:8, the $[Ca^{2+}]_{stromal}$
305 peaks lasted for 1 h and then returned to the basal level and that in SD 8:16, the profile of Ca^{2+}
306 $[Ca^{2+}]_{stromal}$ included a smaller peak later in the night that was not observed in the long-day
307 photoperiod (Sai & Johnson, 2002). We observed similar results under long photoperiod (Fig. 4c)
308 where a $[Ca^{2+}]_{stroma}$ increase was present about 25 min after darkness. However, in SD 8:16 we
309 observed a later large and prolonged peak that is not present plants grown under long day
310 conditions (Fig. 4a). Finally, when we compared the differences and similarities in the dynamics
311 between the cytosolic and stromal signals, we found that the differences between the two signals
312 are more apparent at SD 8:16 and LD 16:8 than 12 h:12 h light : dark cycles (Fig. 4). The different
313 responses of the cytosolic and stromal signals to the change in photoperiod might suggest a
314 different mechanistic basis.
315
316 As there was an effect of length of photoperiod on the dark-induced $[Ca^{2+}]_{cyt}$ signal we tested if
317 the magnitude of the Ca^{2+} increase was dependent on the time of transfer to darkness. We compared
318 the photon-counting data captured at different times of the day from plants that were growing in
319 mixed red and blue light-dark cycles (LD 12:12; 12 h light/12 h dark). Data were recorded every
320 2 h during one day in LD 12:12 conditions as shown in Fig. 5a. If plants were in the photoperiod
321 phase, every 2 h plants were transferred to darkness and data were recorded for 1500 s following
322 a wait of 200 s post-illumination to allow light from delayed fluorescence to scatter. After each
323 measurement, lights were turned on and remained on until the next measurement. When plants
324 were in the dark period of the 24 h LD 12:12 cycles, data were recorded every 2 h during 1500 s.
325 The changes in $[Ca^{2+}]_{cyt}$ upon darkness were modulated by the time of the day, being higher at the
326 end of the photoperiod and absent during the night when there was no transition from light to
327 darkness (Fig. 5b, Fig. 6 a and b). A similar behavior was measured for the $[Ca^{2+}]_{stroma}$ (Fig. 5c,
328 Supporting Information Fig. S6). The absence of signal in non-transgenic plants treated with
329 coelenterazine, demonstrated that the time-of-day change in luminescence was due to changes in
330 $[Ca^{2+}]$ (Supporting Information Fig. S7).
331
332 The pattern of the $[Ca^{2+}]_{cyt}$ signature was different if plants were transferred to darkness every 2 h
333 (Fig. 5b) or only at the end of the photoperiod (Fig. 1a). At dusk, the sustained increase observed
334 in the camera after the first transient spike (Fig. 1a) was absent if plants were assayed every 2 h

(Fig. 5b). The difference between these signals suggests there is some effect of integrated amount of light on the pattern of the signal, in addition to that of time-of-day.

The dark-induced cytosolic and stroma free calcium transients are modulated by the circadian clock

The time-of-day dependent gating of the dark-induced $[Ca^{2+}]_{cyt}$ increase suggests that the circadian timekeeper could be involved. To test this hypothesis, we measured dark-induced Ca^{2+} increases in plants maintained without a prolonged dark of night (constant light (LL) with dark interruptions, a protocol standard for circadian luminescence such as promoter::luciferase fusions measurements, because the short dark breaks do not interfere with functioning of the circadian oscillator (Millar et al., 1995)). Similar to LD cycles, from the second day in LL during the subjective mornings the dark-induced $[Ca^{2+}]_{cyt}$ increase was detected, being higher at later phases of the circadian subjective days, demonstrating that $[Ca^{2+}]_{cyt}$ can respond to darkness in the absent of the dark of night (Fig. 6 a and b (Col-0)). Additionally, this time of day dependent modulation of the magnitude of the dark-induced increases in $[Ca^{2+}]_{cyt}$ in LL, demonstrates that the signal observed is due to gating by the circadian oscillator rather than as a consequence of a compounding effect of the signal due to multiple stimulation or initiated in the light itself. Thus, at 2 h after the onset of dark (14 h) in the LD cycle, there was no increase in $[Ca^{2+}]_{cyt}$ (Fig. 5b), whereas at 14 h in the 1st LL cycle a dark induced-increase of $[Ca^{2+}]_{cyt}$ was measured lower than the one detected at 12 h in the same condition (Fig. 6 a and b (Col-0)), demonstrating that the changes in $[Ca^{2+}]_{cyt}$ are specific for the transition from light to darkness. To further investigate the circadian regulation of $[Ca^{2+}]_{cyt}$ upon darkness, we measured $[Ca^{2+}]_{cyt}$ in the circadian arrhythmic triple mutant *prr7*-11 *prr5*-11 *prr9*-10 (Nakamichi et al., 2005). In the LD cycle and the first day in LL there are no differences between the mutant and the WT in terms of gating, however in free running conditions in which the mutant is arrhythmic, the mutant failed to gate the dark-induced $[Ca^{2+}]_{cyt}$ response (Fig. 6b, Supporting Information Fig. S8). These data demonstrate that modulation of dark-induced increases of $[Ca^{2+}]_{cyt}$ is an output of the nuclear circadian oscillator.

A previous report concluded that dark-induced regulation of $[Ca^{2+}]_{stroma}$ is not regulated by the nuclear circadian oscillator (Sai & Johnson, 2002). In that study, plants were transferred to dark at different times after five days in constant light (Sai and Johnson, 2002). Because the circadian

366 regulation of $[Ca^{2+}]_{cyt}$ damps over time we decided to examine again whether $[Ca^{2+}]_{stroma}$ is under
367 circadian control. We found that circadian-gating persisted for at least two cycles in LL suggesting
368 that the dark-induced $[Ca^{2+}]_{stroma}$ increase is modulated by the circadian oscillator in the nucleus
369 and the previous conclusion of no role for the circadian oscillator might be incorrect (Fig. 7,
370 Supporting Information Fig. S9).
371
372 **The dark-induced transient of $[Ca^{2+}]_{cyt}$ is superimposed on daily and circadian $[Ca^{2+}]_{cyt}$**
373 **oscillations**
374 Aequorin-based luminescence measurements must be performed in the dark because the intensity
375 of photons released by aequorin is many orders of magnitude lower than the light required in the
376 standard growth conditions for plants. The method used here to determine the daily and circadian
377 control of the dark-induced $[Ca^{2+}]_{cyt}$ signal is the same one used to determine the daily and
378 circadian $[Ca^{2+}]_{cyt}$ oscillations (Love et al., 2004), which is based on standard protocols for
379 measurement of circadian regulation of luminescent reporters such as promoter::luciferase fusions
380 (Milllar et al, 1995). Taking this into account and the finding of that the circadian timekeeper gated
381 the dark-induced $[Ca^{2+}]_{cyt}$ increase, we decided to investigate whether the measured daily and
382 circadian oscillations of basal $[Ca^{2+}]_{cyt}$ are a consequence of the measurement protocol or occur in
383 addition to the dark induced $[Ca^{2+}]_{cyt}$ increases.
384
385 We used three different approaches to investigate the circadian control of basal $[Ca^{2+}]_{cyt}$. Firstly,
386 we measured the 24 h oscillations of $[Ca^{2+}]_{cyt}$ using the same method that we used to measure the
387 daily and circadian control of the dark-induced $[Ca^{2+}]_{cyt}$ signal (Fig. 5 and 6). We first attempted
388 to separate the measurement of basal circadian $[Ca^{2+}]_{cyt}$ oscillations from the dark-induced increase
389 in $[Ca^{2+}]_{cyt}$ by investigating the signal generated by a dark break every 2 h in the photoperiod,
390 because this protocol induces a dark Ca^{2+} signal that is not sustained. We compared the integrated
391 photon counts obtained from 0 to 1500 s (including the dark-induced $[Ca^{2+}]_{cyt}$ signal) and the last
392 800 s of each integration (not including the dark-induced $[Ca^{2+}]_{cyt}$ signal) to determine if we could
393 detect basal circadian $[Ca^{2+}]_{cyt}$ oscillations in addition to the dark-induced signal. In both, the first
394 1500 s (representing dark-induced and basal signals) and the last 800 s (representing only basal
395 signals) $[Ca^{2+}]_{cyt}$ increased during the day, peaked several hours after dawn and then decreased
396 reaching the minimum value during the night (Fig. 8a, Supporting Information Fig. S10). When

the circadian parameters were determined similar values were found for both signals (0 - 1500 s, period = 23.6 h, R.A.E = 0.18; 800 - 1500 s, period = 23.6 h, R.A.E = 0.23).

We next investigated the circadian regulation of $[Ca^{2+}]_{cyt}$ in constant darkness (DD), a condition that does not have a light to dark transition during measurement but is complicated by the dampening of circadian rhythms of $[Ca^{2+}]_{cyt}$ in prolonged DD in Arabidopsis (Johnson et al., 1995; Xu et al., 2007). This dampening occurs after ~16 h in DD because the machinery that returns $[Ca^{2+}]_{cyt}$ to resting values does not operate in extended dark (Xu et al., 2007 and Fig. 8b). The persistence of rhythmic $[Ca^{2+}]_{cyt}$ in the subjective morning of the first DD cycle suggests the presence of basal $[Ca^{2+}]_{cyt}$ oscillations that are not dependent on dark-induced $[Ca^{2+}]_{cyt}$ signals (Fig. 8, b and c, Supporting Information Fig. S10). Finally, we investigated the control of $[Ca^{2+}]_{cyt}$ in DD in the presence of sucrose because sucrose sustains circadian rhythms in DD (Dalchau et al., 2011). When sucrose was included in the media, we detected sustained circadian low amplitude oscillations of $[Ca^{2+}]_{cyt}$ in DD (period = 27.4 h, R.A.E = 0.59) (Fig. 8d, Supporting Information Fig. S10), in which there are no light to dark transitions. These oscillations of basal $[Ca^{2+}]_{cyt}$ in DD occurred in the absence of dark-induced of $[Ca^{2+}]_{cyt}$ transients (Fig. 8d, Supporting Information Fig. S10) demonstrating that a light to dark transition is not the cause of basal oscillations of $[Ca^{2+}]_{cyt}$. It also shows that sucrose can have different effects on $[Ca^{2+}]_{cyt}$ in the light and dark. In LL sucrose abolishes circadian oscillations of $[Ca^{2+}]_{cyt}$ (Johnson et al.,1995), whereas in DD rhythms are sustained. All these results suggest that circadian signaling is involved in both the regulation of daily and circadian oscillations of $[Ca^{2+}]_{cyt}$ and the $[Ca^{2+}]_{cyt}$ changes that occur upon a light to dark transition.

