The Calcium Rhythms of Different Cell Types Oscillate with Different Circadian Phases

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Transgenic tobacco (*Nicotiana plumbaginifolia*) seedlings containing the Ca^{2+}-sensitive luminescent protein aequorin have been shown to exhibit circadian variations in cytosolic calcium. Concomitant measurements of cytosolic and nuclear calcium show that circadian variations in the cytoplasm are not expressed in the nucleus. To investigate whether all cells of transgenic seedlings contribute equally to circadian variations in cytosolic calcium, different promoters eliciting different expression patterns have been placed upstream of aequorin and used for transformation. The circadian peak occurred at different times in the three transgenic lines constructed. Luminescence imaging of these transgenic lines indicated that aequorin was differentially accumulated among the main tissues and cells of the seedlings and overcoat technology with applied epidermal strips indicated that the surface cell layers contribute the vast majority of luminescent light. We conclude that the Ca^{2+} rhythmicities of cells and tissues oscillate with distinct differences in phase, that this might represent different underlying cellular control mechanisms and that these observations have significant implications for our understanding and study of Ca^{2+} mediated signal transduction in plant cells.

Circadian rhythmicities, entrained by daily changes in light regime and, to a lesser extent, associated changes in temperature and humidity, have been documented in a multitude of biological phenomena at every level of eukaryotic organization and in some prokaryotes (Hastings, 1991; Kondo et al., 1993; McClung and Kay, 1994). Reported rhythmicities in plants include those in leaflet and petal movements, stomatal conductance, photosynthetic rate, ion fluxes, and gene expression (Gorton et al., 1989; McClung and Kay, 1994).

Present opinion suggests that the clock is comprised of three components. The first component (the entrainment pathway) couples the second component (the autonomous oscillator) to the environmental periodicity and determines the phase of the free-running rhythm. The third component (the output pathway directed by the oscillator) gives rise to the overt biological rhythm. This conceptual dissection of the molecular clock enables investigation of one of the key questions in circadian biology: Do organisms possess a master clock directing a multitude of diverse rhythmic outputs or do multiple circadian oscillators exist, either within single cells or distinct morphological structures for the rhythmic expression of multiple outputs?

Understanding of the processes of signal transduction in plant cells has undergone a revolution in the last 10 to 15 years. Because circadian rhythms are entrained by signals, involvement of transduction pathways in the mechanisms of rhythmic phenomena are directly indicated. Cytosolic Ca^{2+} ($[Ca^{2+}]_c$), a prominent second messenger in plant cells, is known to play a substantive regulatory role in the transduction of many signals (Gilroy et al., 1990; Neuhaus et al., 1993; Malhò et al., 1998). Moreover, red light, often used to entrain circadian rhythms, transiently elevates cytosolic Ca^{2+} (Shacklock et al., 1992; Ermolayeva et al., 1996).

We have previously described the production of transgenic plants containing apoaequorin. In the presence of the luminophore, coelenterazine, the Ca^{2+}-sensitive luminescent protein aequorin can be reconstituted generating plants whose luminescence directly reports $[Ca^{2+}]_c$ (Knight et al., 1991). These plants enable long-term continuous recording of $[Ca^{2+}]_c$. In continuous white or red light, such plants exhibit robust circadian variations in $[Ca^{2+}]_c$ (Johnson et al., 1995). These initial experiments used transgenic plants in which aequorin was placed downstream of the powerful cauliflower mosaic virus (CaMV) 35S promoter. However, because the peak of

[1] This work was supported by the Carnegie Trust for the Universities of Scotland (to N.T.W.), by a fellowship in the Biotechnology programme funded by the European Commission (to M.V.-M.), by the U.S. National Institute of Mental Health (grant no. NIMH MH43836 to C.H.J.), and in part by the Biotechnology and Biological Science Research Council (to A.H., A.J.T., and A.H.v.d.L.).

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the luminescence rhythm was relatively broad it was considered possible that smaller subpopulations of cells could exist with differing rhythmicities. Detection of such cells might enable a re-appraisal of the hypothesis of a central, controlling circadian oscillator.

Previously, only the chloroplast and cytosol compartments were investigated (Johnson et al., 1995). Recent studies have emphasized that nuclei might independently regulate nuclear Ca^{2+} ($[Ca^{2+}]_n$; Badminton et al., 1998). Expression of genes such as *cab*, which exhibit circadian rhythmicity can be regulated by Ca^{2+}, conceivably nuclear Ca^{2+} (Neuhaus et al., 1993). Could circadian variations in nuclear Ca^{2+} exist and might additional investigation indicate separate circadian control?

To address these issues we have generated new transgenic plants using promoters upstream of apoaequorin, which differ in their cell and tissue expression compared with CaMV 35S. By imaging the distribution of luminescence expression with these new promoters and monitoring emitted luminescence over many days, the notion that circadian variation in $[Ca^{2+}]_c$ may, in part, be cell and tissue specific could be tested. The use of transgenic plants containing nuclear targeted aequorin (Van der Luit et al., 1999) enables testing of the nuclear Ca^{2+} hypothesis.

