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Circadian Oscillations of Cytosolic and Chloroplastic Free Calcium in Plants

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Tobacco and *Arabidopsis* plants, expressing a transgene for the calcium-sensitive luminescent protein apoaequorin, revealed circadian oscillations in free cytosolic calcium that can be phase-shifted by light-dark signals. When apoaequorin was targeted to the chloroplast, circadian chloroplast calcium rhythms were likewise observed after transfer of the seedlings to constant darkness. Circadian oscillations in free calcium concentrations can be expected to control many calcium-dependent enzymes and processes accounting for circadian outputs. Regulation of calcium flux is therefore fundamental to the organization of circadian systems.

The circadian clock controls critical daily behavioral and reproductive activities in plants and animals. This timing mechanism is an oscillator that orchestrates a variety of circadian outputs in processes as diverse as ion fluxes, behavior, and gene expression (*1*). Recognition of the extraordinary diversity of calcium-regulated processes (*2*) has led to speculations that intracellular free calcium ($[Ca^{2+}]_i$) acts as a component of the central circadian clockworks (*1*); that it entrains circadian clocks in plants, molluscs, and mammals (*3*); and that it regulates circadian outputs, including the expression of specific genes (*1*, *4*, *5*). Technical limitations of procedures (*6*) for in vivo $[Ca^{2+}]_i$ measurement have hindered direct tests of these speculations. We have developed a method to monitor $[Ca^{2+}]_i$ in plants genetically transformed with the complementary DNA (cDNA) for apoaequorin (*7*). When reconstituted to aequorin, this calcium-sensitive luminescent protein enables continuous noninvasive reporting of $[Ca^{2+}]_i$ for many weeks in tissues, single cells, or whole seedlings (*7*, *8*).

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For measurements of the cytosolic free calcium concentration ($[Ca^{2+}]_c$), tobacco (*Nicotiana plumbaginifolia*) was genetically transformed with the cDNA for apoaequorin downstream of the cauliflower mosaic (CaMV) 35S promoter [strain MAQ 2.4, see (*7*)]. After reconstitution of aequorin with the luminophore coelenterazine (*9*), the luminescence of these seedlings (which reports $[Ca^{2+}]_c$) was monitored after transfer to continuous light (LL), and a circadian pattern was observed (Fig. 1). The luminescence peak occurred shortly after dawn and recurred at circadian intervals. This luminescence rhythm could be phase-shifted by an extension of the last dark interval (compare Fig. 1, A and B), which demonstrates that this rhythm is genuinely circadian. The luminescence rhythm could also be entrained by light-dark (LD) cycles; the two rhythms depicted in Fig. 1D come from separate groups of seedlings that were entrained to reversed-phase LD cycles and then were measured simultaneously.