DISCUSSION

In several previous studies $[Ca^{2+}]_{cyt}$ changes upon transition to darkness have not been detected using either the luminescent reporter protein aequorin (Nomura et al., 2012; Sello et al., 2016; Frank et al., 2019) or the ratiometric reporter protein Yellow Cameleon YC 3.6 (Loro et al., 2016), despite the fact that they were previously reported by Sai and Johnson, 2002. Therefore, it has been a matter of conjecture whether they occur or whether they cannot be resolved. Here we demonstrate that light to dark transitions generate a consistent and reproducible transient in $[Ca^{2+}]_{cyt}$. We have used an ICCD 225 photon-counting camera that optimized the detection of aequorin luminescence from specifically the leaves and because when compared to luminometer experiments, using a camera the signal to noise ratio can be increased by focusing the light from a number of plants to small area on the array and using time series integration. Thus, even though luminometers are more sensitive photon counting devices, using a camera permitted us to detect at the end of the photoperiod a prolonged increase in $[Ca^{2+}]_{cyt}$. To ensure that the signal we were measuring did not originate from the stroma we compared to measurements of changes in $[Ca^{2+}]_{stroma}$ after transition into darkness (Sai and Johnson, 2002; Nomura et al., 2012; Sello et al., 2016). The $[Ca^{2+}]_{cyt}$ transient consists of a peak at around 10 min after the onset of darkness and a sustained increase peaking 30 min after the onset of darkness which decays over the next 2 h, whereas the $[Ca^{2+}]_{stroma}$ consists of two peaks at approximately 8 and 25 min lasting 40 min and a prolonged increase that last about 2 h after dark (Fig.1c). The $[Ca^{2+}]_{stroma}$ signature upon darkness obtained in the photon-counting camera was slightly different to the one previously reported in other studies (Sai and Johnson 2002; Nomura et al., 2012; Sello et al., 2016; Sello et al., 2018) and also here using a luminometer (Supporting Information Fig. S3). Using a intensified photon counting camera as the detector allowed higher resolution in the $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{stroma}$ response to this stimulus than a luminometer, describing more complex $[Ca^{2+}]$ dynamics in both compartments. These results highlight the importance of the detection methods when aequorin is the reporter, and reveal that even though the dark-induced $[Ca^{2+}]_{cyt}$ signal was detected in the luminometer, we were close to the detection limit (Supporting Information Fig. S4).

Sunset and shading are two environmental stimuli that are represented by the transition to darkness. We have observed that similar to the dark-induced $[Ca^{2+}]_{stroma}$ changes (Sai & Johnson, 2002), $[Ca^{2+}]_{cyt}$ signatures upon darkness are affected by the duration of the photoperiod (Fig.4),

suggesting that these $[Ca^{2+}]_{cyt}$ changes could be a mechanism by which plants can distinguish between long and short days and thereby induce photoperiodic responses. The gating of the signal by the time of day (Fig. 5) and circadian oscillator (Fig. 6) might suggest that the $[Ca^{2+}]_{cyt}$ changes after transition to darkness also encode information about time of day. Additionally, we found that the $[Ca^{2+}]_{cyt}$ changes after transition to darkness are specific for photosynthetic organs, which are normally in the light (Fig. 2) and that $[Ca^{2+}]_{cyt}$ changes upon darkness involve at least the *PHYA* photoreceptors (Fig. 3c). The finding about the involvement of *PHYA* in the dark-induced $[Ca^{2+}]_{cyt}$ signal is supported by our previous studies of circadian regulation of Ca^{2+} (Dalchau et al., 2010) and by the maximum expression of the *PHYA* gene close to the end of the light interval which is characteristic of light signals that regulate important physiological responses (e.g. end-of-the-day far-red response) (Toth et al., 2001).

Additionally, the comparison of the $[Ca^{2+}]_{cyt}$ traces recorded when Ga^{3+} or La^{3+} were added before the transition into darkness (Fig. 3), suggests that the dark-induced cytosolic $[Ca^{2+}]_{cyt}$ increase may derived from internal stores rather than extracellular space. Furthermore, the diminution/abolishment of the $[Ca^{2+}]_{cyt}$ upon darkness in the presence of cPTIO and nicotinamide, which inhibit ADPRc activity (Fig. 3), might involve the vacuole and the ER in the generation/intensity of the $[Ca^{2+}]_{cyt}$ signal, similarly to $[Ca^{2+}]_{cyt}$ circadian oscillations (Dodd et al., 2007). The source of the dark-induced $[Ca^{2+}]_{stroma}$ spike has been a matter of conjecture. It has been suggested that it is not dependent on photosynthetic electron transport because DCMU had little or no effect on the magnitude of the dark-stimulated Ca^{2+}-flux (Sai & Johnson, 2002). Additionally, the knockout mutation of BICAT2 which mediates Ca^{2+} uptake across the chloroplast envelope, strongly dampens the dark-induced $[Ca^{2+}]_{stroma}$ signal, suggesting a dark-triggered influx of Ca^{2+} from the cytosol, mediated by this transporter (Frank et al., 2019). In our experiments, inhibition of photosynthetic electron transport by DCMU (Fig. 3) had a negative effect on the dark-induced $[Ca^{2+}]_{cyt}$ signal, suggesting that photosynthesis might be necessary for the plants to respond. We hypothesis that the mechanism by which photosynthesis dampens dark-induced $[Ca^{2+}]_{cyt}$ signal could be associated with the effect of photosynthetic sugars on the abundance of oscillator components (Haydon et al., 2013; Haydon et al., 2017) which also regulate the dark-induced $[Ca^{2+}]_{cyt}$ increase (Fig. 6). The decreased of the light off signal in the $[Ca^{2+}]_{cyt}$ by DCMU and the lack of effect on the $[Ca^{2+}]_{stroma}$, indicates that upon darkness, the regulatory

mechanisms of cytosolic and stromal [Ca^{2+}] signatures might be distinct as previously suggested by Sai & Johnson, 2002; Sello et al., 2018. This conclusion is also supported by the fact that the $[Ca^{2+}]_{cyt}$ signal is larger when plants were grown in LD compared to plants grown in SD and interestingly, the dark-induced stromal [Ca^{2+}] increase has a larger peak in SD compared to LD (Fig. 4).

A striking result of our study is that not only $[Ca^{2+}]_{cyt}$ but also $[Ca^{2+}]_{stroma}$ signatures upon darkness are under the control of the circadian clock (Fig. 7) because it was previously concluded that daily dark-stimulated $[Ca^{2+}]_{stroma}$ spikes are not gated by the circadian clock (Sai & Johnson, 2002). The failure to detect gating of $[Ca^{2+}]_{stroma}$ signatures in the previous study might be because the dark-induced signal was measured only after five days in LL when the circadian system might have become damped. It has been suggested dampening might be due to the clock damping in all cells or cell desynchronization in term of phase or period, or desynchronization due to stochasticity in clock activity (Komin et al., 2011; Guerriero et al., 2012).

Circadian $[Ca^{2+}]_{cyt}$ oscillations in Arabidopsis are robust in LL but are absent in DD when sucrose is not included in the media (Johnson et al., 1995; Love et al., 2004), therefore they are usually determined by performing measurement breaks of darkness every 2 h in otherwise LL. Our discovery of the dependence on the time of the day of the dark-induced $[Ca^{2+}]_{cyt}$ changes due to circadian-gating, forced us to consider whether circadian and daily oscillations of basal $[Ca^{2+}]_{cyt}$ might arise as a consequence of repeated dark-induced increases in $[Ca^{2+}]_{cyt}$, which might not occur in the absence of the measuring protocol. We conclude that there are two modes of circadian regulation of $[Ca^{2+}]_{cyt}$, the circadian-gating of dark-induced increases in $[Ca^{2+}]_{cyt}$ and 24 h oscillations in basal $[Ca^{2+}]_{cyt}$ that are not a consequence of transfer to darkness, commonly called circadian oscillations of $[Ca^{2+}]_{cyt}$ (Johnson et al., 1995; Love et al., 2004; Xu et al., 2007). We reached that conclusion by calculating the total photon counts including and excluding the dark-induced $[Ca^{2+}]_{cyt}$ signal in LL and by the measurement of $[Ca^{2+}]_{cyt}$ in DD in the presence and absence of sucrose (Fig. 8). The detection of circadian oscillations of $[Ca^{2+}]_{cyt}$ in DD in the presence of sucrose demonstrates unequivocally that basal $[Ca^{2+}]_{cyt}$ can free run with a circadian period.

Since their first discovery by Johnson et al (1995) the purpose of basal circadian oscillations of $[Ca^{2+}]_{cyt}$ have been a mystery because Ca^{2+} signalling is usually considered to work on timescales much shorter than circadian timescales. Our finding of similarities between circadian and dark-induced increases in $[Ca^{2+}]_{cyt}$ suggests that they might have a common basis and function. Basal circadian oscillations of $[Ca^{2+}]_{cyt}$ and circadian-gated dark-induced $[Ca^{2+}]_{cyt}$ signals have their greatest magnitude near dusk, suggesting that their purpose could be associated with dusk sensing and/or day length sensing. This dusk-associated timing is consistent with the sensing of circadian $[Ca^{2+}]_{cyt}$ signals by Calmodulin-like 24 Ca^{2+} sensors that genetically interact with TOC1 to form part of the circadian oscillator because TOC1 is expressed maximally near dusk (Marti et al., 2018). One possibility is that the oscillation of basal $[Ca^{2+}]_{cyt}$ is indicative of the relaxing of the "gate" by which the circadian oscillator times dark-induced increases in $[Ca^{2+}]_{cyt}$ to the end of the photoperiod. The existence of the dark-induced $[Ca^{2+}]_{cyt}$ signal reported here and the methodology described to characterize it, together with the novel aequorin reporters for chloroplast subcompartments (Sello et al., 2018) and the discovery of the two chloroplast-targeted Ca^{2+} transporters in *Arabidopsis thaliana*, BIVALENT CATION TRANSPORTER 1 (BICAT1) and BICAT2 (Frank et al., 2019), which determine the amplitude of the prolonged and sustained dark-induced $[Ca^{2+}]_{stroma}$, will pave the way to understand the function and unravel the mechanisms responsible for $[Ca^{2+}]$ fluxes during light-dark transitions. Here, our data also demonstrate that the circadian oscillator in the nucleus, which is of Eukaryotic origin, can regulate the timing of stimulus-induced increases of $[Ca^{2+}]_{stroma}$ in the chloroplast, which is of bacterial origin, suggesting that a mechanism has evolved that allows temporal information to be communicated between these two organelles, resulting in time of day dependent Ca^{2+} signals adding a new sub-cellular spatial dimension to the circadian network of plants.

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AUTHORS CONTRIBUTION

M.C.M.R. and H.J.J. carried out the experiments and data analyses. M.C.M.R. and A.A.R.W. conceived the research, designed the experiments and wrote the manuscript.