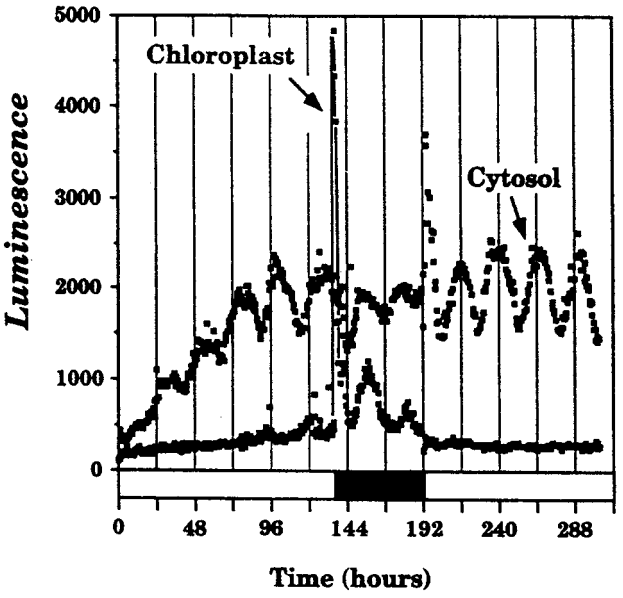


Figure 1. Variation in Ca^{2+}-dependent luminescence from transgenic seedlings containing cytosolic or chloroplastic aequorin. Light emission from 10- to 14-d-old seedlings was monitored over 13 d. Individual days are marked with bars. The light-dark regime is indicated at the bottom of the figure.

RESULTS

Cytosolic Ca^{2+} But Not Nuclear Ca^{2+} Exhibits Circadian Rhythmicity

Figure 1 shows the circadian oscillations in luminescence emitted by MAQ2.4 (cytoplasmic aequorin) and MAQ 6.3 (chloroplast aequorin) seedlings run coincidentally for 13 d. As described previously the cytoplasm generates a distinct luminescence rhythm in continuous light, which diminishes in darkness. Resumption of illumination induces a peak in luminescence, followed by a phase shift. In contrast the onset of darkness is accompanied by a large peak of luminescence emitted by the MAQ6.3 chloroplast aequorin with indications of subsidiary circadian peaks damping rapidly.

We have also constructed plants expressing a fusion protein between nucleoplasmin and aequorin, which as judged by immune analysis targets at least 85% into the nucleus (Van der Luit et al., 1999). This line, designated MAQ7.11, has been used in a variety of experimental treatments, which demonstrate that nuclear Ca^{2+}, $[Ca^{2+}]_n$, is regulated independently of $[Ca^{2+}]_c$. MAQ 2.4 seedlings (Fig. 2a), wild-type seedlings incubated with coelenterazine (Fig. 2b), and MAQ7.11 seedlings (Fig. 2c) were measured coincidentally under the white-light/dark regime illustrated for 8 d. The dark period was again set for a length 6 h out of phase from the initial period, and the data in Figure 2a indicate the re-establishment of the rhythm when re-illuminated, as in Figure 1. The resulting $[Ca^{2+}]_c$ peak after re-initiation of the new light regime is 2-fold higher than the previous $[Ca^{2+}]_c$ oscillations but finishes within 12 h, perhaps reflecting the phase shift induced by the extended dark period. The subsequent oscillations in the white light after the dark period exhibit the effects of the phase shift.

Peak and trough values of $[Ca^{2+}]_c$ were estimated as described previously (Johnson et al., 1995) as 159 ± 17.2 nM in the trough (n = 3) rising to 536 ± 27.6 nM at the peak (n = 3). Due to continued apoaequorin synthesis and discharge we are unable to provide proper $[Ca^{2+}]_c$ scaling for these experiments. These estimates of $[Ca^{2+}]_c$ however, assume that all cells and tissues contribute equally to the detected luminescence, a supposition that is at present difficult to justify.

No circadian variation could be discerned in the wild-type/coelenterazine control (Fig. 2b) and the nuclear transformed seedlings (Fig. 2c). Although we may be unable to detect tiny variations in $[Ca^{2+}]_n$, currently these data do not support the hypothesis that $[Ca^{2+}]_n$ and $[Ca^{2+}]_c$ are freely exchangeable through the nuclear pores.

Differences in Circadian $[Ca^{2+}]_c$ Variation between Different Transgenic Lines

Transgenic plants were generated in which apoaequorin expression was driven by either the abscisic acid (ABA)-inducible, cDeT6–19 promoter from *Craterostigma plantagineum* (Michel et al., 1994), designated line MAQ15, or the constitutive lipid transfer

