

# Time of day modulates low-temperature $\text{Ca}^{2+}$ signals in Arabidopsis

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## Summary

We tested the hypothesis that the circadian clock modulates  $\text{Ca}^{2+}$ -based signalling pathways, using low-temperature (LT)-induced  $\text{Ca}^{2+}$  signals. We investigated the relationship between diurnal and circadian modulation of LT-induced increases in cytosolic-free calcium ( $[\text{Ca}^{2+}]_{\text{cyt}}$ ), and regulation of  $[\text{Ca}^{2+}]_{\text{cyt}}$ -dependent outputs of the LT-signalling network (*RD29A* transcript abundance and stomatal closure). We measured  $[\text{Ca}^{2+}]_{\text{cyt}}$  non-invasively using aequorin, and targeted aequorin to the guard cell using a guard cell-specific GAL4-green fluorescent protein enhancer trap line. LT caused transient increases in whole plant and guard cell  $[\text{Ca}^{2+}]_{\text{cyt}}$ . In guard cells, the LT-induced  $[\text{Ca}^{2+}]_{\text{cyt}}$  elevation preceded stomatal closure. In whole plants, the magnitude of LT-induced  $[\text{Ca}^{2+}]_{\text{cyt}}$  transients, measured from the entire plant or specifically the guard cell, varied with the time of day: LT-induced  $[\text{Ca}^{2+}]_{\text{cyt}}$  transients were significantly higher during the mid-photoperiod than at the beginning or end. Diurnal variation in LT-induced guard cell  $[\text{Ca}^{2+}]_{\text{cyt}}$  increases was not correlated to diurnal variation in LT-induced stomatal closure. There was circadian modulation of LT-induced whole plant  $[\text{Ca}^{2+}]_{\text{cyt}}$  increases, which were correlated to the circadian pattern of *RD29A* induction. In order to understand the significance of LT-induced  $[\text{Ca}^{2+}]_{\text{cyt}}$  increases, we used a computer simulation to demonstrate that, in guard cells, LT-induced  $[\text{Ca}^{2+}]_{\text{cyt}}$  increases measured from a population of cells are likely to represent the summation of cold-induced single-cell  $[\text{Ca}^{2+}]_{\text{cyt}}$  oscillations.

**Keywords:** circadian, calcium, cold, guard cell, signalling.

## Introduction

Circadian clocks synchronize plant physiology with the external light–dark cycle, which provides both physiological and competitive advantages (Dodd *et al.*, 2005). Circadian control is believed to derive from several interlocking and autoinhibitory loops of gene expression that incorporate time-delay steps (Alabadi *et al.*, 2001; Mizuno and Nakamichi, 2005), and become entrained to day/night cycles by signals from photoreceptors (Devlin, 2002; Millar, 2004). In contrast to the molecular clock and its entrainment, the signalling pathways that communicate the estimate of time from this clock to clock-controlled physiology are poorly understood (Webb, 2003). We hypothesize that one mechanism by which the clock could regulate cell physiology is by

time modulation of stimulus-induced signalling events. We have tested this hypothesis by investigating cold-induced  $\text{Ca}^{2+}$  signalling in the whole plant and guard cell.

$\text{Ca}^{2+}$  is an important second messenger in plant cells that regulates numerous physiological and developmental responses, such as stomatal movements (Ng *et al.*, 2001), pollen tube development (Holdaway-Clarke and Hepler, 2003), and root hair growth (Dolan and Davies, 2004). In single cells, many extracellular stimuli induce oscillations in the concentration of cytosolic-free  $\text{Ca}^{2+}$  ( $[\text{Ca}^{2+}]_{\text{cyt}}$ ), with periods ranging from minutes to tens of minutes (Evans *et al.*, 2001). These stimulus-induced  $[\text{Ca}^{2+}]_{\text{cyt}}$  oscillations are believed to enable this ubiquitous signalling molecule to

encode and transduce stimulus-specific information (Allen *et al.*, 2000, 2001; Hetherington *et al.*, 1998; McAinsh *et al.*, 1995; Staxén *et al.*, 1998). We wished to investigate whether stimulus-induced  $[Ca^{2+}]_{cyt}$  signals are modulated by the circadian clock, and used low-temperature (LT) signalling as a model system with which to test this hypothesis, as LT signalling incorporates  $[Ca^{2+}]_{cyt}$  signals and is circadian-regulated (Fowler *et al.*, 2005; Knight *et al.*, 1991). We examined circadian gating of LT-induced  $[Ca^{2+}]_{cyt}$  signals in relation to two  $[Ca^{2+}]_{cyt}$ -regulated outputs from the LT-signalling pathway: cold-induced stomatal closure (Allen *et al.*, 2000), and *RD29A* expression (Henriksson and Trewavas, 2003).