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FIGURE LEGENDS

Figure 1: Dark-induced $[Ca^{2+}]_{cyt}$ transient occurs after light off in 12h-12h light-dark cycles.
Luminescence from reconstituted aequorin in Arabidopsis seedlings expressing aequorin targeted to the cytosol (black) (a and c) or the stroma (green) (c) and from non-transgenic seedlings (blue) (b). The ecotypes used were Col-0 and Ws-2 (green-c). Data were recorded immediately after plants were transferred to darkness at the end of the photoperiod (12 h after the lights went on) or after being 6 h in the dark (a, D to D) as shown in the bars above or below the figures. Data represent the mean luminesce ± SEM from 8 (a, L to D), 4 (a, D to D), 2 (b) and 3 (c, stroma) experiments. Each experiment consisting of 24 clusters of seedlings, each cluster contained 7 - 10 seedlings. See supplemental figures 1, 2 and 3 for the replicates. (*) in (a), denotes $p<0.05$ after Student´s t-test for equal variances of the areas under the traces.

Figure 2: The dark induced $[Ca^{2+}]_{cyt}$ transient occurs in green tissues.
Three Col-0 Arabidopsis transgenic seedlings expressing aequorin targeted to the cytosol were grown in white light-dark cycles. On the night of the 11^{th} day of growth, the root and leaves were separated and incubated with coelenterazine. Aequorin luminescence was recorded in a luminometer from reconstituted aequorin when the tissues were 12 days old. Traces represent the data obtained in one experiment. Experiments were repeated 4 times.

Figure 3: **Identification of the signalling pathways leading to the cytosolic-free calcium transient induced by dark.**
Luminescence from reconstituted aequorin in Ler, *cry1*, *cry2*, *phyA*, *phyB* mutants and Col-0 Arabidopsis seedlings expressing aequorin targeted to the cytosol was recorded immediately after plants were transferred to darkness at the end of the photoperiod (12 h after the lights went on) as shown in the bars above the figures. In (a) plants were grown in white light and transferred to monochromatic red or blue light four days before the measurements. In (b-d), all treatments were applied 30 min before sunset, but nicotinamide that was applied when plants were seven days old

and DCMU that was applied 24 h before measurements. Data represent the mean ± SEM luminesce from three experiments consisting of 24 clusters of seedlings, each cluster contained 7 - 10 seedlings. Error bars are indicated every 10 min for clarity.

Figure 4: Dark-induced $[Ca^{2+}]_{cyt}$ signature is affected by photoperiod length.
Luminescence from reconstituted aequorin in Col-0 (black) or Ws-2 (green) Arabidopsis seedlings expressing aequorin targeted to the cytosol (black) or the stroma (green). Plants were grown in white light-dark cycles (8h:16h, 12h:12h and 16h:8h, (a), (b) and (c) respectively) (100 µmol m^{-2} s^{-1}) for 12-13 days. Data were recorded immediately after plants were transferred to darkness at the end of the photoperiod (8h (a), 12 h (b) and 16 h (c) after the lights went on). The data represent the mean luminescence ± SEM from three experiments consisting of 24 clusters of seedlings, each cluster contained 7 - 10 seedlings. Error bars are indicated every 10 min for clarity.

Figure 5: The dark-induced $[Ca^{2+}]_{cyt}$ transient depends on the time of the day.
Arabidopsis transgenic seedlings expressing aequorin targeted to the cytosol (Col-0) or the stroma (Ws-2) were grown in white light-dark cycles (12h:12h) (100 µmol m^{-2} s^{-1}). On the night of the 11th day of growth, seedlings were incubated with coelenterazine and aequorin luminescence was recorded from reconstituted aequorin since they were 12 days old as shown in (a). (b) and (c) show data of changes in $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{stroma}$, respectively, every 2 h during 1 LD cycle. The data represent one experiment consisting of 80 clusters of seedlings and each cluster contained 7-10 seedlings. Experiments were repeated at least six times (b) and twice (c).

Figure 6: **The dark-induced $[Ca^{2+}]_{cyt}$ transient is gated by the circadian clock.**
Col-0 and *prr7*-11 *prr5*-10 *prr9*-11 triple mutant Arabidopsis transgenic seedlings expressing aequorin targeted to the cytosol were grown in white light-dark cycles (12h:12h) (100 µmol m^{-2} s^{-1}). On the night of the 11th day of growth, seedlings were incubated with coelenterazine and aequorin luminescence was recorded from reconstituted aequorin since they were 12 days old. (a) shows data of changes in $[Ca^{2+}]_{cyt}$ every 2 h during LL cycles in Col-0 plants. (b and c) show data of changes in $[Ca^{2+}]_{cyt}$, every 2 h during 1 LD and 4 LL cycles in Col-0 and *prr7*-11 *prr5*-10 *prr9*-11 triple mutant plants, respectively. The data represent one experiment consisting of 80 (a)

and 8 (b and c) clusters of seedlings, each cluster contained 7 - 10 seedlings. Experiments were repeated at least seven times (a) and twice (b).

Figure 7: **The dark-induced $[Ca^{2+}]_{stroma}$ transient is gated by the circadian clock.**
Ws-2 Arabidopsis transgenic seedlings expressing aequorin targeted to the stroma were grown in white light-dark cycles (12h:12h) (100 µmol m^{-2} s^{-1}). On the night of the 11th day of growth, seedlings were incubated with coelenterazine and aequorin luminescence was recorded from reconstituted aequorin since they were 12 days old. On the 13th day, plants were transferred to LL. The data show changes in $[Ca^{2+}]_{stroma}$ every 2 h during 2 LL cycles. The data represent one experiment consisting of 80 clusters of seedlings, each cluster contained 7 - 10 seedlings. Experiments were repeated at least twice.

Figure 8: Dark-induced transients in $[Ca^{2+}]_{cyt}$ are superimposed on daily and circadian $[Ca^{2+}]_{cyt}$ oscillations.
(a) Daily and circadian $[Ca^{2+}]_{cyt}$ oscillations measured in LL using different time integration intervals. Col-0 Arabidopsis transgenic seedlings expressing aequorin targeted to the cytosol were grown in LD 12h:12h cycles and then transferred to LL. The data represent one experiment consisting of 80 clusters of seedlings and each cluster contained 7 - 10 seedlings. (b) Daily and circadian $[Ca^{2+}]_{cyt}$ oscillations measured in DD without (b) 3% (w/v) sucrose using different time integration intervals. (c) Changes in $[Ca^{2+}]_{cyt}$ recorded every 2 h and used in (b). (d) Daily and circadian $[Ca^{2+}]_{cyt}$ oscillations measured in DD with 3% (w/v) sucrose. Black are the raw time series data, showing no dark to light transitions. In red is the integrated photon counts obtained during the 1500 s of recording. (b-d) Col-0 Arabidopsis transgenic seedlings expressing aequorin targeted to the cytosol were grown in LD 12h:12h cycles and then transferred to DD. (b-d) the data represent one experiment consisting of 8 clusters of seedlings and each cluster contained 7 - 10 seedlings. Experiments were repeated at least six times (a) and (b-d) twice.

SUPPORTING INFORMATION
The following supplemental material are available.

Supplemental Figure 1: Dark-induced $[Ca^{2+}]_{cyt}$ changes upon darkness using a photon counting camera.

Luminescence from reconstituted aequorin in Col-0 Arabidopsis seedlings expressing aequorin targeted to the cytosol. Data were recorded immediately after plants were transferred to darkness at the end of the photoperiod (12 h after the lights went on) as shown in the bar above figure. Each experiment represents the luminescence from 24 clusters of seedlings, each cluster contained 7 - 10 seedlings.

Supplemental Figure 2: Dark-induced $[Ca^{2+}]_{cyt}$ transient does not occur 6 after light off or in non-transgenic plants in 12h-12h light-dark cycles.

Luminescence from reconstituted aequorin in Arabidopsis seedlings expressing aequorin targeted to the cytosol (black) (a) and from non-transgenic seedlings (blue) (b). The ecotype used was Col-0. Data were recorded immediately after plants were transferred to darkness at the end of the photoperiod (12 h after the lights went on) (b) or after being 6 h in the dark (a) as shown in the bars above the figures. Data represent the luminescence from one experiment consisting of 24 clusters of seedlings, each cluster contained 7 - 10 seedlings. These experiments are a repeat of the one showed in Fig. 1.

Supplemental Figure 3: The signature of the dark-induced $[Ca^{2+}]_{stroma}$ changes upon darkness depends on the detection method.

Luminescence or $[Ca^{2+}]$ from reconstituted aequorin in Ws-2 Arabidopsis seedlings expressing aequorin targeted to the stroma. Data were recorded immediately after plants were transferred to darkness at the end of the photoperiod (12 h after the lights went on) as shown in the bars above the figures. Camera data represent the luminescence from 24 clusters of seedlings, each cluster contained 7 - 10 seedlings. Luminometer data represent the luminesce from three plants. Experiments were repeated at least twice and three are represented in the figure.

Supplemental Figure 4: Calibration of the dark-induced $[Ca^{2+}]_{cyt}$ changes upon darkness using a luminometer.

$[Ca^{2+}]_{cyt}$ from reconstituted aequorin in Col-0 Arabidopsis seedlings expressing aequorin targeted to the cytosol. Data were recorded immediately after plants were transferred to darkness at the end of the photoperiod (12 h after the lights went on) as shown in the bars above the figures. Each graph represents the luminescence from three plants. Experiments were repeated 14 times with each trace presented in the figure.

Supplemental Figure 5: The dark-induced $[Ca^{2+}]_{cyt}$ transient occurs in green tissues.
Three Col-0 Arabidopsis transgenic seedlings expressing aequorin targeted to the cytosol were grown in white light-dark cycles. On the night of the 11th day of growth, the root and leaves were separated and incubated with coelenterazine. Aequorin luminescence was recorded in a luminometer from reconstituted aequorin when tissues were 12 days old. Traces represent the data obtained from one experiment. This is a repeat of figure 2 experiment.

Supplemental Figure 6: The dark-induced $[Ca^{2+}]_{cyt}$ transient depends on the time of the day.
Arabidopsis transgenic seedlings expressing aequorin targeted to the stroma (Ws-2) were grown in white light-dark cycles (12h:12h) (100 µmol m^{-2} s^{-1}). On the night of the 11th day of growth, seedlings were incubated with coelenterazine and aequorin luminescence was recorded from reconstituted aequorin since they were 12 days old. (A) shows data of changes in $[Ca^{2+}]_{stroma}$, every 2 h during 1 LD cycle. The data represent one experiment consisting of 80 clusters of seedlings and each cluster contained 7 - 10 seedlings. Experiments were repeated at least twice. This experiment is a repeat of the one in Figure 5b.