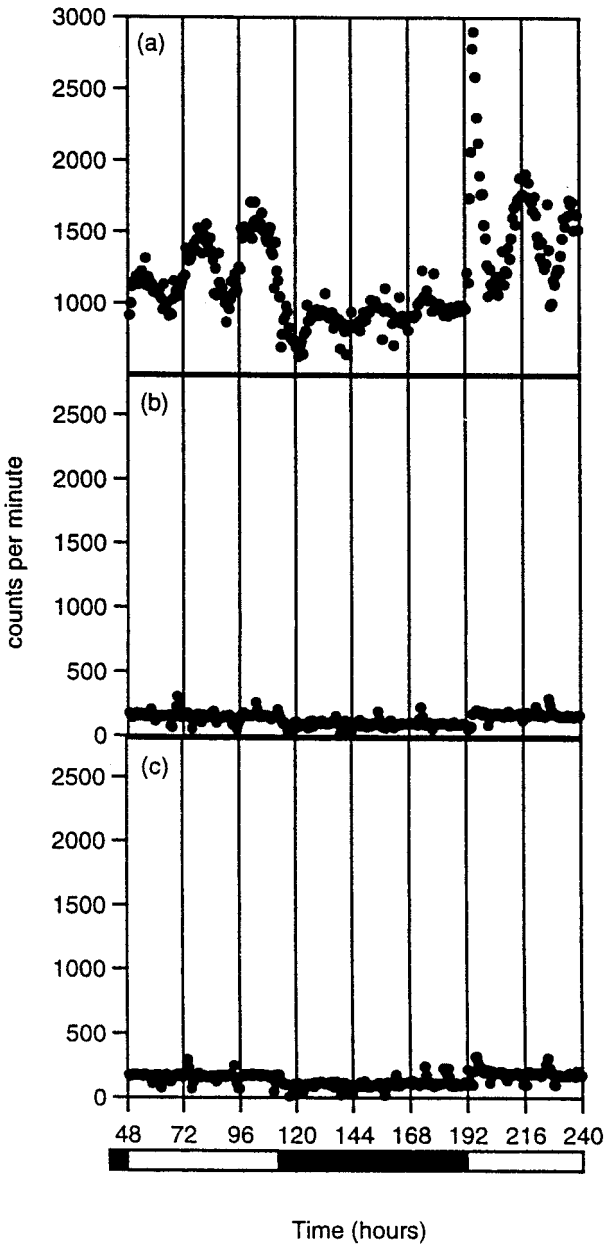


Figure 2. Variation in Ca^{2+}-dependent luminescence from transgenic seedlings containing cytosolic or nuclear aequorin. a, MAQ2.4 plants expressing cytoplasmic aequorin; b, untransformed; c, MAQ7.11 plants expressing nuclear aequorin. The light-dark regime is indicated at the bottom of the figure.

protein (LTP) promoter from Arabidopsis (Thoma et al., 1994), designated MAQ16. The expression patterns of both promoters in leaves using β-glucuronidase as a reporter have been described elsewhere (Thoma et al., 1994; Taylor et al., 1995) and should certainly be sufficiently different from those driven by CaMV 35S to test the basic hypothesis outlined in the introduction.

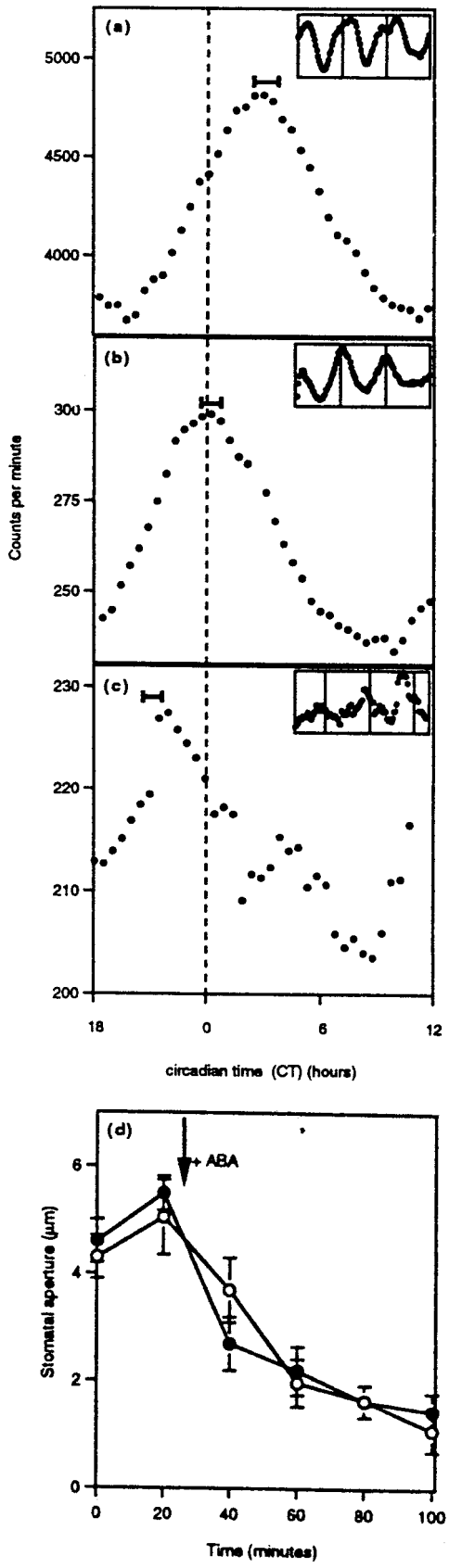
Figure 3 shows the circadian variations in luminescence emitted by each transgenic line throughout one circadian day. The horizontal line over the peak represents the mean time of the highest peak value and the SE of the mean. The inset shows 3 successive days, illustrating that the oscillations are robust in free-running conditions. Figure 3a shows the MAQ2.4 (CaMV 35S-aequorin) seedlings in which the variation is relatively symmetrical, although a slight shoulder at dawn can sometimes be detected. Figure 3b summarizes data for MAQ15 (cDeT6–19-aequorin) seedlings. The major peaks occur 3.39 ± 0.69 h earlier than those of the MAQ2.4 line. Luminescence values are much lower than MAQ2.4 plants, but the circadian variation is still relatively symmetrical around the mean value. The expression of this construct requires pretreatment with ABA. ABA was removed by placing the seedlings on water for 1 d prior to experimentation. Seedlings grew normally and stomata functioned in response to further additions of ABA by closure (Fig. 3d). Addition of ABA to MAQ2.4 plants did not alter the phase of the rhythm (data not shown). The oscillations damp to zero after 5 d, and measurements of aequorin discharge suggest that continued aequorin turnover finally leads to loss of the reporter. Expression from the cDeT6–19 promoter requires continuous exposure to drought or ABA (Michel et al., 1994).

Figure 3c shows the circadian variation in MAQ16 (LTP-aequorin) seedlings. There is a pronounced shoulder in the emitted luminescence that corresponds with the peak shown in Figure 3a and could represent equivalent cell types in both cases. The main peak occurs 6.62 ± 0.64 h prior to that of MAQ2.4 seedlings.