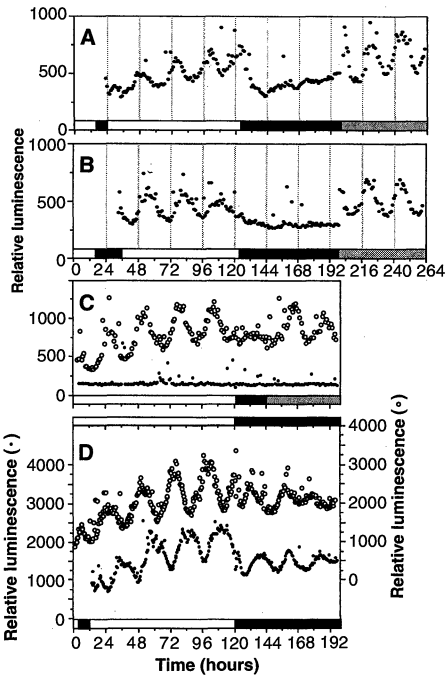

Fig. 1. Luminescence of transgenic tobacco seedlings containing cytoplasmic aequorin (MAQ 2.4) under various illumination regimes (*9*). White bars along the abscissas indicate white light background (22 $\mu E\ m^{-2}\ s^{-1}$), black bars indicate darkness, and gray bars indicate red light (18 $\mu E\ m^{-2}\ s^{-1}$). (**A**) Seedlings (two per vial) grown on a light-dark cycle of 16 hours light, 8 hours dark (LD 16:8); the last light cycle is shown. (**B**) Same as in (A), except that the final dark period was extended by 12 hours, resulting in a delay of the subsequent rhythm. (**C**) Open circles, transgenic MAQ 2.4 seedlings; closed circles, nontransgenic wild-type tobacco seedlings. Both groups were treated with coelenterazine (two seedlings per vial, grown on LD 16:8). (**D**) Separate cultures of MAQ 2.4 grown on reversed LD 16:8 cycles (10 seedlings per vial). The last light cycle for each is shown. The upper bar is for the open-circle trace, and the lower bar is for the closed-circle trace. Seedlings were initially in LL, then transferred to DD at hour 120.

Luminescence was not detected in either nontransgenic wild-type seedlings incubated with coelenterazine (Fig. 1C) or transgenic seedlings that were not exposed to coelenterazine (*10*). The rhythms of strain MAQ 2.4 were measured in both white and red LL (Fig. 1, A to C). In constant darkness (DD), the rhythm usually damped rapidly (Fig. 1, A and B), but in a few experiments, an oscillation was observed in DD, albeit at a reduced amplitude (Fig. 1D). The greater amplitude of the luminescence rhythm in LL may reflect the common observation that circadian expression in plants is more robust in LL (*11*), or that the circadian pacemaker modulates the sensitivity of its photoreceptive pathway (*11*), resulting in rhythmic phytochrome-mediated calcium flux (*12*).

Aequorin luminescence is specific for Ca^{2+} and is sensitive to free calcium in the

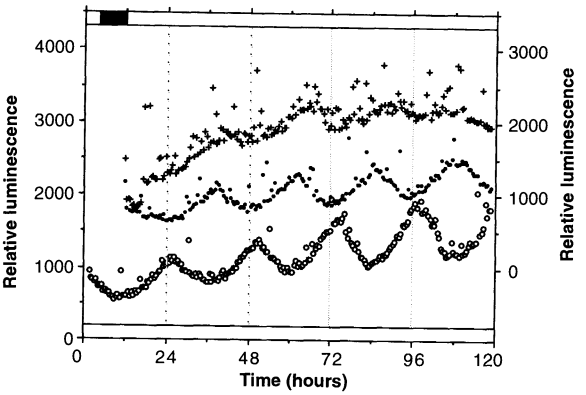

Fig. 2. Rhythmic variations in luminescence of transgenic *Arabidopsis* seedlings containing cytoplasmic aequorin (*29*) after entrainment to reversed-phase LD cycles. Separate cultures of *Arabidopsis* were grown in reversed LD 16:8 cycles on medium without sucrose; the last LD cycle for each is shown. The upper bar and right ordinate is for the upper two traces, and the lower bar and left ordinate is for the lowest trace. After reconstitution of aequorin (*7, 9*), 7-day-old seedlings were transferred to vials containing media with or without sucrose (three seedlings per vial), then exposed to LL (22 $\mu E\ m^{-2}\ s^{-1}$) at 22°C to monitor luminescence. Lower two traces, 0.5 M&S medium without sucrose; upper trace, 0.5 M&S medium plus 1% sucrose.

physiological range (*6*). Calibration of the $[Ca^{2+}]_c$ changes requires knowledge of the total amount of reconstituted aequorin in the plants, which necessitates a destructive assay (*6*). An estimate was therefore made by using seedlings of equivalent age and overnight aequorin reconstitution for calibration (*6*). We estimate that the luminescence peaks in Fig. 1 represent $[Ca^{2+}]_c$ values between 500 and 700 nM in the different experiments; the troughs represent approximately 100 to 150 nM. An oscillation between 100 and 600 nM free calcium will affect calcium-dependent activities in plants (*5, 13*). Furthermore, these values could be an underestimate of the relevant calcium concentrations if the signals reported here come from a subset of cells in the seedlings, an issue that could be resolved by luminescence imaging (*8*). However, imaging of the luminescence in these seedlings at 600 nM $[Ca^{2+}]_i$ would require higher levels of circadian light emission than those currently produced by strain MAQ 2.4.