Supplemental Figure 7: Dark-induced increases of luminescence were not detected from plants not carrying the Aequorin transgene.
Non-transgenic Col-0 Arabidopsis seedlings were grown in white light-dark cycles (12h:12h) (100 µmol m^{-2} s^{-1}). On the night of the 11th day of growth, seedlings were incubated with coelenterazine and luminescence was recorded when they were 12 days old. Graphs shows data during 1 LD 12h:12h cycle taken every 2 h during 1500 s. There were 80 clusters of seedlings per plate and each cluster contained 7 - 10 seedlings.

Supplemental Figure 8: The dark-induced $[Ca^{2+}]_{cyt}$ transient is gated by the circadian clock.
Col-0 and *prr7*-11 *prr5*-10 *prr9*-11 triple mutant Arabidopsis transgenic seedlings expressing aequorin targeted to the cytosol were grown in white light-dark cycles (12h:12h) (100 µmol m^{-2} s^{-1}). On the night of the 11th day of growth, seedlings were incubated with coelenterazine and aequorin luminescence was recorded from reconstituted aequorin since they were 12 days old. Figures show data of changes in $[Ca^{2+}]_{cyt}$ every 2 h during LL cycles in Col-0 and *prr7*-11 *prr5*-10 *prr9*-11 triple mutant plants, respectively. The data represent one experiment consisting of 8 clusters of seedlings, each cluster contained 7 - 10 seedlings. Experiments were repeated at least twice. This is a repeat of the experiment in Figure 6.

Supplemental Figure 9: The dark-induced $[Ca^{2+}]_{stroma}$ transient is gated by the circadian clock.

Ws-2 Arabidopsis transgenic seedlings expressing aequorin targeted to the stroma were grown in white light-dark cycles (12h:12h) (100 µmol m^{-2} s^{-1}). On the night of the 11th day of growth, seedlings were incubated with coelenterazine and aequorin luminescence was recorded from reconstituted aequorin since they were 12 days old. The data show changes in $[Ca^{2+}]_{stroma}$ every 2 h during 2 LL cycles. The data represent one experiment consisting of 80 clusters of seedlings, each cluster contained 7 - 10 seedlings. This experiment is a repeat of the one in Figure 7.

Supplemental Figure 10: Dark-induced transients in $[Ca^{2+}]_{cyt}$ are superimposed on daily and circadian $[Ca^{2+}]_{cyt}$ oscillations.

(a) Daily and circadian $[Ca^{2+}]_{cyt}$ oscillations measured in LL using different time integration intervals. Col-0 Arabidopsis transgenic seedlings expressing aequorin targeted to the cytosol were grown in LD 12h:12h cycles and then transferred to LL. The data represent one experiment consisting of 80 clusters of seedlings and each cluster contained 7 - 10 seedlings. (b) Daily and circadian $[Ca^{2+}]_{cyt}$ oscillations measured in DD without (b) 3% (w/v) sucrose using different time integration intervals. (c) Changes in $[Ca^{2+}]_{cyt}$ recorded every 2 h and used in (B). (d) Daily and circadian $[Ca^{2+}]_{cyt}$ oscillations measured in DD with 3% (w/v) sucrose. Black are the raw time series data, showing no dark to light transitions. In red is the integrated photon counts obtained during the 1500 s of recording.

(b-d) Col-0 Arabidopsis transgenic seedlings expressing aequorin targeted to the cytosol were grown in LD 12h:12h cycles and then transferred to DD. (b-d) the data represent one experiment consisting of 8 clusters of seedlings and each cluster contained 7 - 10 seedlings. These experiments are a repeat of the ones in Figure 8.

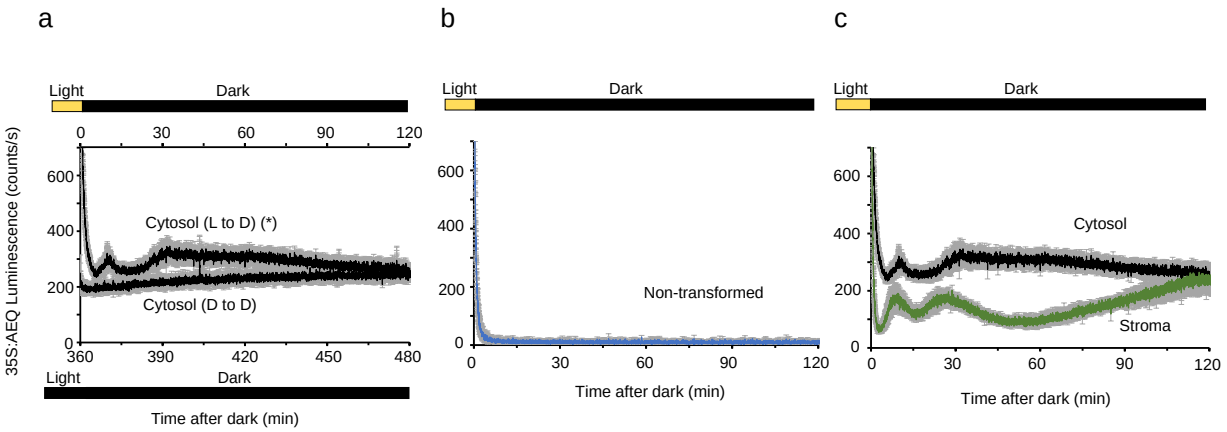

Figure 1: Dark-induced $[Ca^{2+}]_{cyt}$ transient occurs after light off in 12h-12h light-dark cycles.

Luminescence from reconstituted aequorin in Arabidopsis seedlings expressing aequorin targeted to the cytosol (black) (a and c) or the stroma (green) (c) and from non-transgenic seedlings (blue) (b). The ecotypes used were Col-0 and Ws-2 (green-c). Data were recorded immediately after plants were transferred to darkness at the end of the photoperiod (12 h after the lights went on) or after being 6 h in the dark (a, D to D) as shown in the bars above or below the figures. Data represent the mean luminescence ± SEM from 8 (a, L to D), 4 (a, D to D), 2 (b) and 3 (c, stroma) experiments. Each experiment consisting of 24 clusters of seedlings, each cluster contained 7 - 10 seedlings. See supplemental figures 1, 2 and 3 for the individual experiments. (*) in (a), denotes $p<0.05$ after Student's t-test for equal variances of the areas under the traces.

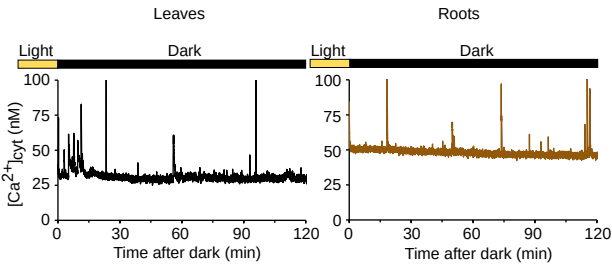

Figure 2: The dark-induced $[Ca^{2+}]_{cyt}$ transient occurs in green tissues.

Three Col-0 Arabidopsis transgenic seedlings expressing aequorin targeted to the cytosol were grown in white light-dark cycles. On the night of the 11th day of growth, the root and leaves were separated and incubated with coelenterazine. Aequorin luminescence was recorded in a luminometer from reconstituted aequorin when tissues were 12 days old. Traces represent the data obtained from one experiment. Experiments were repeated 4 times.

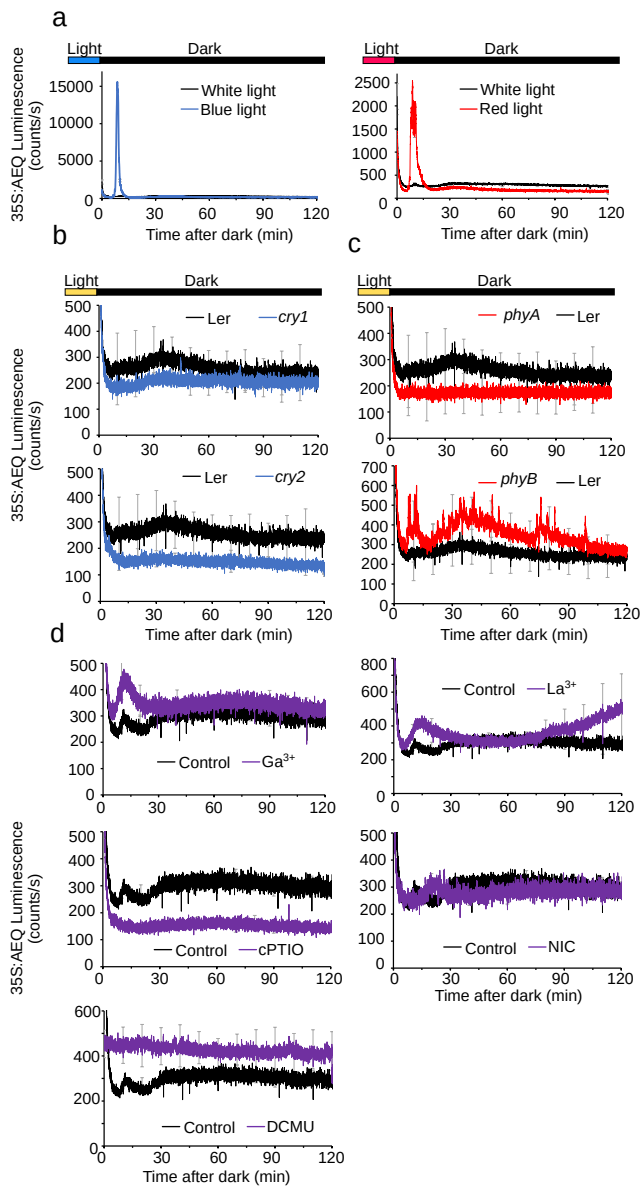

Figure 3: Identification of the signalling pathways leading to the cytosolic-free calcium transient induced by dark.

Luminescence from reconstituted aequorin in Ler, *cry1*, *cry2*, *phyA*, *phyB* mutants and Col-0 Arabidopsis seedlings expressing aequorin targeted to the cytosol was recorded immediately after plants were transferred to darkness at the end of the photoperiod (12 h after lights went on) as shown in the bars above the figures. In (a) plants were grown in white light and transferred to monochromatic red or blue light four days before the measurements. In (b-d) all treatments were applied 30 min before sunset, but nicotinamide that was apllied when plants were 7 days old and DCMU that was applied 24 h before the measurements. Data represent the mean luminescence ± SEM from three experiments consisting of 24 clusters of seedlings, each cluster contained 7 - 10 seedlings. Error bars are indicated every 10 min for clarity.