Acclimation to freezing and non-freezing LT conditions involves alterations in membrane lipid composition, synthesis of cryoprotective proteins, and expression of drought-responsive genes (Artus *et al.*, 1996; Steponkus *et al.*, 1988). One genetic pathway by which LT responses are induced is the CBF pathway (Thomashow, 2001). LT-induction of the CRT/DRE Binding Factor 1 (CBF1), CBF2 and CBF3 transcription factors induces over 200 transcripts that are believed to contribute to cold tolerance (Fowler and Thomashow, 2002; Maruyama *et al.*, 2004; Vogel *et al.*, 2005). The concentration of cytosolic-free calcium ( $[Ca^{2+}]_{cyt}$ ) is also elevated by LT exposure (Knight *et al.*, 1991), and LT-induced  $[Ca^{2+}]_{cyt}$  signals induce some CBF-responsive genes that are associated with cold acclimation, such as *RD29A* (also described as *COR78* or *LT178*; Henriksson and Trewavas, 2003; Horvath *et al.*, 1993; Nordin *et al.*, 1993; Yamaguchi-Shinozaki and Shinozaki, 1993). LT-induction of *RD29A* is reduced, but not completely abolished, by the  $[Ca^{2+}]_{cyt}$ -signalling antagonists lanthanum and EGTA, which also partially abolish LT-induced increases in  $[Ca^{2+}]_{cyt}$  (Henriksson and Trewavas, 2003).  $[Ca^{2+}]_{cyt}$  oscillates in guard cells following LT treatment, and these oscillations may encode the signals that cause cold-induced stomatal closure (Allen *et al.*, 2000). LT activation of *CBF* genes is gated by the circadian clock, such that LT induction of *CBF* genes is greatest 4 h after the beginning of the subjective day, and reduced during the subjective night (Fowler *et al.*, 2005). Circadian gating enables the output of environment-sensing pathways to be graded to a magnitude that is appropriate for the time of day. As *CBF* targets such as *RD29A* exhibit weak circadian gating (Fowler *et al.*, 2005), and the clock-related gene *GIGANTEA* participates in cold signalling (Cao *et al.*, 2005), it is likely that low temperature-induced alterations in cell physiology are circadian-modulated.

Aequorin is a recombinant bioluminescent reporter of  $[Ca^{2+}]_{cyt}$  that has been widely used to measure changes in  $[Ca^{2+}]_{cyt}$  in whole seedlings (Knight *et al.*, 1991). Using this technique, it has been demonstrated that  $[Ca^{2+}]_{cyt}$  oscillates with a circadian period (Johnson *et al.*, 1995; Love *et al.*, 2004). We have targeted aequorin to the guard cell using GAL4:VP16-mediated transactivation of aequorin in a guard

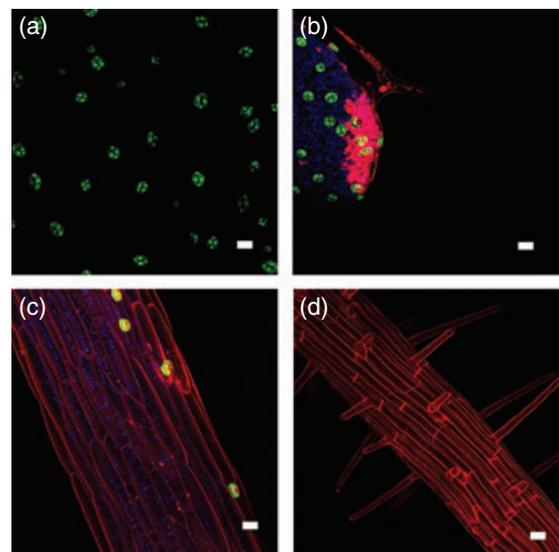
cell-specific enhancer trap line (Haseloff *et al.*, 1997; Kiegle *et al.*, 2000). This allows non-invasive measurement of cell type-specific changes in  $[Ca^{2+}]_{cyt}$  in the intact plant (Kiegle *et al.*, 2000).

In this study, we demonstrate that the circadian clock can modulate cold-induced  $Ca^{2+}$  signals. The day–night cycle of illumination modulates cold-induced  $Ca^{2+}$  signals, and may gate *RD29A* cold-induction. We show that temporal modulation of cold-induced  $Ca^{2+}$  signals could occur via alterations in cell-specific  $[Ca^{2+}]_{cyt}$  oscillations.

## Results

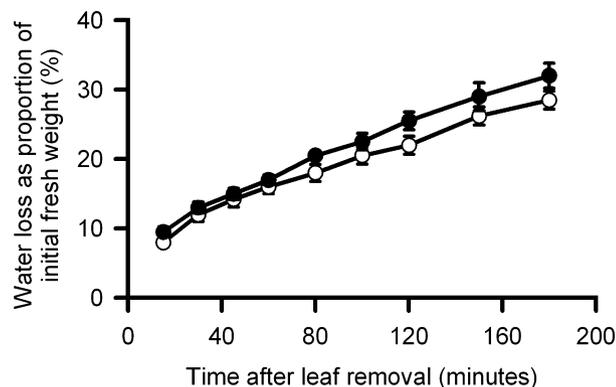
### *E1728 is a guard cell-specific GAL4-GFP enhancer trap line and a tool for guard cell-specific gene expression*

To investigate LT signalling in guard cells, we expressed aequorin in guard cells of the enhancer trap line E1728. E1728 was selected from a library of GAL4-GFP enhancer trap lines (<http://enhancertraps.bio.upenn.edu>). Green fluorescent protein (GFP) was expressed only in the guard cells of E1728 (Figure 1). GFP was expressed in mature guard cells but not in meristemoids, guard mother cells or any other cells. We did not detect developmental control of GFP expression because its expression pattern did not change during the first 3 weeks of growth (data not shown). The GFP expression pattern was unaffected by a range of abiotic and biotic stresses, and remained guard cell-specific