Differences in phase between these transgenic lines are not a consequence of mutations caused by transgene insertion since the luminescence rhythms of two other transformant lines harboring the pMAQ15 construct also peaked significantly nearer circadian time (CT) 0 than line MAQ2.4. When monitored under identical conditions the luminescence peaks of lines MAQ15.11, 15.18, and 15.27 occur at 3.39 ± 0.69, 2.67 ± 0.35, and 3.02 ± 0.25 h earlier respectively than those of MAQ2.4. Neither do the oscillations reflect circadian variations in the synthesis of apoaequorin (Fig. 4). We therefore conclude that the oscillations represent real changes in $[Ca^{2+}]_c$.

The Three Transgenic Lines Exhibit Spatially Distinct Patterns of Aequorin Reconstitution

Given the variations in $[Ca^{2+}]_c$ rhythmicities between transgenic lines, it was essential to determine whether this could be due to differential cell or tissue expression of aequorin (despite frequent attempts to image the light emitted during circadian variation illustrated in Figs. 1 and 2a, the background is too high and the emitted intensity of fluence too low for imaging, in part the result of the log/log relationship of light to $[Ca^{2+}]_c$). Several possibilities such as use of a reporter gene with the promoter or immune



staining were considered. However, expression patterns using reporter genes can mislead if the stability of the reporter gene product differs from the original protein. The stability of apoaequorin (and aequorin) compared with β-glucuronidase is not known and could vary in different cell types. Similarly, whereas the distribution of apoaequorin, judged by immunolocalization, could be used as an indicator of aequorin reconstitution, we do not know the extent of coelenterazine penetration and eventual aequorin reconstitution or the rate of turnover in different tissues. Furthermore, no data is available that would indicate the depth of tissue from which luminescent light could penetrate. For these reasons we decided to image where aequorin was expressed and reconstituted as the best indicator of the putative source of circadian luminescence. We have previously developed a means of aequorin localization in whole seedlings (Wood et al., 2000). The procedure involves rupturing of the cellular membranes by a single freeze-thaw cycle in the presence of 10 mM $CaCl_2$. Penetration of Ca^{2+} under these conditions discharges more than 90% of reconstituted aequorin. Imaging the resulting luminescence indicates the location of active aequorin present in vivo. We have imaged luminescence resulting from freeze/thaw at a whole tissue level (Fig. 5, a–f) and at a cellular level (Fig. 5, g–n). Luminescence is only observed in the freeze part of the cycle and is not detected during the thaw (data not shown).

Figure 5 (a and b) shows bright field and luminescent images of a MAQ2.4 (35S-aequorin) seedling. The seedlings we routinely use contain cotyledons and a small first leaf. Luminescence is apparent throughout the whole seedling but is nonuniform, being highest in the cotyledons and first leaf, the root and root/shoot junction ($n = 20$). Figure 5 (c and d) shows bright field and corresponding luminescent images for the ABA-induced MAQ15 (cDeT6–19-aequorin) line. Expression levels as judged by luminescence are substantially lower than in the MAQ2.4 line (confirmed by homogenization and discharge of aequorin) and are completely limited to the first leaf and cotyledons and occasional regions of the upper stem ($n = 20$). No luminescence was observed in the roots, root/shoot junction, or lower stem of this transgenic line.

Figure 3. Variation in luminescence from transgenic seedlings through 1 d and inset showing multiple days. Luminescence of MAQ2.4 (a), MAQ15 (b), and MAQ16 (c) seedlings was monitored in continuous white light. The oscillation of 1 d with the mean ± SE as a horizontal bar over the peak ($n = 20$) is shown with further cycles of each rhythm shown inset. The dotted line indicates subjective dawn (CT 0) in each case. d, Shows the effects of the addition of exogenous ABA (at the arrow) on the stomatal behavior of seedlings that have undergone a 2-d pretreatment with 100 μM ABA followed by 1 d free of ABA (●) compared with non-ABA treated controls (○). These pretreatment conditions mimic the treatment of MAQ15 plants.

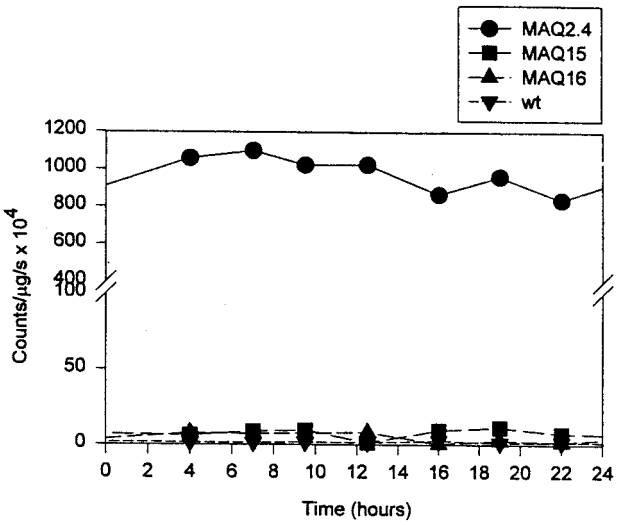


Figure 4. Variation in seedling apoaequorin content throughout 1 circadian d. MAQ2.4, MAQ15, MAQ16, and wild-type tobacco seedlings were collected throughout one complete 24-h period. After homogenisation and reconstitution, the aequorin was discharged with an excess of $CaCl_2$.