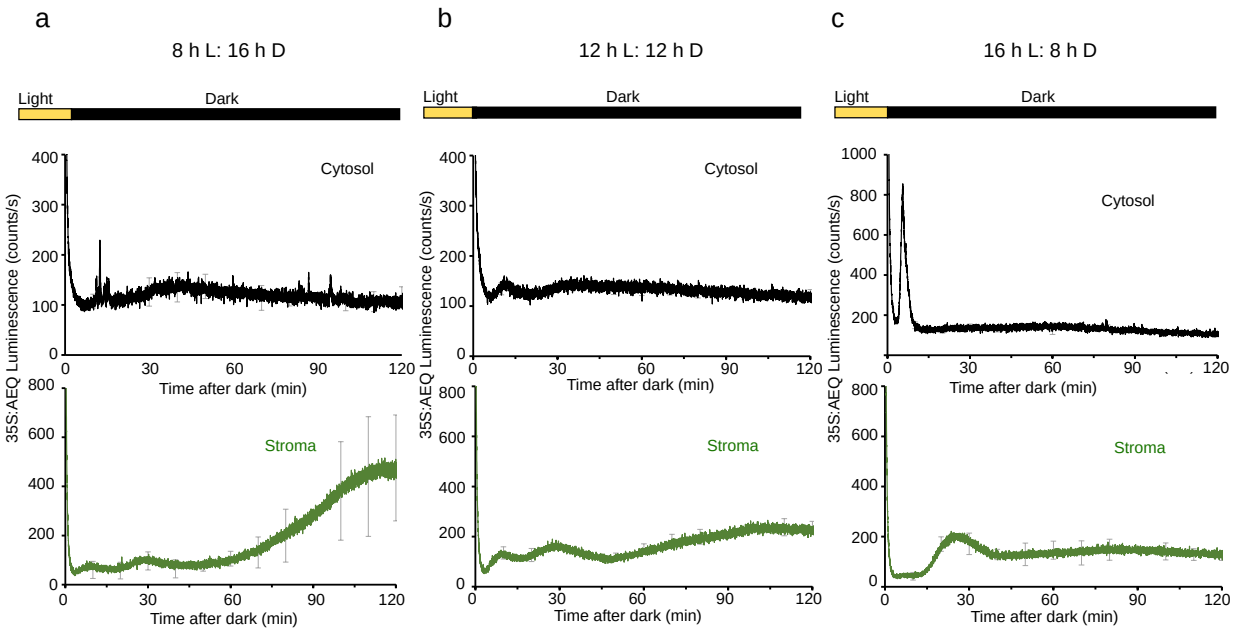

Figure 4: Dark-induced $[Ca^{2+}]_{cyt}$ signature is affected by photoperiod length.

Luminescence from reconstituted aequorin in Col-0 (black) or Ws-2 (green) Arabidopsis seedlings expressing aequorin targeted to the cytosol (black) or the stroma (green). Plants were grown in light-dark cycles (8h:16h, 12h:12h, 16h:8h, (a), (b) and (c), respectively) (100 µmol $m^{-2}s^{-1}$) for 12 - 13 days. Data were recorded immediately after plants were transferred to darkness at the end of the photoperiod (8 h (a), 12 h (b) and 16 h (c) after the lights went on). The data represent the mean luminescence ± SEM from three experiments consisting of 24 clusters of seedlings, each cluster contained 7 - 10 seedlings. Error bars are indicated every 10 min for clarity.

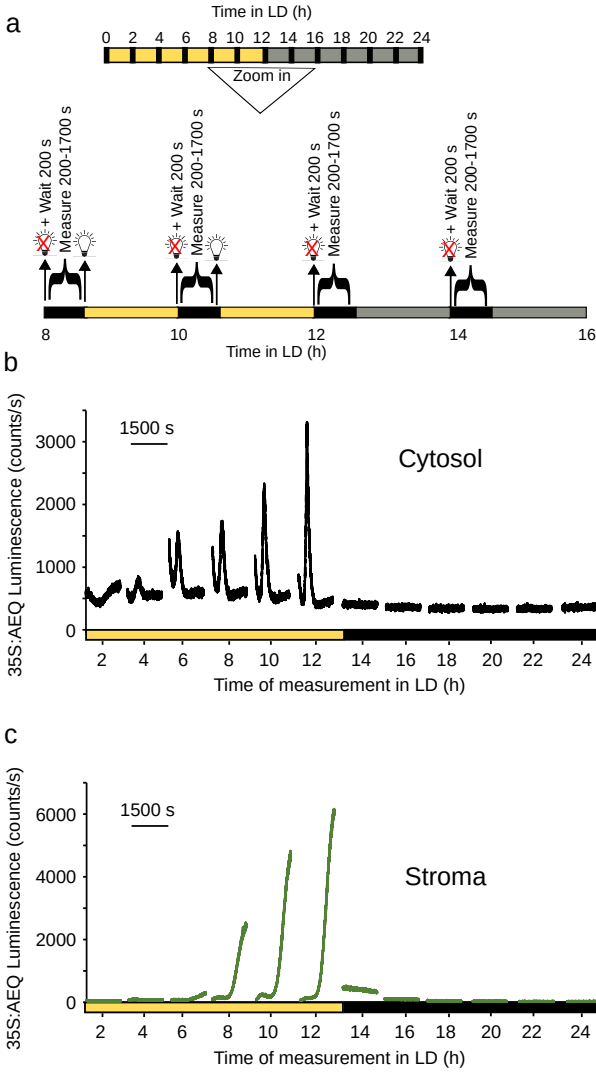

Figure 5: The dark-induced $[Ca^{2+}]_{cyt}$ transient depends on the time of the day.

Arabidopsis transgenic seedlings expressing aequorin targeted to the cytosol (Col-0) or the stroma (Ws-2) were grown in white light-dark cycles (12h:12h) (100 µmol m^{-2} s^{-1}). On the night of the 11th day of growth, seedlings were incubated with coelenterazine and aequorin luminescence was recorded from reconstituted aequorin since they were 12 days old as shown in (a). (b) and (c) show data of changes in $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{stroma}$, respectively, every 2 h during 1 LD cycle. The data represent one experiment consisting of 80 clusters of seedlings and each cluster contained 7 - 10 seedlings. Experiments were repeated at least seven times (b) and twice (c).

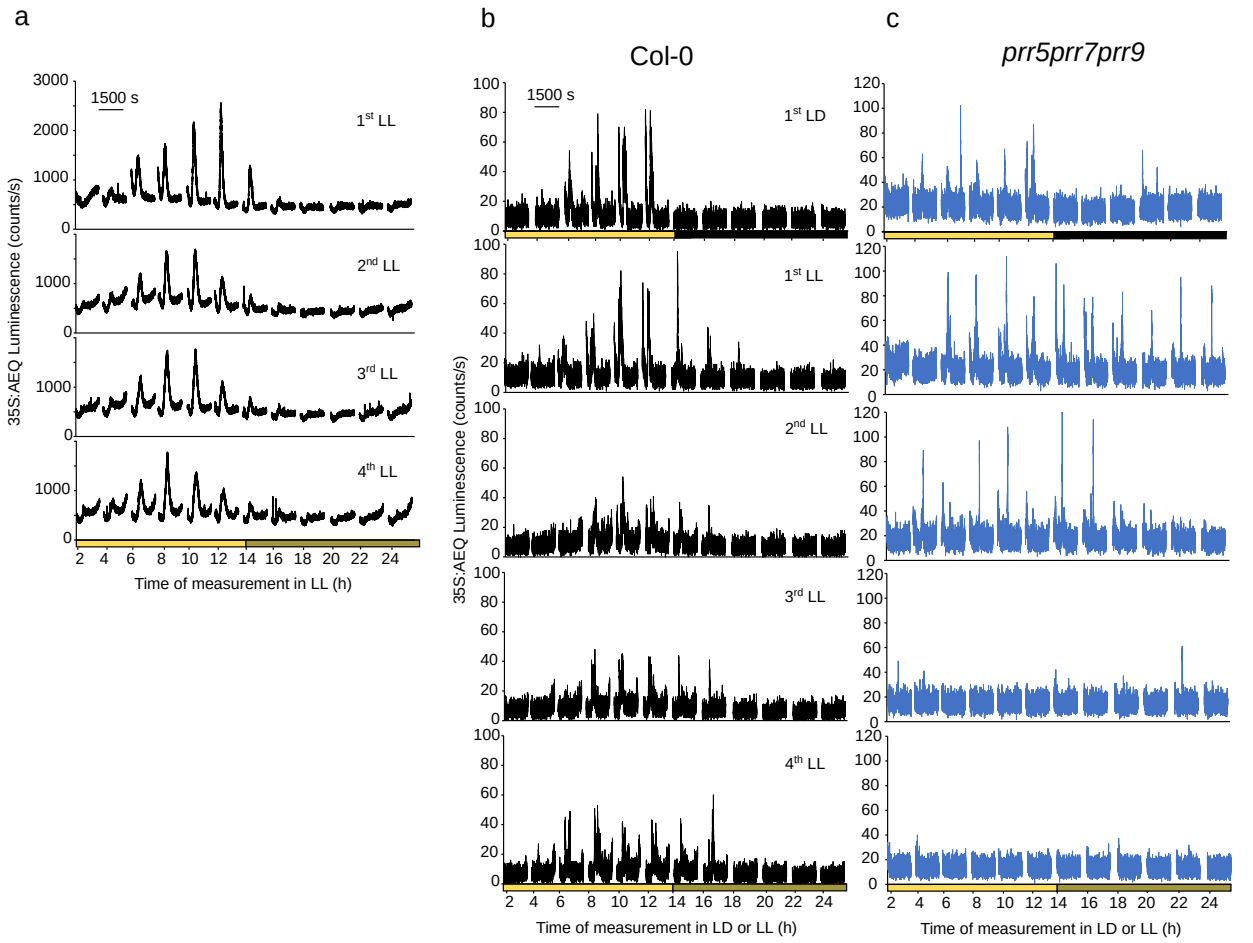

Figure 6: The dark-induced $[Ca^{2+}]_{cyt}$ transient is gated by the circadian clock.

Col-0 and *prr7*-11 *prr5*-10 *prr9*-11 triple mutant Arabidopsis transgenic seedlings expressing aequorin targeted to the cytosol were grown in white light-dark cycles (12h:12h) (100 µmol m^{-2} s^{-1}). On the night of the 11th day of growth, seedlings were incubated with coelenterazine and aequorin luminescence was recorded from reconstituted aequorin since they were 12 days old. (a) shows data of changes in $[Ca^{2+}]_{cyt}$ every 2 h during LL cycles in Col-0 plants. (b and c) show data of changes in $[Ca^{2+}]_{cyt}$, every 2 h during 1 LD and 4 LL cycles in Col-0 and *prr7*-11 *prr5*-10 *prr9*-11 triple mutant plants, respectively. The data represent one experiment consisting of 80 (a) and 8 (b and c) clusters of seedlings, each cluster contained 7 - 10 seedlings. Experiments were repeated at least seven times (a) and twice (b).