**Figure 1.** Green fluorescent protein (GFP) was expressed exclusively in guard cells of E1728.

Images are 3D projections of confocal scanning laser microscopy (CLSM) optical sections of the enhancer trap line E1728. GFP fluorescence is shown in green; propidium iodide staining the cell walls is red; chlorophyll autofluorescence is blue. (a,b) Abaxial and adaxial surfaces of a cotyledon and leaf, respectively; (c) hypocotyl; (d) part of a root. The number of optical sections comprising each projection and the distance separating them was (a) 6, 5.82  $\mu\text{m}$ ; (b) 14, 7.49  $\mu\text{m}$ ; (c) 14, 7.49  $\mu\text{m}$ ; (d) 16, 6.68  $\mu\text{m}$ . Scale bars, 20  $\mu\text{m}$ .



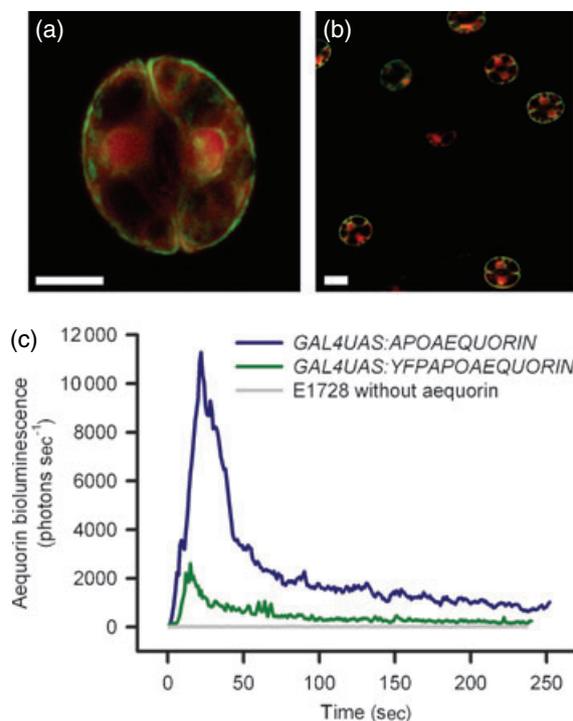
**Figure 2.** Rate of water loss from detached leaves of Col-0 (open circles) and the guard cell-specific enhancer trap E1728 (closed circles);  $n = 12 \pm \text{SE}$ .

during growth in the dark, at 4°C, in the presence of 20 nM 2,4-dichlorophenoxyacetic acid (2,4-D), 1 µM kinetin and 0.5 µM abscisic acid. Guard cell-specific GFP expression was inherited in the  $T_3$ ,  $T_4$  and  $T_5$  generations. Southern analysis demonstrated that the cell-specific GFP expression was driven by a tandem T-DNA insert at a single locus in E1728 (data not shown).

We did not detect physiological or developmental phenotypes due to insertion of the T-DNA. Stomata of E1728 closed normally (Figure 2), the morphology of E1728 was indistinguishable from the Col-0 background, flowering time was unaffected, and the responses of root growth to LT (4°C), 20 nM 2,4-D, 1 µM kinetin and 0.5 µM abscisic acid (ABA) were not significantly different from Col-0 (data not shown). The stomatal density on abaxial surfaces of leaves and cotyledons was unaffected by T-DNA insertion in E1728 (data not shown). Stomatal development and function, and whole-plant development, were therefore normal in E1728. Guard cell size and pore aperture were representative of previous measurements from Col-0 plants grown under identical conditions (Webb and Baker, 2002; Webb *et al.*, 2001). These results indicate that E1728 is an attractive system for GAL4-mediated gene transactivation in the guard cell.

#### *GAL4 transactivation of aequorin in E1728 reports guard cell $[\text{Ca}^{2+}]_{\text{cyt}}$*

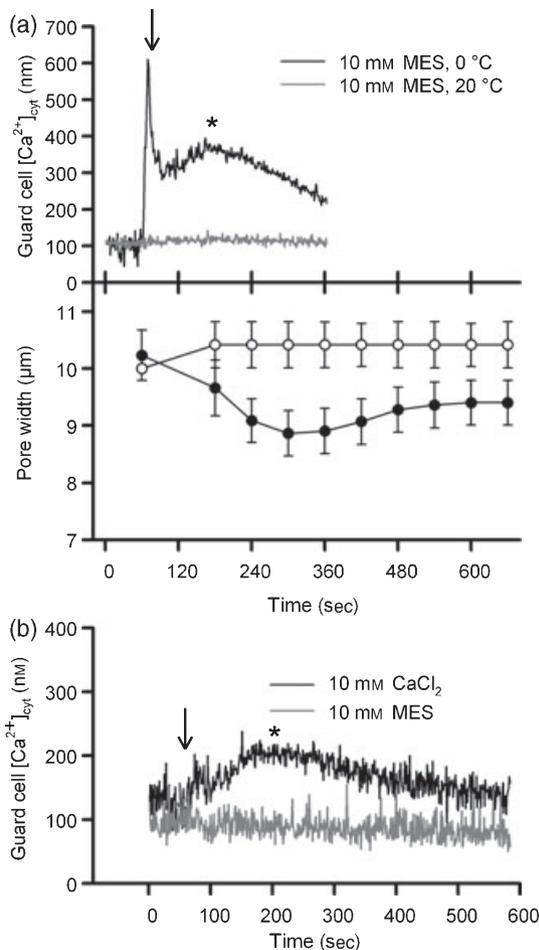
We confirmed that aequorin was targeted exclusively to the guard cell by examining the distribution of a yellow fluorescent protein (YFP)–apoequorin fusion in the E1728 enhancer trap line. Transformation of homozygous E1728 plants with pBINYFPAEQ (Kiegle *et al.*, 2000) resulted in guard cell-specific expression of the YFP–apoequorin fusion (Figure 3a,b). GFP fluorescence arising from the enhancer trap construct was localized to the endoplasmic reticulum due to ER targeting of the GFP construct (Haseloff *et al.*, 1997). In contrast, YFP fluorescence was