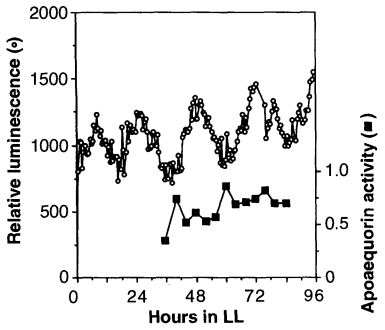

Fig. 3. Comparison of luminescence expression of MAQ 2.4 seedlings with total extractable apoaequorin activity. Open circles, luminescence rhythm measured as in Fig. 1; closed squares, specific activity (normalized to protein concentration) of apoaequorin extracted (*30*) at the indicated times from seedlings under the same conditions as the vial used for luminescence monitoring.

A transgenic line of thale-cress (*Arabidopsis thaliana*) harboring the same chimaeric transgene as the tobacco strain MAQ 2.4 also expressed a circadian rhythm of luminescence which could be entrained to reversed-phase LD cycles (Fig. 2). Sucrose in the medium (1%) reduced the amplitude of the luminescence rhythm in *Arabidopsis* (Fig. 2). It is possible that this response is related to the effect of sucrose on the expression of genes (*14*) regulated by the circadian clock and $[Ca^{2+}]_i$ (*5*). Sucrose (1%) did not affect luminescence rhythms in tobacco MAQ 2.4 seedlings (*10*).

The luminescence rhythms cannot be attributed to rhythmic concentrations of apoaequorin or of reconstituted aequorin. Apoaequorin content assayed from extracts of MAQ 2.4 seedlings at various circadian times during a 48-hour period displayed no circadian oscillations that could account for the rhythm of luminescence (Fig. 3). There appeared to be a progressive increase in apoaequorin concentrations (Fig. 3) which might result from seedling growth and continued apoaequorin synthesis and could account for the increases in overall luminescence seen in Fig. 1, C and D, and Fig. 2. In other experiments, we estimated the in vivo amounts of reconstituted aequorin by extracting seedlings that had been loaded with coelenterazine at the peak (50 hours) and trough (62 hours). We found that more aequorin activity could be extracted from seedlings at the trough than at the peak (*10*). We suggest that the differences in the extractable amounts are due to the monotonic increase of aequorin content (as in Fig. 3). The apparently higher aequorin amounts at the trough phases of the luminescence could only lead to an underestimate of the true circadian variation in $[Ca^{2+}]_c$ and certainly cannot account for the circadian changes in luminescence levels that we observed. In agreement with the data shown here, the activity of the CaMV 35S promoter has been shown to be constitutive over the circadian cycle in LL and DD in *Arabidopsis* (*4*). We therefore conclude that the oscillations of luminescence are due to oscillations in $[Ca^{2+}]_c$.

Light signals have been suggested to alter $[Ca^{2+}]_c$ by regulating the Ca^{2+} flux into the chloroplast (*15*). We have tested the possibility that the circadian $[Ca^{2+}]_c$ variations were directly regulated by flux across the chloroplast membrane by using the transgenic tobacco line MAQ 6.3, which contains the 35S promoter–driven apoaequorin transgene with the coding sequence of the transit peptide of the small subunit of ribulose-1,5-bisphosphate carboxylase–oxygenase (RuBisCO) (*rbcS*) fused to the 5′ end of the apoaequorin coding region. These tranformants permit measurement of chloroplast Ca^{2+} ($[Ca^{2+}]_{chl}$) in vivo (Fig. 4). With this construct, effectively 100% of apoaequorin was targeted to intact chloro-