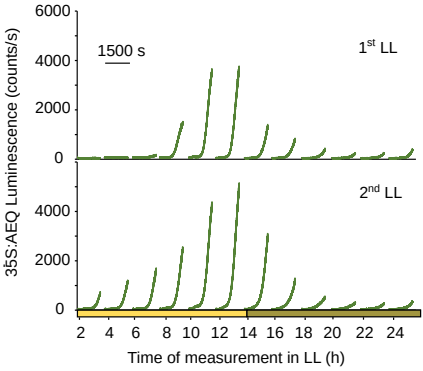

Figure 7: The dark-induced $[Ca^{2+}]_{stroma}$ transient is gated by the circadian clock.

Ws-2 Arabidopsis transgenic seedlings expressing aequorin targeted to the stroma were grown in white light-dark cycles (12h:12h) (100 µmol m^{-2} s^{-1}). On the night of the 11th day of growth, seedlings were incubated with coelenterazine and aequorin luminescence was recorded from reconstituted aequorin since they were 12 days old. On the 13th day, plants were transferred to LL. The data show changes in $[Ca^{2+}]_{stroma}$ every 2 h during 2 LL cycles. The data represent one experiment consisting of 80 clusters of seedlings, each cluster contained 7 - 10 seedlings. Experiments were repeated at least twice.

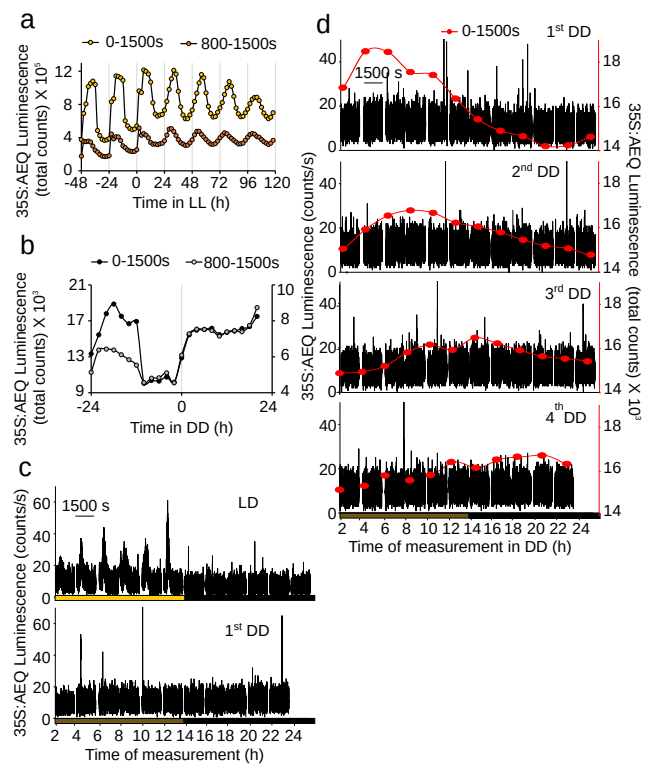

Figure 8: Dark-induced transients in $[Ca^{2+}]_{cyt}$ are superimposed on daily and circadian $[Ca^{2+}]_{cyt}$ oscillations.

(a) Daily and circadian $[Ca^{2+}]_{cyt}$ oscillations measured in LL using different time integration intervals. Col-0 Arabidopsis transgenic seedlings expressing aequorin targeted to the cytosol were grown in LD 12h:12h cycles and then transferred to LL. The data represent one experiment consisting of 80 clusters of seedlings and each cluster contained 7 - 10 seedlings. (b) Daily and circadian $[Ca^{2+}]_{cyt}$ oscillations measured in DD without (B) 3% (w/v) sucrose using different time integration intervals. (c) Changes in $[Ca^{2+}]_{cyt}$ recorded every 2 h and used in (b). (d) Daily and circadian $[Ca^{2+}]_{cyt}$ oscillations measured in DD with 3% (w/v) sucrose. Black are the raw time series data, showing no dark to light transitions. In red is the integrated photon counts obtained during the 1500 s of recording. (b-d) Col-0 Arabidopsis transgenic seedlings expressing aequorin targeted to the cytosol were grown in LD 12h:12h cycles and then transferred to DD. (b-d) the data represent one experiment consisting of 8 clusters of seedlings and each cluster contained 7 - 10 seedlings. Experiments were repeated at least six times (a) and (b-d) twice.

New Phytologist

***New Phytologist* Supporting Information**

Article title: **Circadian gating of dark-induced increases in chloroplast- and cytosolic-free calcium in Arabidopsis**

Authors: María Carmen Martí Ruiz, Hyun Ju Jung and Alex A. R. Webb

Article acceptance date: Click here to enter a date.

The following Supporting Information is available for this article:

Fig. S1 Dark-induced $[Ca^{2+}]_{cyt}$ changes upon darkness using a photon counting camera

Fig. S2 Dark-induced $[Ca^{2+}]_{cyt}$ transient does not occur 6 after light off or in non-transgenic plants in 12h-12h light-dark cycles

Fig. S3 The signature of the dark-induced $[Ca^{2+}]_{stroma}$ changes upon darkness depends on the detection method

Fig. S4 Calibration of the dark-induced $[Ca^{2+}]_{cyt}$ changes upon darkness using a luminometer

Fig. S5 The dark-induced $[Ca^{2+}]_{cyt}$ transient occurs in green tissues

Fig. S6 The dark-induced $[Ca^{2+}]_{cyt}$ transient depends on the time of the day

Fig. S7 Dark-induced increases of luminescence were not detected from plants not carrying the Aequorin transgene

Fig. S8 The dark-induced $[Ca^{2+}]_{cyt}$ transient is gated by the circadian clock

Fig. S9 The dark-induced $[Ca^{2+}]_{stroma}$ transient is gated by the circadian clock

Fig. S10 Dark-induced transients in $[Ca^{2+}]_{cyt}$ are superimposed on daily and circadian $[Ca^{2+}]_{cyt}$ oscillations

Fig. S1 Dark-induced $[Ca^{2+}]_{cyt}$ changes upon darkness using a photon counting camera. Luminescence from reconstituted aequorin in Col-0 Arabidopsis seedlings expressing aequorin targeted to the cytosol. Data were recorded immediately after plants were transferred to darkness at the end of the photoperiod (12 h after the lights went on) as shown in the bar above figure. Each experiment represents the luminesce from 24 clusters of seedlings, each cluster contained 7 - 10 seedlings.

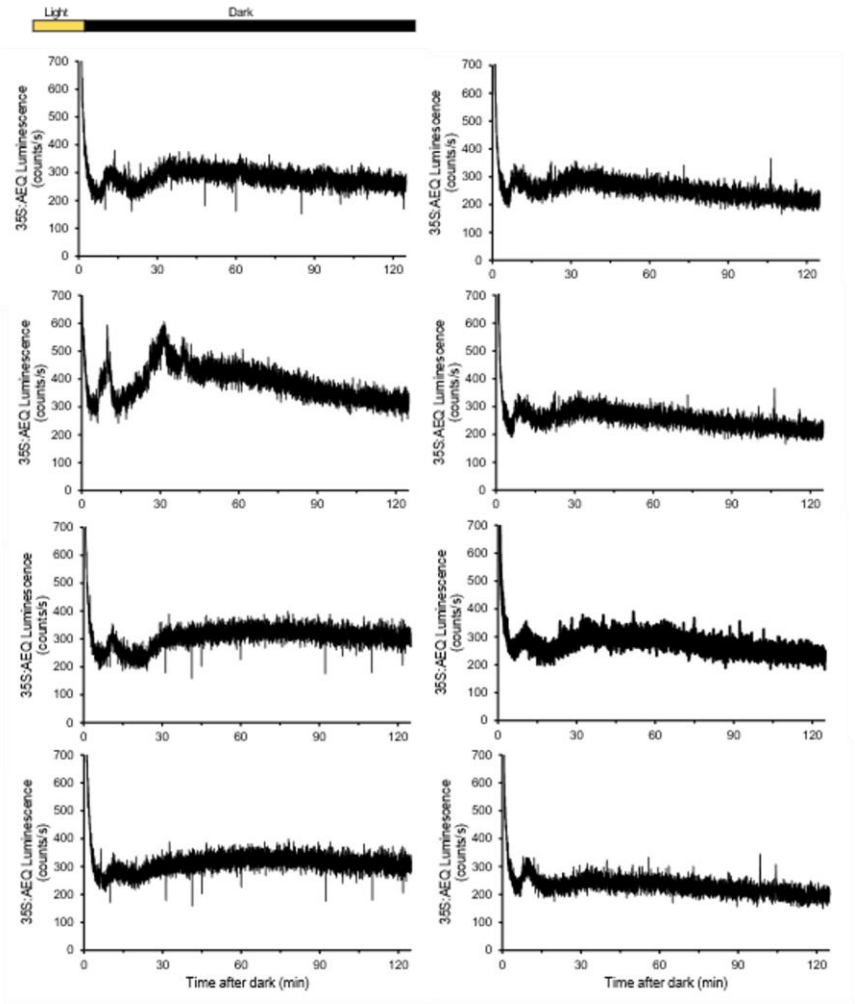

Fig. S2 Dark-induced $[Ca^{2+}]_{cyt}$ transient does not occur 6 after light off or in non-transgenic plants in 12h-12h light-dark cycles. Luminescence from reconstituted aequorin in Arabidopsis seedlings expressing aequorin targeted to the cytosol (black) (a) and from non-transgenic seedlings (blue) (b). The ecotype used was Col-0. Data were recorded immediately after plants were transferred to darkness at the end of the photoperiod (12 h after the lights went on) (b) or after being 6 h in the dark (a) as shown in the bars above the figures. Data represent the luminescence from one experiment consisting of 24 clusters of seedlings, each cluster contained 7 - 10 seedlings. These experiments are a repeat of the one showed in Fig. 1.