**Figure 3.** Localisation of YFPAPOAEQUORIN to the cytosol of guard cells in the E1728 enhancer trap line. (a,b) 3D projections of confocal scanning laser microscopy (CLSM) optical sections from (a) a single stoma; (b) leaf epidermis of the E1728 enhancer trap line transformed with *GAL4UAS:YFPAPOAEQUORIN*. Green, green fluorescent protein fluorescence (ER-targeted); red, yellow fluorescent protein fluorescence from the YFPAPOAEQUORIN fusion protein. Scale bars: (a) 10 µm, (b) 30 µm. (c) Total dischargeable aequorin luminescence from individual seedlings transformed with *GAL4UAS:APOAEQUORIN* (blue); *GAL4UAS:YFPAPOAEQUORIN* (green); untransformed E1728 (grey).

present in the cytosol and nucleus. YFP fluorescence was not present in any other parts of the plant (Figure 3b), therefore aequorin expression was consistent with the enhancer trap expression pattern, and localized exclusively to guard cells.

Lines with the highest total aequorin activity were selected from 18 lines transformed independently with *GAL4UAS:YFPAEQ*, and 26 independent lines of *GAL4UAS:AEQ*. In the  $T_3$  line transformed with each of these constructs that had the highest total aequorin activity, total aequorin was fivefold higher in the line expressing apoaequorin under the control of the GAL4 UAS than the line expressing the YFP–apoequorin fusion protein (Figure 3c). This suggests that higher levels of aequorin luminescence can be achieved in the absence of the YFP tag. A line lacking the YFP tag was therefore used for  $[\text{Ca}^{2+}]_{\text{cyt}}$  measurements, while a line with the YFP tag provided confirmation of guard cell targeting of aequorin. E1728 plants that were not transformed with pBIBUASAEQ or pBINYFPAEQ did not contain dischargeable aequorin activity or exhibit stimulus-increases in luminescence (data not shown).



**Figure 4.** Guard cell  $[Ca^{2+}]_{cyt}$  in individual seedlings. Guard cell  $[Ca^{2+}]_{cyt}$  in individual seedlings measured after (a) cold shock; (b) 10 mM  $[Ca^{2+}]_{ext}$ . Black traces are stimulated plants; grey traces control plants. (a) Incorporates cold-induced stomatal movements on a parallel time course in the lower panel; solid and open circles are stomatal pore widths of cold shocked and control epidermal strips, respectively; for stomatal measurements  $n = 60 \pm SE$ . Vertical arrows indicate time of stimulus; asterisks, occurrence of secondary  $[Ca^{2+}]_{cyt}$  peaks.