Figure 5 (e and f) shows the bright field and luminescent images of the MAQ16 (LTP-aequorin) line. Expression at this stage of development is lower again but substantial light emission is limited just to the petioles. Some light emission can be detected from the cotyledons and first leaf but is faint and is not easily detected at this magnification ($n = 20$). No luminescence was observed emanating from the stem or roots of these seedlings.

Because the main observation common to all three transgenic lines was luminescence in the cotyledons and first leaf (although to differing degrees) we have examined these tissues at higher magnification. Figure 5 (g–j) shows MAQ2.4 cotyledons at two different magnifications. The primary light source in the images investigated ($n = 50$) are guard cells. There is very faint light production by other cells, but these cannot be clearly distinguished. It should be emphasized that what is measured here is aequorin content, and content does not necessarily relate directly to gene expression. Given the well-documented constitutive nature of the CaMV 35S promoter it is unlikely that the guard cells exhibit localized expression. Higher rates of aequorin turnover in epidermal cells, or perhaps the peripheral nature of the epidermal cell cytosol as compared with the denser guard cell cytosol, could account for the apparent absence of substantial light emission. Compared with the intensity of light emanating from guard cells, peripheral epidermal cell luminescence may be harder to detect in this line. In comparison, the MAQ15 cotyledons analyzed ($n = 20$) reveal much more uniform light emission (Fig. 5, k and l) and are substantially different to MAQ2.4. Much of the imaged light, although weaker than MAQ2.4, follows the characteristic shape of the surface epidermal cells. Occasionally discrete luminescing guard cell pairs have been distinguished. Finally, the cotyledon light emission from the MAQ16 line (Fig. 5, m and n) seems to be totally uniform but is very low and not easy to detect. Luminescence of guard cell pairs has never been detected. Some epidermal shapes can be seen although these again are very weak. We have also imaged the main source of light in MAQ16 in the petiole (data not shown), but a characteristic cell structure was not easy to discern probably because of the optical difficulties with imaging a thin cylindrical tissue. Low expression levels in lines MAQ 15 and MAQ 16 precluded more detailed imaging studies at higher magnifications.

Do these images in Figure 5 (g–n) represent the major sources of luminescent light or is there much more light emitted from deeper within tissues that is diffracted or reflected by the outer layers making it difficult to image and discriminate its cellular source? To test this possibility we prepared epidermal peels (containing a single layer of cells) from untransformed *N. plumaginifolia* leaves and placed them on top of one cotyledon before subjecting a MAQ2.4 seedling to freeze-thaw (Fig. 6) and estimating the numbers of photons emitted from each individual cotyledon. This epidermal "overcoat" treatment indicated that the covered cotyledon emitted only 66 ± 4% ($n = 20$) of the light of its uncovered partner. The maximal light collecting efficiency from these seedlings will therefore be from the surface layers of the tissue.

In summary, we conclude that the three transgenic lines exhibit spatially distinct patterns of aequorin accumulation and that the major sources of luminescent light are from the roots, stem, guard cells (and most likely other cells within the epidermis) of MAQ2.4, epidermal cells in MAQ15 and the petiole cells in MAQ16. We propose therefore there are distinct differences between the transgenic lines regarding which cell populations are able to contribute (i.e. contain fully reconstituted aequorin) to the detected circadian rhythms in Ca^{2+}-dependent aequorin luminescence and that the variations in rhythmicities reported reflect tissue/cell specific circadian patterns.

DISCUSSION

We have developed an approach that enables the direct, noninvasive monitoring of $[Ca^{2+}]_c$ in different cells/tissues of intact seedlings. Circadian changes in $[Ca^{2+}]$-dependent aequorin luminescence, originally described for whole seedlings (Johnson et al., 1995), seem likely to emanate from a number of different cell or tissue locations within the plant. Furthermore, the circadian character in each of these sources appears different. In some way, which at present can only be speculated, different tissues and cells are generating different rhythmic patterns in $[Ca^{2+}]_c$, an