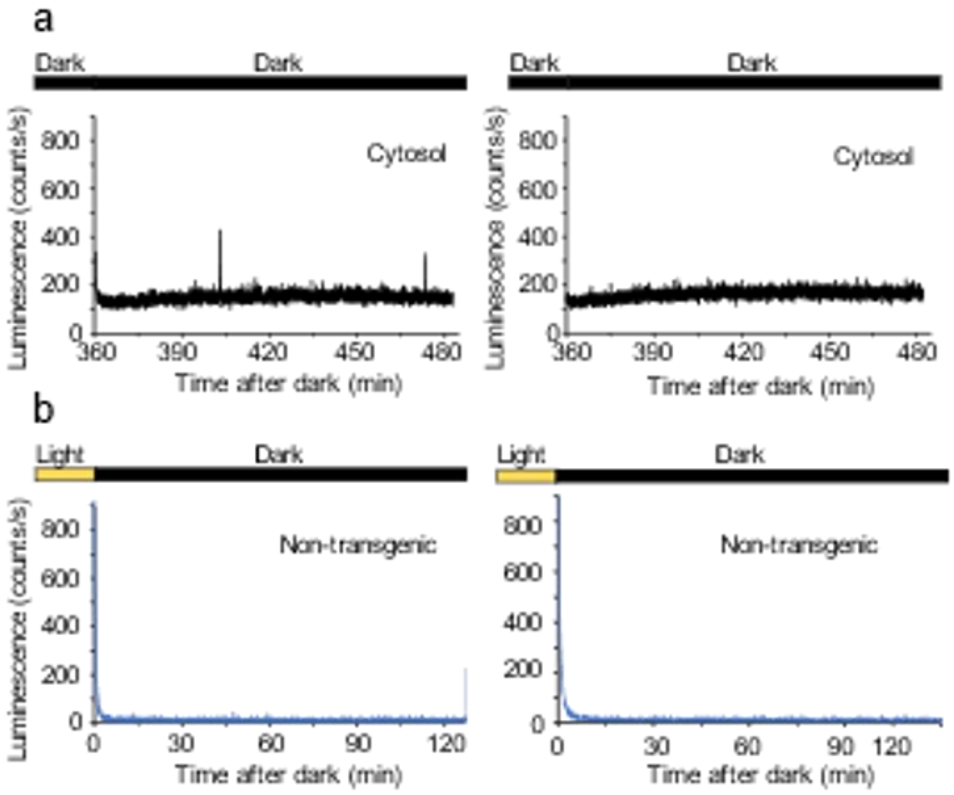

Fig. S3 The signature of the dark-induced $[Ca^{2+}]_{stroma}$ changes upon darkness depends on the detection method. Luminescence or $[Ca^{2+}]$ from reconstituted aequorin in Ws-2 Arabidopsis seedlings expressing aequorin targeted to the stroma. Data were recorded immediately after plants were transferred to darkness at the end of the photoperiod (12 h after the lights went on) as shown in the bars above the figures. Camera data represent the luminescence from 24 clusters of seedlings, each cluster contained 7 - 10 seedlings. Luminometer data represent the luminescence from three plants. Experiments were repeated at least twice and three are represented in the figure.

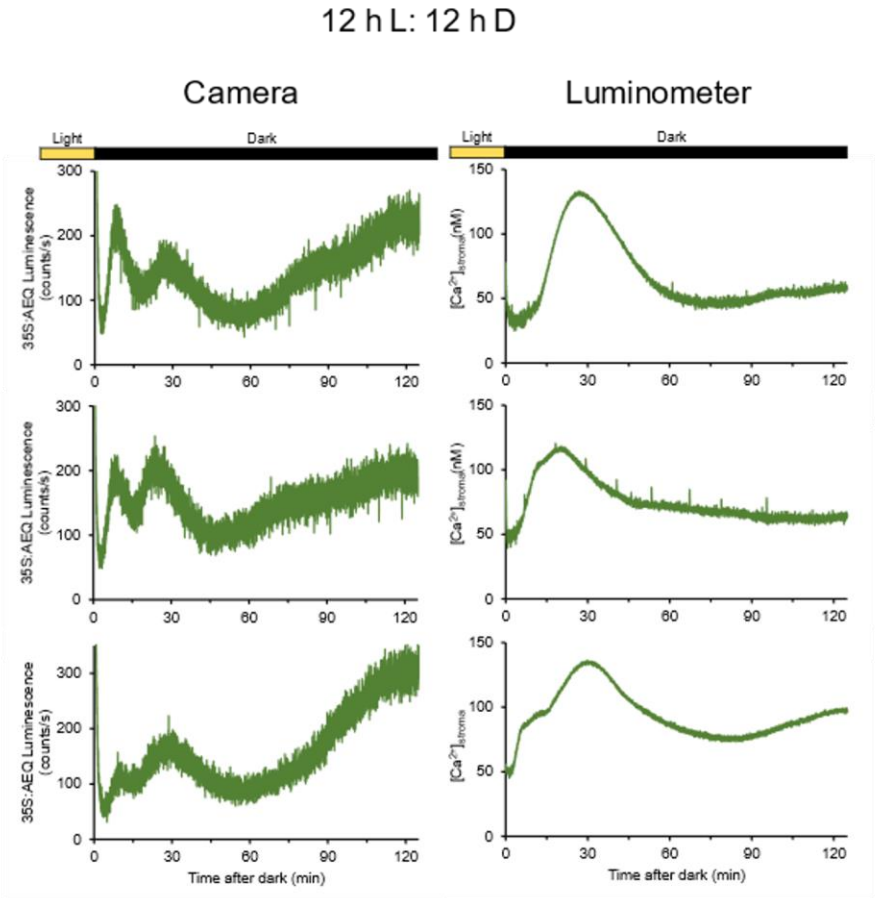

Fig. S4 Calibration of the dark-induced $[Ca^{2+}]_{cyt}$ changes upon darkness using a luminometer. $[Ca^{2+}]_{cyt}$ from reconstituted aequorin in Col-0 Arabidopsis seedlings expressing aequorin targeted to the cytosol. Data were recorded immediately after plants were transferred to darkness at the end of the photoperiod (12 h after the lights went on) as shown in the bars above the figures. Each graph represents the luminescence from three plants. Experiments were repeated 14 times with each trace presented in the figure.

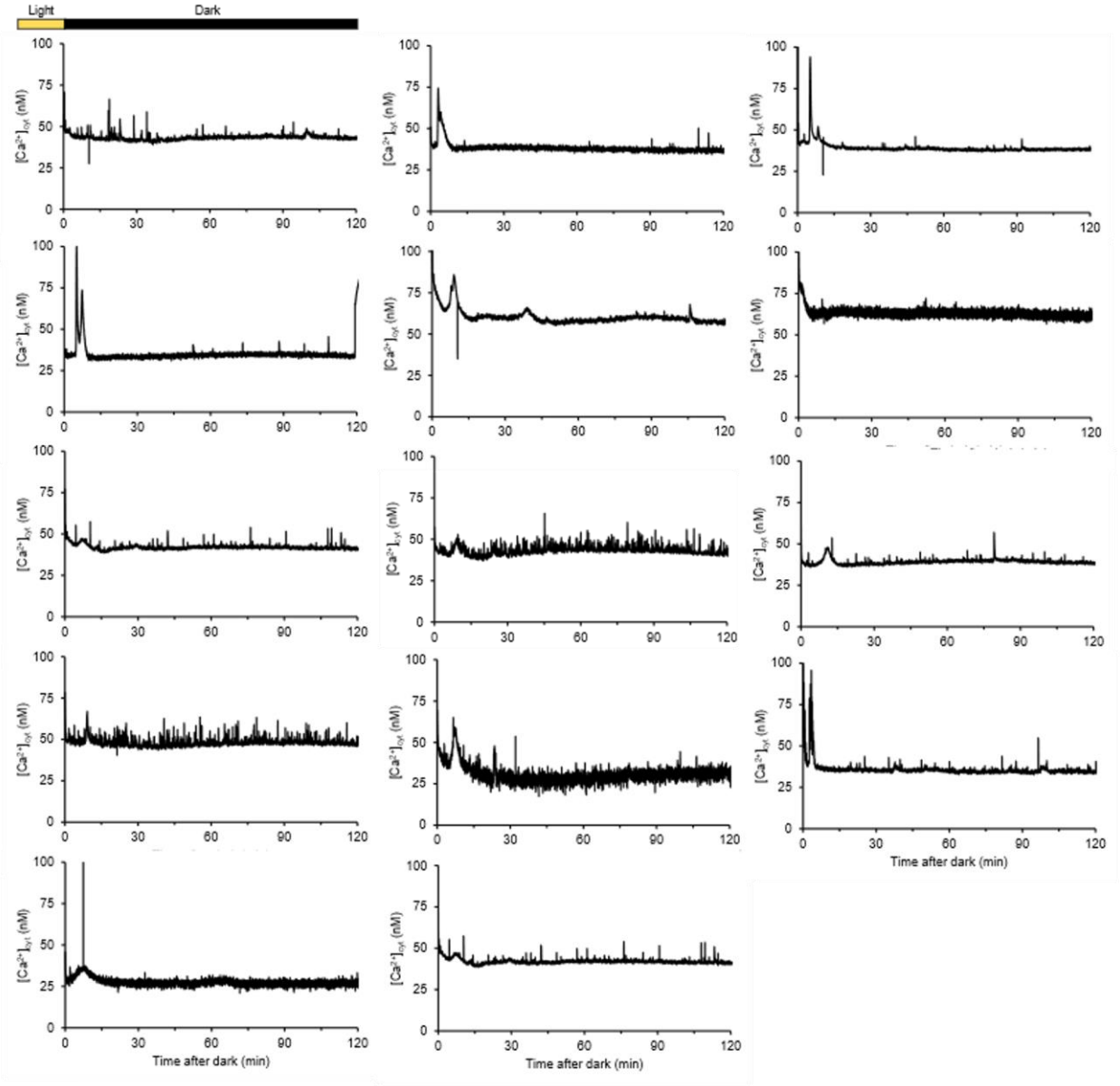

Fig. S5 The dark-induced $[Ca^{2+}]_{cyt}$ transient occurs in green tissues. Three Col-0 Arabidopsis transgenic seedlings expressing aequorin targeted to the cytosol were grown in white light-dark cycles. On the night of the 11th day of growth, the root and leaves were separated and incubated with coelenterazine. Aequorin luminescence was recorded in a luminometer from reconstituted aequorin when tissues were 12 days old. Traces represent the data obtained from one experiment. This is a repeat of figure 2 experiment.