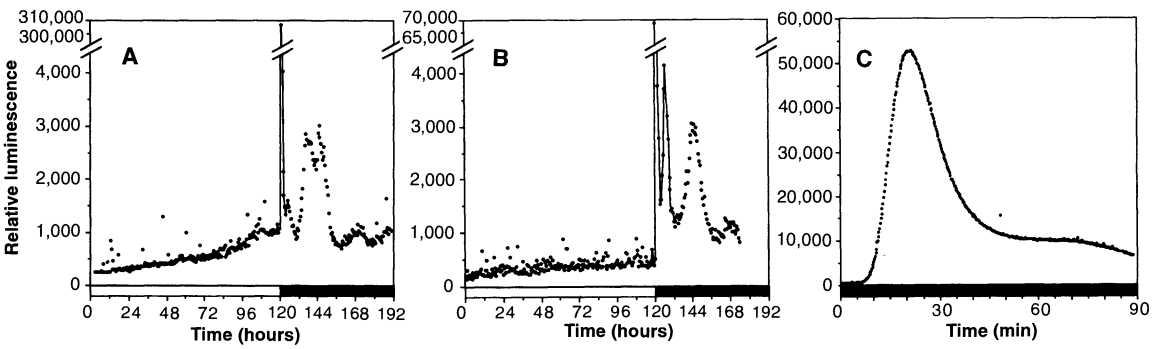

Fig. 4. Luminescence from reconstituted aequorin in tobacco seedlings expressing chloroplastic aequorin [MAQ 6.3; see (*16*)]. In (A) and (B), seedlings treated with coelenterazine (*9*) were in LL from time 0 to hour 120, and thereafter in DD. Each panel shows the luminescence pattern from an independent sample. (**A**) Ten seedlings per vial, 10-day-old seedlings. (**B**) Five seedlings per vial, 22-day-old seedlings. (**C**) Ten seedlings per vial, 10-day-old seedlings measured every 15 s; time 0 is the time of the LL-to-DD transition.

plasts, most probably to the stroma (*16*). In contrast to rhythms expressed in $[Ca^{2+}]_c$, the $[Ca^{2+}]_{chl}$ of seedlings did not exhibit detectable circadian variation in LL (Fig. 4, A and B). On transfer to DD, we reproducibly observed a large transient increase that peaked about 20 to 25 min ($n = 7$) after the LL-to-DD transition (Fig. 4). We estimate that this transient peak represents 5 to 10 μM chloroplast free calcium rising from basal concentrations of less than 150 nM. The timing of this peak after the LL-to-DD transition (Fig. 4C) suggests that this calcium spike may play a role in the recognition of lights-off. Thereafter, in DD damped oscillations of luminescence were often observed that had a circadian period (Fig. 4, A and B). Because the transgenes MAQ 2.4 and MAQ 6.3 share the same 35S promoter, these data support the conclusion that the circadian luminescence oscillations of MAQ 2.4 result from rhythmic variations in $[Ca^{2+}]_c$. The variations in $[Ca^{2+}]_{chl}$ acquire significance from the reported detection of a number of calcium binding proteins in the chloroplast (*15*) and the observations that a number of chloroplast activities have a circadian basis (*1*). Calcium is apparently regulated differentially by the clock in distinct compartments, or there may be different circadian oscillators in the cytoplasm and the chloroplast, as previously suggested (*17*).

Oscillations of free calcium within the range reported here will affect the function of many cellular systems. Plant circadian processes that involve movements of stems, petioles, or stomatal apertures are known to respond to turgor pressure which is regulated by movements of potassium ions across the plasma membrane through Ca^{2+}-gated potassium channels (*18*). Other known $[Ca^{2+}]_c$-regulated processes include growth and mitosis (*19*), plant protein kinases (*13*), and expression of genes such as those encoding chlorophyll a/b binding proteins (*4, 5, 11*). All of these processes also exhibit circadian variation. If calcium is involved in both circadian inputs (*3*) and outputs (this study), then it could well be an integral component of the central mechanism itself (*1*). Finally, the photoperiodic timing mechanism that regulates seasonal aspects of flowering, reproduction, and dormancy involves interactions of red light with a circadian clock (*20*). Our results implicate $[Ca^{2+}]_c$ in the transduction of red light–activated phytochrome events (*12*) and in circadian rhythmicity. The discovery of this link aids understanding of circadian pacemakers and their role in the timing of flowering, movements, gene expression, and other aspects of plant development.