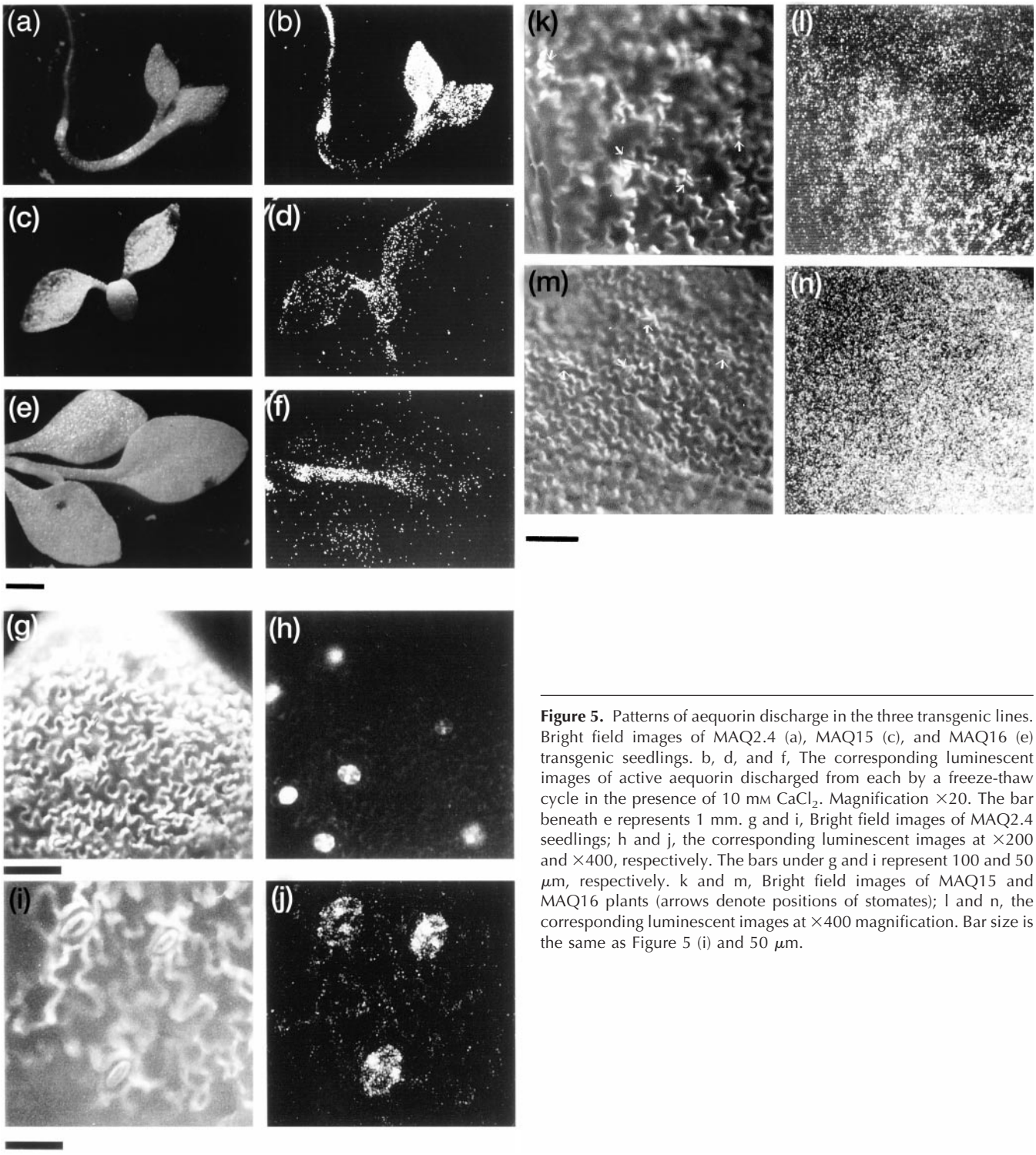


Figure 5. Patterns of aequorin discharge in the three transgenic lines. Bright field images of MAQ2.4 (a), MAQ15 (c), and MAQ16 (e) transgenic seedlings. b, d, and f, The corresponding luminescent images of active aequorin discharged from each by a freeze-thaw cycle in the presence of 10 mM $CaCl_2$. Magnification ×20. The bar beneath e represents 1 mm. g and i, Bright field images of MAQ2.4 seedlings; h and j, the corresponding luminescent images at ×200 and ×400, respectively. The bars under g and i represent 100 and 50 μm, respectively. k and m, Bright field images of MAQ15 and MAQ16 plants (arrows denote positions of stomates); l and n, the corresponding luminescent images at ×400 magnification. Bar size is the same as Figure 5 (i) and 50 μm.

effect that is masked in MAQ 2.4 seedlings by the inevitable whole seedling averaging. Either there are different oscillators for each cell or tissue type or each cell type interprets the same fundamental oscillator in different ways (see below). Data obtained using the nuclear-targeted MAQ7.11 plants do not support the hypothesis that circadian variation in Ca^{2+}-regulated genes is transcriptionally controlled by $[Ca^{2+}]_n$, although a cytoplasmic transduction mechanism operating through Ca^{2+} is not precluded.

Some differences to the reported expression patterns of the promoters used here became apparent during this study. CDeT6–19 promoter activity for example, was more uniform throughout the epider-

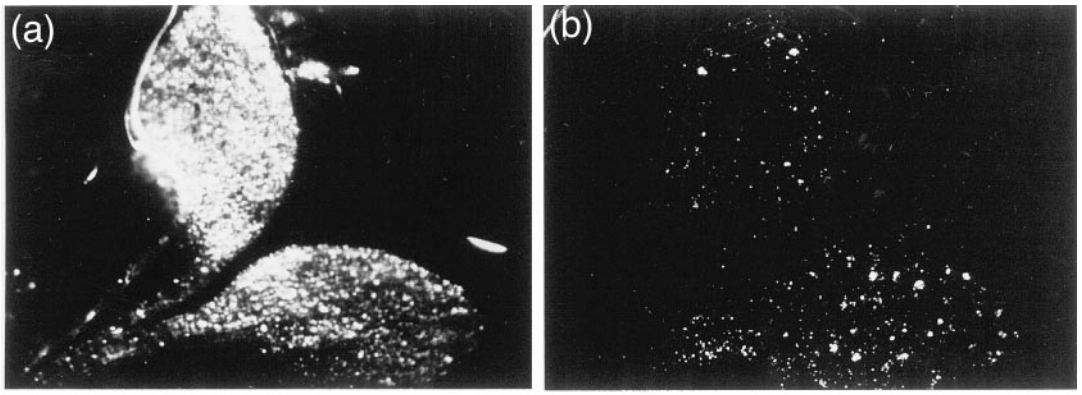


Figure 6. Effect of an epidermal "overcoat" on the detection of freeze-thaw aequorin luminescence from MAQ2.4 cotyledons. Bright-field image (a) and the associated luminescence image with the epidermal peel draped over the upper of the two cotyledons (b). While not visible on top of the cotyledon in the bright field picture, traces of the epidermal strip can be distinguished on either side. Bar = 0.4 mm.

mis than had been previously reported (Taylor et al., 1995). Similarly with the LTP promoter, although our detection of slight aequorin activity in cotyledons of line MAQ16 seedlings was consistent with findings of the original authors (Thoma et al., 1994), we found that aequorin was predominantly localized in the petioles. Given the complex tissue-specific developmental regulation of both promoters (Michel et al., 1994; Thoma et al., 1994), it is likely that these discrepancies reflect differences in the ages and morphology of plant species used. With respect to the cDe-T6–19 promoter different basal levels of constitutive activity and variable levels of endogenous ABA have also been previously reported in experimental plant material (Michel et al., 1994; Taylor et al., 1995). It is important to emphasize however, as previously noted, that what we have imaged is the location of reconstituted aequorin rather than apoaequorin expression.