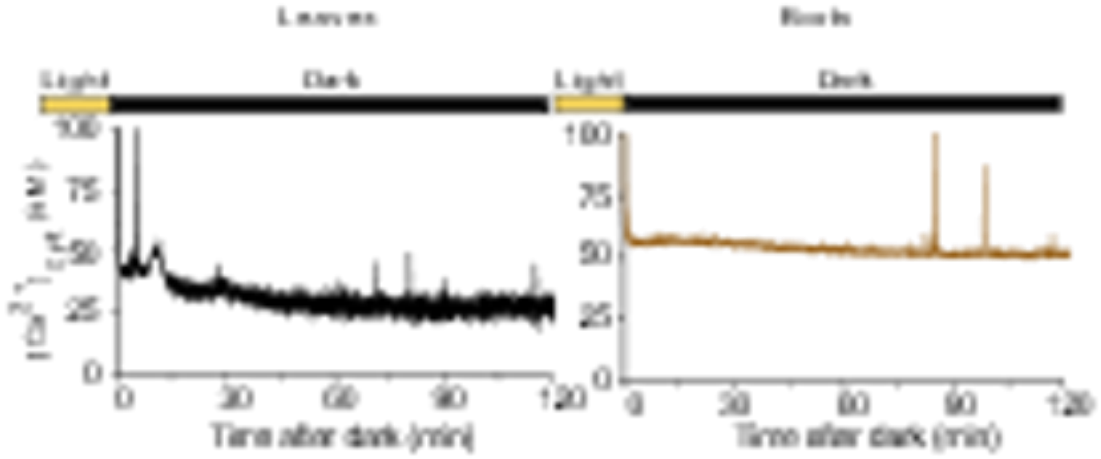

Fig. S6 The dark-induced $[Ca^{2+}]_{cyt}$ transient depends on the time of the day. Arabidopsis transgenic seedlings expressing aequorin targeted to the stroma (Ws-2) were grown in white light-dark cycles (12h:12h) (100 µmol m^{-2} s^{-1}). On the night of the 11th day of growth, seedlings were incubated with coelenterazine and aequorin luminescence was recorded from reconstituted aequorin since they were 12 days old. (A) shows data of changes in $[Ca^{2+}]_{stroma}$, every 2 h during 1 LD cycle. The data represent one experiment consisting of 80 clusters of seedlings and each cluster contained 7 - 10 seedlings. Experiments were repeated at least twice. This experiment is a repeat of the one in Figure 5b.

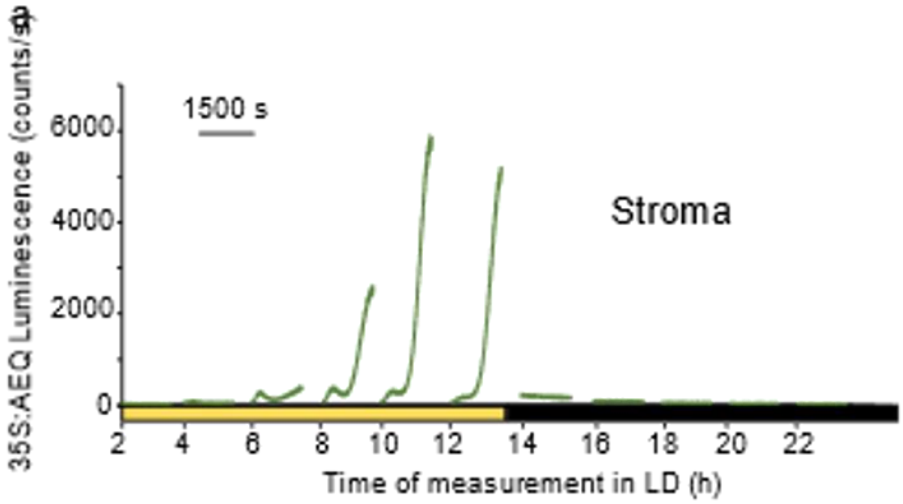

Fig. S7 Dark-induced increases of luminescence were not detected from plants not carrying the Aequorin transgene. Non-transgenic Col-0 Arabidopsis seedlings were grown in white light-dark cycles (12h:12h) (100 µmol m^{-2} s^{-1}). On the night of the 11th day of growth, seedlings were incubated with coelenterazine and luminescence was recorded when they were 12 days old. Graphs shows data during 1 LD 12h:12h cycle taken every 2 h during 1500 s. There were 80 clusters of seedlings per plate and each cluster contained 7 - 10 seedlings.

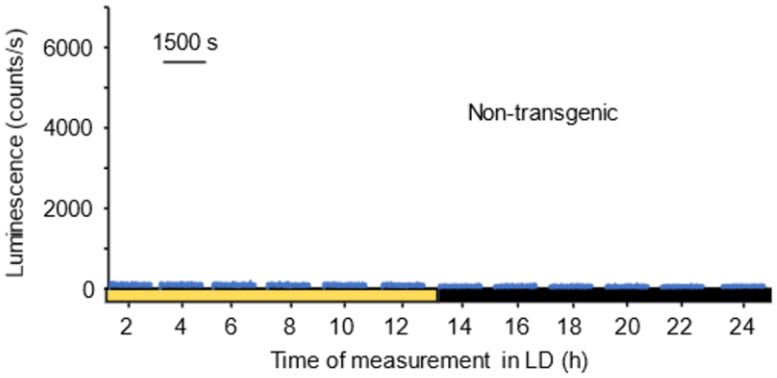

Fig. S8 The dark-induced $[Ca^{2+}]_{cyt}$ transient is gated by the circadian clock. Col-0 and *prr7*-11 *prr5*-10 *prr9*-11 triple mutant Arabidopsis transgenic seedlings expressing aequorin targeted to the cytosol were grown in white light-dark cycles (12h:12h) (100 µmol m^{-2} s^{-1}). On the night of the 11th day of growth, seedlings were incubated with coelenterazine and aequorin luminescence was recorded from reconstituted aequorin since they were 12 days old. Figures show data of changes in $[Ca^{2+}]_{cyt}$ every 2 h during LL cycles in Col-0 and *prr7*-11 *prr5*-10 *prr9*-11 triple mutant plants, respectively. The data represent one experiment consisting of 8 clusters of seedlings, each cluster contained 7 - 10 seedlings. Experiments were repeated at least twice. This is a repeat of the experiment in Figure 6.

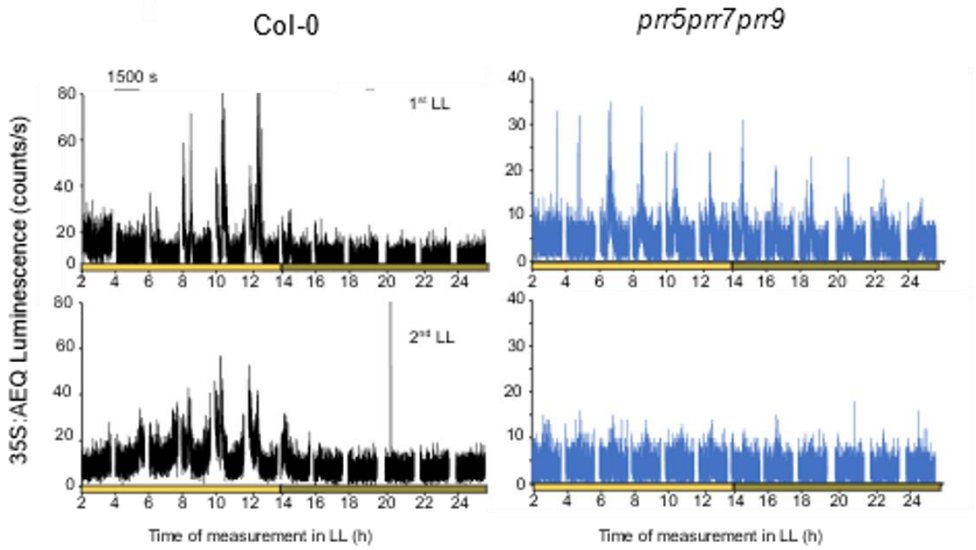

Fig. S9 The dark-induced $[Ca^{2+}]_{stroma}$ transient is gated by the circadian clock. Ws-2 Arabidopsis transgenic seedlings expressing aequorin targeted to the stroma were grown in white light-dark cycles (12h:12h) (100 µmol m^{-2} s^{-1}). On the night of the 11th day of growth, seedlings were incubated with coelenterazine and aequorin luminescence was recorded from reconstituted aequorin since they were 12 days old. The data show changes in $[Ca^{2+}]_{stroma}$ every 2 h during 2 LL cycles. The data represent one experiment consisting of 80 clusters of seedlings, each cluster contained 7 - 10 seedlings. This experiment is a repeat of the one in Figure 7.

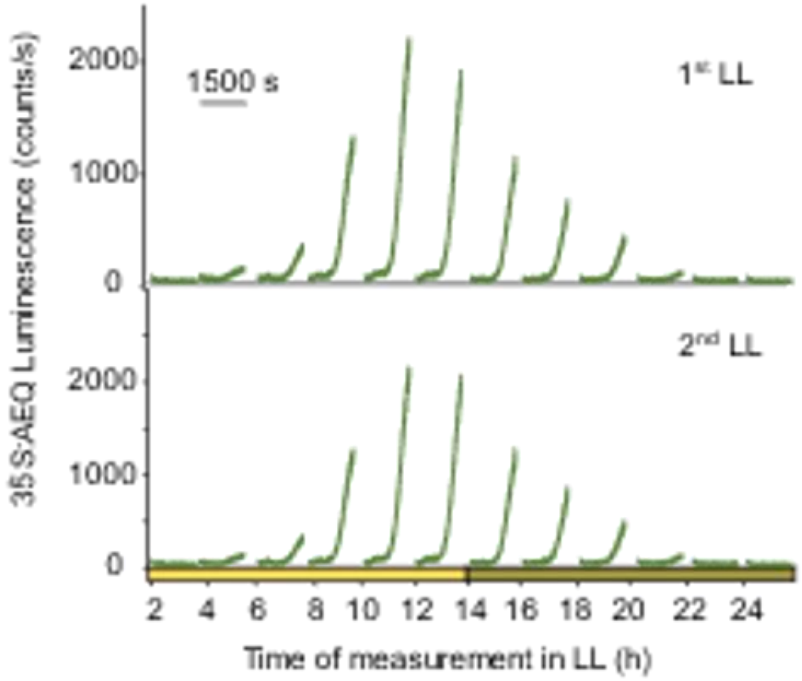

Fig. S10 Dark-induced transients in $[Ca^{2+}]_{cyt}$ are superimposed on daily and circadian $[Ca^{2+}]_{cyt}$ oscillations. (a) Daily and circadian $[Ca^{2+}]_{cyt}$ oscillations measured in LL using different time integration intervals. Col-0 Arabidopsis transgenic seedlings expressing aequorin targeted to the cytosol were grown in LD 12h:12h cycles and then transferred to LL. The data represent one experiment consisting of 80 clusters of seedlings and each cluster contained 7 - 10 seedlings. (b) Daily and circadian $[Ca^{2+}]_{cyt}$ oscillations measured in DD without (b) 3% (w/v) sucrose using different time integration intervals. (c) Changes in $[Ca^{2+}]_{cyt}$ recorded every 2 h and used in (B). (d) Daily and circadian $[Ca^{2+}]_{cyt}$ oscillations measured in DD with 3% (w/v) sucrose. Black are the raw time series data, showing no dark to light transitions. In red is the integrated photon counts obtained during the 1500 s of recording. (b-d) Col-0 Arabidopsis transgenic seedlings expressing aequorin targeted to the cytosol were grown in LD 12h:12h cycles and then transferred to DD. (b-d) the data represent one experiment consisting of 8 clusters of seedlings and each cluster contained 7 - 10 seedlings. These experiments are a repeat of the ones in Figure 8.