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6. For our calibrations of the change in cytosolic calcium concentration, seedlings incubated 8 hours with coelenterazine were either homogenized in 5 mM EGTA and 0.1 M Hepes (pH 7) and the aequorin discharged with excess Ca^{2+}, or the coelenterazine-treated seedlings were immersed in 0.9 M $CaCl_2$ and 10% ethanol. Both methods give identical total aequorin estimations after the introduction of a slight correction (about 10%) for the effect of ethanol on aequorin. Cytosolic calcium concentrations were then estimated with the calibration curve reported by P. M. Cobbold and T. J. Rink [*Biochem. J.* **248**, 313 (1987)].
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9. *Nicotiana plumbaginifolia* strain MAQ 2.4 T3 plants and *Arabidopsis* plants expressing cytosolic apoaequorin [homozygous for the transgene (*7*)] were grown on 0.5 Murashige and Skoog (M&S) medium (Sigma) in LD 16:8 for 8 to 10 days, then incubated with 10 μM coelenterazine in darkness for 8 hours to reconstitute the calcium-sensitive luminescent protein aequorin (*7, 8*). The seedlings were rinsed, placed on 2 ml of 0.5 M&S and 0.8% (w/v) agar at 22°C, and luminescence was measured by an automated photomultiplier tube apparatus (*21*) in which the sensitivity was increased by routing the output of a low-noise photomultiplier tube (Hamamatsu R2693P) to a photon counter (Hamamatsu C3866) to detect weak luminescence. Luminescence was measured for 1 min in the middle of a 3-min dark interruption of the background illumination. Interruption of LL by brief repetitive dark pulses does not interfere with circadian expression (*21*).
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16. The chloroplast-targeted aequorin coding region sequence of MAQ 6.3 was produced by fusing a Hind III–Sal 1 fragment from the plasmid pSPTP19 (*22*) encoding the first 62 amino acids of pea *rbcS* (the small subunit of RuBisCO) gene to the apoaequorin coding region. The junction at the Sal 1 site was in translational frame and at the eighth NH_2-terminal amino acid of apoaequorin. This fused coding sequence was inserted between the cauliflower mosaic 35S transcriptional promoter and terminator in pDH51 (*23*) and the whole chimæric gene transferred to the *Agrobacterium* binary vector pBIN19 (*7*). *Nicotiana plumbaginifolia* was genetically transformed and plants homozygous for the transgene selected. Independent transformants were tested for high expression and correct proteolytic processing with a combination of aequorin reconstitutions and immunoblot analysis (*10*). MAQ 6.3 was identified as expressing the highest amounts of aequorin and so was used for subsequent analysis. To assess targeting efficiency, we isolated chloroplasts from MAQ 6.3 protoplasts made from leaves of 6-week-old plants by a procedure modified from (*24*). The protoplasts were lysed in the light phase after removal of samples for total aequorin (*7*) and chlorophyll (*25*) estimation. After centrifugation through a 50% Percoll gradient (*26*) containing 50 mM Mops-NaOH (pH 7.8), 2 mM EDTA, 0.15% bovine serum albumin, and 330 mM mannitol, the chloroplast band was isolated and, with differential interference contrast microscopy (×100 magnification), was found to contain 93.1% intact chloroplasts. Aequorin was reconstituted in these isolated chloroplasts (and protoplast extracts) by incubation in 2 μM coelenterazine (*7*) and 93.8% of the total protoplast aequorin was located in the intact chloroplasts.
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29. Apoaequorin-expressing *A. thaliana* plants (No-0 ecotype) were generated by *Agrobacterium*-mediated T-DNA (transfer DNA) transformation (*27*) with the MAQ 2.4 construct (*7*) which specifies cytosolic apoaequorin expression. The seedlings used in these experiments were homozygous for a single T-DNA insert and expressed high amounts of apoaequorin.
30. Ten-day-old MAQ 2.4 tobacco seedlings were grown and treated with coelenterazine as in (*9*). At each time point, 10 MAQ 2.4 seedlings were collected, quick frozen in liquid nitrogen, homogenized in 0.5 ml of 0.5 M NaCl, 5 mM EDTA, 5 mM dithiothreitol, 0.1% gelatin, and 50 mM Pipes (pH 7.2) and samples taken for protein and apoaequorin estimation. Apoaequorin was reconstituted into aequorin with a standard incubation of 3 hours in coelenterazine (*7*), and activity was estimated by discharge with an excess of 20 mM $CaCl_2$ in buffer (*7, 28*) in an Aminco photometer.

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