The question of whether organisms possess a master clock or whether multiple circadian oscillators exist, has long interested circadian biologists. Recent evidence indicates the presence of multiple oscillators in *Drosophila melanogaster* (Plautz et al., 1997) and in the unicellular alga *Gonyaulax polyhedra* (Roenneberg and Morse, 1993). In higher plants, support for the concept of multiple oscillators has come primarily from reports of free-running rhythms oscillating with different circadian periods (Hennessey and Field, 1992; Sai and Johnson, 1999). More recently, in a study using tobacco (*Nicotiana plumbaginifolia*) and Arabidopsis seedlings expressing luciferase, Thain et al. (2000) have demonstrated not only that the luminescence rhythms of excised petioles can be reset following isolation from the plant, but also that individual organs of intact seedlings, and even distal versus proximal ends of the same leaf, can be stably entrained to different light/dark regimes. These data strongly suggest that multiple copies of independent oscillators reside in different plant tissues.

The indication in this study that, although entrained by the same photic regime, the free-running Ca^{2+} rhythmicities of different cells/tissues oscillate with different phases may support this conclusion. However, it should be noted that such phase differences do not necessarily confirm circadian control by more than one oscillator. In studies using transgenic cyanobacteria expressing luciferase from different promoters, it has been found that although the phase of the luminescence rhythm varies considerably between promoters (Kondo et al., 1993; Liu et al., 1995), the similarity of effects of mutations of the central clock Kai proteins on their activities indicate that they are being driven by the same oscillator (Ishiura et al., 1998). The phase differences in Ca^{2+} rhythmicities reported here therefore may equally arise due to the activity of a single controlling oscillator, the rhythm being cued by the oscillator at different phases of the clock in different cells/tissues.

The observations presented here clearly have considerable significance for Ca^{2+} signaling studies. Varying basal levels of $[Ca^{2+}]_c$ throughout the circadian cycle have implications for the thresholds and kinetics of Ca^{2+}-mediated signal transduction processes in different cells/tissues, making the time of day an important consideration for experimental interpretation.

Limitations of the techniques presented here are associated with low aequorin expression levels in some transgenic lines. However in view of these data and the growing body of evidence that Ca^{2+} plays an integral role in a number of rhythmic phenomena in plants (Neuhaus et al., 1993; Gomez and Simon, 1995; Anderson and Kay, 1996), we propose that, with development, $[Ca^{2+}]_c$-dependent aequorin luminescence will prove to be a useful rhythmic marker phenotype in transgenic plants. In conjunction with the use of tissue or cell specific promoters to target the expression of apoaequorin to different locations within the plant, and topogenic sequences to direct

expression to specific subcellular locations, this approach will facilitate the continuing investigations into both the role of Ca^{2+} in circadian rhythmicities and the functional organization of the circadian clock mechanism in higher plants.

MATERIALS AND METHODS

Germination and Growth of Plant Material

Tobacco (*Nicotiana plumbaginifolia*) MAQ2.4 and MAQ7.11 seedlings were germinated from F_3 seeds, and MAQ15 and MAQ16 were germinated from F_1 seeds. Seeds were sterilized for 12 min in 10% to 14% (v/v) sodium hypochlorite, incubated in 1 mM gibberellic acid overnight at 4°C, and grown on 0.5× Murashige and Skoog medium (Murashige and Skoog, 1962) containing 400 μg/mL kanamycin at 22°C to 24°C with a 16-h photoperiod for 10 to 14 d. Prior to the reconstitution of aequorin in vivo, apoaequorin expression was induced in MAQ15 seedlings by floating on 100 μM (±)-cis, trans ABA for 32 h, starting from the beginning of the 8-h dark period on the 8 to 10 d of growth.

In Vivo Reconstitution of Aequorin

Seedlings were floated on 5 μM coelenterazine (in distilled water) during the 8-h dark period for two successive days prior to experimentation.