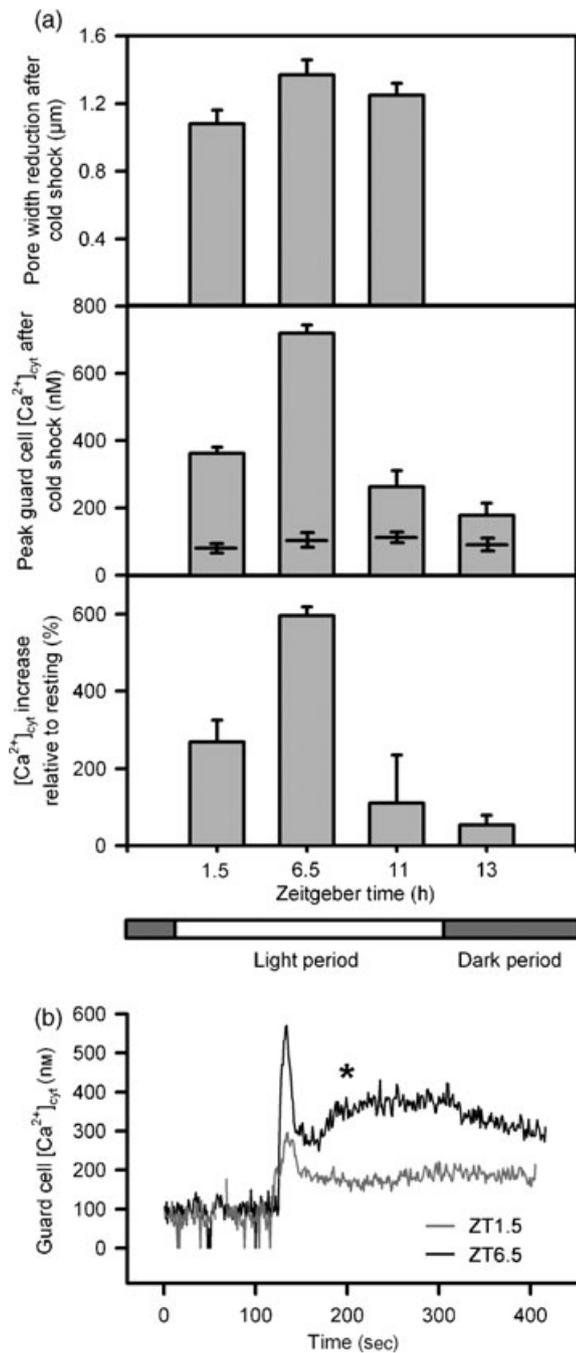
We verified that the guard cell  $[Ca^{2+}]_{cyt}$  measurements obtained using guard cell-targeted aequorin were consistent with previous studies of stimulus-induced changes in guard cell  $[Ca^{2+}]_{cyt}$ . Prior to stimulation, mean resting guard cell  $[Ca^{2+}]_{cyt}$  was  $128 \pm 9$  nM ( $n = 16$ ) measured using aequorin at ZT3. LT caused an immediate increase in guard cell  $[Ca^{2+}]_{cyt}$  that peaked between 300 and 1100 nM (mean  $486 \pm 73$  nM;  $n = 28$ ). After LT treatment,  $[Ca^{2+}]_{cyt}$  slowly decreased to resting concentrations, which always took more than 6 min and frequently incorporated a secondary  $[Ca^{2+}]_{cyt}$  peak (Figure 4a). Application of exogenous  $Ca^{2+}$  at 10 mM caused a slow increase in guard cell  $[Ca^{2+}]_{cyt}$  to between 200 and 295 nM after 2–3 min (mean  $249 \pm 14$  nM;  $n = 5$ ), after which guard cell  $[Ca^{2+}]_{cyt}$  decreased slowly to the resting concentration (Figure 4b).

#### Time of day modulates LT-induced guard cell $Ca^{2+}$ signals

To test whether there was diurnal variation in the magnitude of LT-induced guard cell  $[Ca^{2+}]_{cyt}$  increases and stomatal closure, we measured guard cell  $[Ca^{2+}]_{cyt}$  and LT-induced stomatal closure at ZT1.5, ZT6.5 and ZT11, under light–dark conditions (ZT, zeitgeber time or time from dawn, in h). Exposure of epidermal strips to 0°C for 1 min caused transient and partial stomatal closure (Figure 4a). The maximum degree of closure occurred 5 min after LT, and pores started to reopen 6–7 min after LT treatment (two-way ANOVA comparison identified significant aperture change following LT,  $P < 0.001$ ; and significant difference between LT and control samples;  $P = 0.004$ ). In the middle of the photoperiod (ZT6.5), LT promoted a numerically greater degree of stomatal closure ( $1.37 \pm 0.08$   $\mu m$ ) than LT at the beginning or end of the photoperiod (Figure 5a; ZT1.5,  $1.08 \pm 0.09$   $\mu m$ ). However, these diurnal variations were not statistically significant ( $P = 0.06$  in two-sample  $t$ -test). Stomata remained fully open in control epidermal fragments that were processed identically, but without LT shock (Figure 4a).

We tested whether the time of day modulates the LT-induced guard cell  $Ca^{2+}$  signal. Peak LT-induced guard cell  $[Ca^{2+}]_{cyt}$  was significantly higher in the middle of the photoperiod than at the start or end of the photoperiod, or during the dark period (Figure 5a; variation significant through time course;  $P < 0.02$ ,  $F = 4.32$ , two-way ANOVA). At ZT6.5, LT caused guard cell  $[Ca^{2+}]_{cyt}$  to peak at  $719 \pm 25$  nM, whereas at ZT1.5 h, peak LT-induced  $[Ca^{2+}]_{cyt}$  was  $362 \pm 18$  and  $263 \pm 48$  nM at ZT11. By 1 h after dusk, guard cell  $[Ca^{2+}]_{cyt}$  peaked at  $179 \pm 36$  nM after LT. The LT-induced  $[Ca^{2+}]_{cyt}$  increases differed significantly between every time point, with the lowest level of significance between ZT1.5 and ZT6.5 ( $P = 0.04$ , two-sample  $t$ -test). The occurrence of a secondary  $[Ca^{2+}]_{cyt}$  peak within the LT-induced  $Ca^{2+}$ -signature depended on the time of day (Figure 5b). Secondary  $Ca^{2+}$  peaks never occurred at ZT1.5 ( $n = 5$ ); 100% of the time at ZT6.5 ( $n = 5$ ); 40% of the time at ZT11 ( $n = 5$ ); and never at ZT13 ( $n = 5$ ). We did not detect overt diurnal variation in resting guard cell  $[Ca^{2+}]_{cyt}$  (ZT1.5,  $93 \pm 5$  nM; ZT6.5,  $107 \pm 9$  nM; ZT11;  $109 \pm 5$  nM; ZT13,  $119 \pm 10$  nM).