Production of Tobacco Lines MAQ15 and MAQ16

The 971-bp cDeT6–19 promoter (position −889 to 82 of the published sequence; donated by D. Bartels, Max-Planck Institut für Züchtungsforschung, Cologne) was amplified by PCR using oligo M3414 to introduce *Sst*I and *Not*I sites at the 5′ end (CGATTAGAGCTCGCGGCCGCCGGATCTATAGCAACTGA) and M3415 to add a *Sal*I site at the 3′ end (CGGCGCGTCGACT TTCTCTCGTAAATAACAGTTGC). Identical 5′ sites were added to the 1,149-bp LTP promoter fragment (nucleotides 68–1,217 of the published sequence; donated by S. Thoma, Michigan State University) using oligo P2212 (CAGTGAGCTCGCGGCCGCAATCTCAAAACCAAAG) and a *Xho*I site at the 3′ end using oligo P2213 (GATCCTCGAGCATATTGATCTCTTAGG). PCR products were digested as appropriate, and cloned into p7AQ, a modified version of the pART7 (Gleave, 1992) into which the full-length apoaequorin coding sequence had been inserted and the CaMV 35S promoter removed. Expression cassettes were transferred to the binary vector pART27 (Gleave, 1992) as *Not*I inserts to produce pMAQ15 and pMAQ16. The plasmids were purified from *Escherichia coli* JM101 and used to transform *Agrobacterium tumefaciens* LBA4404 (Holsters et al., 1978). Wild-type tobacco was transformed (Horsch et al., 1985) and putative transformants selected for resistance to 400 μg/mL kanamycin. Two-week-old F_1 progeny were tested for apoaequorin expression by western blotting. Five micrograms of total protein extracts were electrophoresed on 12% (w/v) SDS-polyacrylamide gels, electroblotted onto nitrocellulose and challenged with polyclonal mouse anti-aequorin antisera. Immunodetection using an enhanced chemiluminescence protein detection kit (Amersham, Buckinghamshire, UK) was used to determine the highest expressing lines, MAQ15.11 and MAQ16.1, which were used for all subsequent experimentation.

Measurement of Circadian Changes in Luminescence

Groups of 10 seedlings were floated on 2 mL of 0.5× liquid MS media in universal vials and transferred from their normal light/dark regime to free-running conditions (constant white light (22 $\mu Em^{-2}\ s^{-1}$ at 22°C) at CT 0. $[Ca^{2+}]$-dependent aequorin luminescence was monitored using an automated photomultiplier tube apparatus as described by Johnson et al. (1995).

Calibration of Calcium Measurements

In cells containing reconstituted aequorin there is a double logarithmic relationship between the free intracellular calcium concentration and the amount of aequorin remaining in the entire sample at any one point in time (Lum_{max}) (Blinks et al., 1978). At the peaks and troughs of the rhythms of each transgenic line, individual vials of 10 seedlings were removed and luminescence measured using a Hamamatsu photomultiplier tube (R2693P) linked to a Hamamatsu C5410 photon counter. To establish Lum_{max} the seedlings were discharged with 900 mM $CaCl_2$, 10% (v/v) ethanol, and counted over a period of 1 min. To determine calcium concentrations the following equation, originally described by Allen et al. (1977), was used: $L/L_{max} = \{(1+ KR [Ca^{2+}])/(1 + KTR + KR [Ca^{2+}])\}^3$, where L represents light emission from the sample (counts s^{-1}), Lum_{max} is the total amount of light present in the entire sample, $[Ca^{2+}]$ is the calculated calcium concentration, KR is the dissociation constant for the first calcium ion to bind to aequorin, and KTR is the dissociation constant of the second calcium ion. We have found that KR = 2,000,000 M^{-1} and KTR = 147 M^{-1} for coelenterazine by fitting the coelenterazine sensitivity curve (Shimomura et al., 1993) with the equation. This modified formula can be used for the quantification of $[Ca^{2+}]$ values below 700 nM.

Imaging and Measurement of Total Luminescence Discharged by Freeze-Thaw Treatment

Aequorin was discharged from single seedlings by cellular disruption with a single freeze-thaw cycle (down to −4°C in 30 s back up to 8°C after 60 s) on a Peltier temperature stage in a drop of 10 mM $CaCl_2$. Images were collected using a Berthold LB980 intensified tube camera (Hertfordshire, UK), in conjunction with a Nikon Diaphot microscope. Bright field images were collected over 2 s (2 × 1-s integrations) and luminescent images over 6-s intervals (2 × 3-s integrations). To estimate the proportion of total aequorin discharged by a single freeze-thaw treatment, batches of 10 seedlings with reconstituted aequorin were homogenized in 0.5 mL of 0.5 M NaCl, 5 mM EGTA, 5 mM dithiothreitol, 0.1% (w/v) gelatin, 50 mM PIPES (1,4-

piperazinediethanesulfonic acid), pH 7.2, before the aequorin was discharged with an excess of $CaCl_2$ in the luminometer (Knight et al., 1991). Similarly, batches of 10 seedlings subjected to a single freeze thaw were subjected to homogenization and discharge.

Stomatal Aperture Measurements

Apertures of randomly selected stomata were magnified 400× under oil using a Diaphot microscope and measured using an eyepiece micrometer (Nikon, Tokyo). Apertures were measured in non-ABA- and ABA-treated MAQ15 seedlings at 20-min intervals following the addition of 10 μM ABA. Aperture measurements were made from 5 seedlings sample group^{-1}, 5 stomata seedling^{-1}.

Chemicals

All chemical reagents were purchased from BDH Chemicals (Poole, UK) or Sigma (Poole, UK). All enzymes used for DNA manipulations were obtained from Biogene (Bedfordshire, UK), Boehringer Mannheim (East Sussex, UK), or Life Technologies (Paisley, UK). DNA isolation kits were purchased from Promega (Southampton, UK) and Qiagen (Dorking, UK). Coelenterazine was purchased from Cambridge Biosciences (Cambridge, UK). Oligonucleotide primers were synthesized by Oswel DNA services (Southampton, UK).

Statistical Analysis

All tests of statistically significant differences between data sets were performed using either *t*-tests or analysis of variance at $P = 0.05$. Data used for statistical analysis of phase differences were obtained from 3 to 5 seedlings transgenic line^{-1}, 4 peaks seedling^{-1}.

ACKNOWLEDGMENT

We thank Gideon Baum for assistance with calcium calibrations.

Received June 26, 2000; returned for revision August 22, 2000; accepted October 2, 2000.

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