The diurnal pattern of LT-induced  $[Ca^{2+}]_{cyt}$  signals in the guard cell was not comparable with the effect of LT on stomatal closure. The diurnal variation in peak LT-induced  $[Ca^{2+}]_{cyt}$  increases was significant, whereas there was no significant diurnal variation in the magnitude of LT-induced stomatal closure. These data may indicate that diurnal alterations in LT-induced guard cell  $[Ca^{2+}]_{cyt}$  increases relate to aspects of cell physiology other than stomatal closure. We therefore examined the diurnal and circadian relationship between LT-induced  $[Ca^{2+}]_{cyt}$  increases and output genes of the LT-signalling pathway in whole plants.

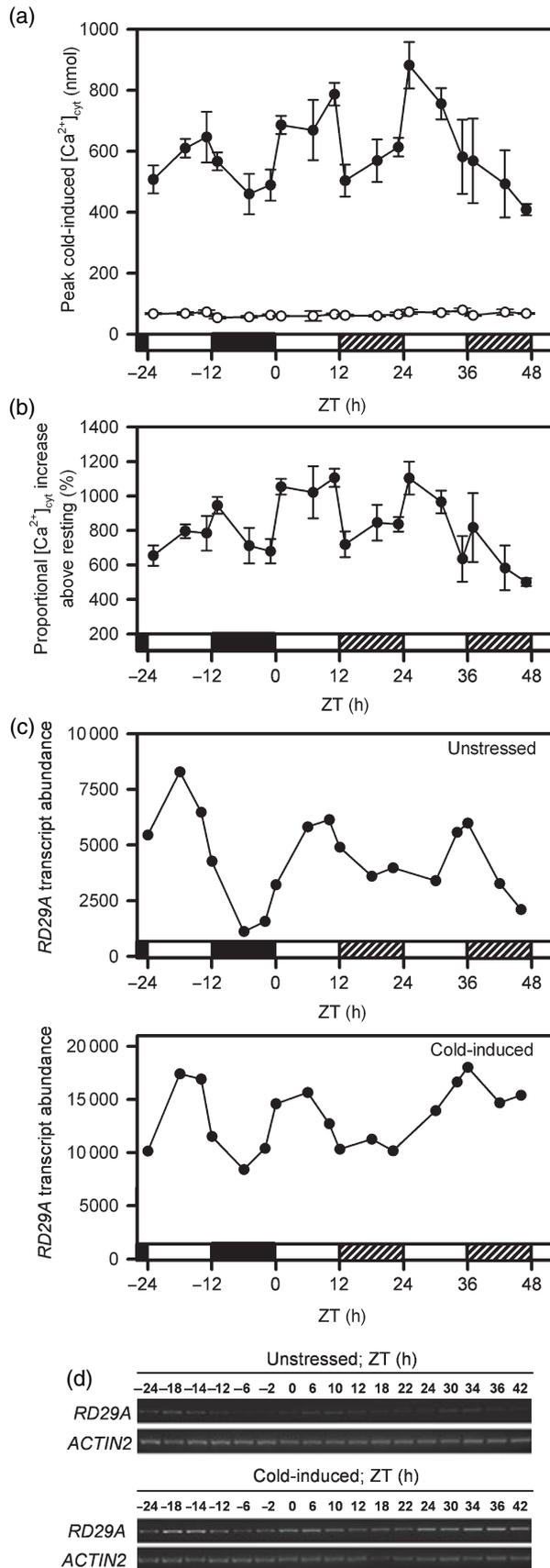


**Figure 5.** Diurnal rhythm in LT-induced  $[Ca^{2+}]_{cyt}$  increased in guard cells. (a) Magnitude of stomatal closure and peak guard cell  $[Ca^{2+}]_{cyt}$  following cold shock at given times from start of photoperiod and 1 h into the subsequent dark period. Bars superimposed on middle graph indicate resting  $[Ca^{2+}]_{cyt}$  prior to low-temperature (LT) treatment. The bottom panel of (a) is the proportional change in  $[Ca^{2+}]_{cyt}$  following LT treatment relative to resting  $[Ca^{2+}]_{cyt}$  at each time point. Shaded bar across bottom of (a) indicates relative times within light-dark period that measurements were obtained (grey areas, dark period; white area, light period). For stomatal aperture measurements,  $n = 60 \pm SE$ ; for  $[Ca^{2+}]_{cyt}$  measurements,  $n = 5 \pm SE$ . (b) Cold-induced  $[Ca^{2+}]_{cyt}$  at ZT1.5 (grey trace) and ZT6.5 (black trace). Primary and secondary  $[Ca^{2+}]_{cyt}$  peaks occurred when seedlings were cold-shocked in the middle of the photoperiod, but not when cold-shocked at the start of the photoperiod; \*, secondary peak.

### Circadian control of cold-induced $Ca^{2+}$ signals

We tested whether there was circadian modulation of (i) LT-induced  $[Ca^{2+}]_{cyt}$  signals in whole plants and guard cells; and (ii) LT induction of the  $[Ca^{2+}]_{cyt}$ -dependent, LT-responsive gene *RD29A*. In whole plants there was circadian and diurnal modulation of LT-induced  $[Ca^{2+}]_{cyt}$  increases, which persisted for 48 h of constant light (Figure 6a). During the first 24 h in constant light (ZT0 to ZT24), LT-induced  $[Ca^{2+}]_{cyt}$  increases during the subjective light period were significantly greater than in the first subjective dark period ( $P = 0.02$ , two-sample *t*-test). This could be indicative of diurnal, rather than true circadian control, caused by transfer to constant light. However, the repetition of this pattern during the second subjective day (ZT24 to ZT48), with significantly greater LT-induced  $[Ca^{2+}]_{cyt}$  increases during the second subjective light than dark period ( $P = 0.01$ , two-sample *t*-test), is indicative of circadian control. During the first and second subjective days, LT-induced  $[Ca^{2+}]_{cyt}$  increases ranged from 600 to 900 nM, whereas LT-induced  $[Ca^{2+}]_{cyt}$  increases during the subjective night were approx. 500 nM (variation statistically significant through time course;  $P < 0.001$ ,  $F = 371$  by two-way ANOVA; least significant difference 96 nM at 5% confidence level). This pattern was therefore consistent with the hypothesis that there is circadian modulation of LT-induced  $[Ca^{2+}]_{cyt}$  signals. Differences in the shape of the circadian waveform of LT-induced  $[Ca^{2+}]_{cyt}$  increases on the first and second subjective day in constant light may indicate that LT-induced  $[Ca^{2+}]_{cyt}$  increases on the first subjective day incorporate both diurnal and circadian information, whereas the temporal variation in LT-induced  $[Ca^{2+}]_{cyt}$  increases on the second subjective day can only be attributed to circadian control. In guard cells, we detected diurnal but not reproducible free-running circadian regulation of LT-induced  $[Ca^{2+}]_{cyt}$  increases (data not shown), and therefore did not investigate whether there was modulation of LT-induced stomatal closure over circadian timescales. We were unable to detect circadian variation in resting guard cell  $[Ca^{2+}]_{cyt}$ .

*RD29A* induction was examined after an LT treatment that comprised 20 min at  $0^{\circ}C$ , followed by 1 h post-treatment gene induction at  $19^{\circ}C$ . There were circadian and diurnal rhythms in the abundance of *RD29A* transcripts in both plants that were unstressed and in those that received LT (Figure 6c,d). In unstressed plants, maximum *RD29A* transcript abundance was during the subjective day, and *RD29A* abundance minima were during the subjective night. Following LT treatment, there were circadian rhythms in *RD29A* transcript abundance that followed a similar pattern to unstressed plants. The abundance and amplitude of circadian fluctuations in *RD29A* transcripts were substantially greater after LT treatment, so fewer PCR cycles were used to detect *RD29A* transcripts after LT. In both LT-treated and unstressed plants, the second circadian cycle was less



**Figure 6.** Low-temperature (LT)-induced increases in  $[Ca^{2+}]_{cyt}$  and  $RD29A$  induction are circadian regulated.

(a) In plants transformed with *35S:APOAEQUORIN*, peak LT-induced  $[Ca^{2+}]_{cyt}$  (solid symbols) and resting  $[Ca^{2+}]_{cyt}$  prior to stimulation (open symbols). Data area mean peak  $[Ca^{2+}]_{cyt} \pm SEM$ ,  $n = 10$ .

(b) Cold-induced increase in  $[Ca^{2+}]_{cyt}$  as a proportion of resting  $[Ca^{2+}]_{cyt}$ ; calculated from (a).

(c)  $RD29A$  transcript abundance following LT induction, and in control plants, across a diurnal and circadian time course.

(d)  $RD29A$  and  $ACTIN2$  transcript abundance across the entire time course. (a–c) Dark bands on x-axis represent dark period; shaded bands, subjective dark period in constant light. (c,d) RT–PCR bands and relative band intensities are arranged according to zeitgeber time (ZT) at which 20-min cold shock commenced. Negative ZT values indicate time (h) prior to constant light; and positive ZT values indicate time (h) since the start of constant light.

robust than the first. In summary, we detected circadian regulation of LT-induced  $[Ca^{2+}]_{cyt}$  increases, and of abundance in the transcripts of the LT-induced and  $[Ca^{2+}]_{cyt}$ -regulated gene  $RD29A$ .

## Discussion

### Diurnal and circadian regulation of signalling

$Ca^{2+}$  participates in signalling events that lead to development of chilling resistance in plants (Monroy *et al.*, 1993).  $RD29A$  induction is well correlated to non-freezing, LT-induced  $[Ca^{2+}]_{cyt}$  increases (Henriksson and Trewavas, 2003), and  $KIN1$ , which encodes an antifreeze-like protein, is  $Ca^{2+}$ -induced (Knight *et al.*, 1996; Kurkela and Borg-Franck, 1992). In the guard cell, cold shock causes  $Ca^{2+}$  oscillations that lead to transient stomatal closure (Allen *et al.*, 2000).

We provide evidence that stimulus-induced  $Ca^{2+}$  signalling events may be regulated by the circadian clock. The most likely explanation for the persistence of 24-h rhythms in the magnitude of LT-induced  $[Ca^{2+}]_{cyt}$  changes, during constant light, is that there was circadian regulation of LT-induced  $[Ca^{2+}]_{cyt}$  increases. Circadian modulation of LT-induced  $[Ca^{2+}]_{cyt}$  increases could exhibit similarities with time-dependent regulation of light signalling. Light signals derived from photoreceptors are modulated (gated) by the time of day, due to circadian control (Devlin, 2002). The genes *EARLY FLOWERING 3* (*ELF3*) and *TIME FOR COFFEE* (*TIC*) are clock-controlled components that feed back to gate light signals that entrain the clock (Hall *et al.*, 2003; McWatters *et al.*, 2000). This may occur by circadian control of photoreceptor gene promoter activity and transcript abundance (Tóth *et al.*, 2001). Circadian gating of light signals is important for optimal growth and development under normal light–dark cycles (Mockler *et al.*, 2003; Salter *et al.*, 2003).

There was circadian regulation of  $RD29A$  transcript abundance in LT-treated and control plants (Figure 6c). In unstressed plants,  $RD29A$  transcript abundance is under circadian control (Edwards *et al.*, 2006; Schaffer *et al.*, 2001;

M. J. Gardner and A.A.R. Webb., unpublished data), and circadian rhythms of *RD29A* transcripts are abolished by overexpression of the oscillator component CCA1 (S. Barak *et al.*, unpublished microarray study, <http://affymetrix.arabidopsis.info/narrays/experimentpage.pl?experimentid=308>). The circadian dynamics of *RD29A* mRNA that we observed (Figure 6c) were consistent with previously reported circadian patterns of *RD29A* abundance. In contrast to Fowler *et al.* (2005), we reproducibly detected circadian rhythms of *RD29A* transcripts in control seedlings, which may be due to the enhanced sensitivity conferred by RT-PCR over Northern analysis. As exposure to extended periods of LT reduces the amplitude of clock outputs and causes oscillator arrhythmia (Gould *et al.*, 2006; Ramos *et al.*, 2005), the weak circadian rhythm in LT-induced *RD29A* abundance reported by Fowler *et al.* (2005), compared with the relatively robust rhythm in our experiments, is likely to reflect circadian arrhythmia caused by the relatively long periods of LT exposure applied by Fowler *et al.* (2005) (up to 24 h), in comparison with the 20-min LT treatment that we used.

Over a circadian time course, we found that LT-induced *RD29A* transcript abundance was correlated with the magnitude of LT-induced  $[Ca^{2+}]_{cyt}$  increases. Importantly, this indicates that circadian modulation of LT-induced  $[Ca^{2+}]_{cyt}$  signals might cause circadian gating of LT-induced *RD29A* expression (Fowler *et al.*, 2005), as *RD29A* is regulated by LT-induced  $Ca^{2+}$ -increases (Henriksson and Trewavas, 2003). Several mechanisms could contribute to circadian modulation of  $Ca^{2+}$  signalling. Circadian expression of genes encoding  $Ca^{2+}$  channels and pumps may lead to varying cold-induced  $Ca^{2+}$  fluxes, depending on the time of day. For example, genes encoding  $Ca^{2+}$ -transporting ATPases, cyclic nucleotide gated ion channels, cation efflux proteins (Harmer *et al.*, 2000) and  $Ca^{2+}$  antiporters (Schaffer *et al.*, 2001) exhibit circadian transcription patterns.

There were differences between the patterns of LT-induced  $[Ca^{2+}]_{cyt}$  increases during the first and second 24 h of constant light (Figure 6a,b). This could suggest that there is cross-talk between the light-signalling and LT-response pathways, as  $[Ca^{2+}]_{cyt}$  participates in red light signalling and there is interaction between phytochrome signalling and LT signalling (Benedict *et al.*, 2006; Shacklock *et al.*, 1992). Alternatively, these variations could reflect damping of the circadian rhythm in LT-induced  $[Ca^{2+}]_{cyt}$  increases, which is comparable with the rapid damping of circadian variation in other acute environmental responses (e.g. light signalling; Millar and Kay, 1996).

#### Guard cell-targeted aequorin reports guard cell $[Ca^{2+}]_{cyt}$

Measurements of resting and stimulus-induced guard cell  $[Ca^{2+}]_{cyt}$  were comparable with previous measurements performed in intact plants and detached epidermis (Table 1).

**Table 1** Comparison of published resting and stimulus induced guard cell cytosolic-free calcium ( $[Ca^{2+}]_{cyt}$ ) in detached epidermal strips and intact plants

	$[Ca^{2+}]_{cyt}$ (nM)	
	Whole plant	Detached epidermis
Guard cell resting $[Ca^{2+}]_{cyt}$	128 ± 9 <sup>a</sup>	140 ± 20 <sup>c</sup>
Cold	100 <sup>b</sup>	80–150 <sup>e</sup> (Commelina)
1 mM extracellular-free $Ca^{2+}$ ( $[Ca^{2+}]_{ext}$ )	250–1000 <sup>a</sup>	260 <sup>d</sup>
10 mM $[Ca^{2+}]_{ext}$	–	160 <sup>d</sup>
		1000 <sup>e</sup>
	100 <sup>a</sup>	1020 <sup>d</sup>

Stimulus-induced  $[Ca^{2+}]_{cyt}$  values specified as concentration above resting, rather than absolute concentration. Superscript letters indicate source: <sup>a</sup>this study; <sup>b</sup>Levchenko *et al.* (2005); <sup>c</sup>Allen *et al.* (1999a); <sup>d</sup>Allen *et al.* (2000); <sup>e</sup>Webb *et al.* (2001).

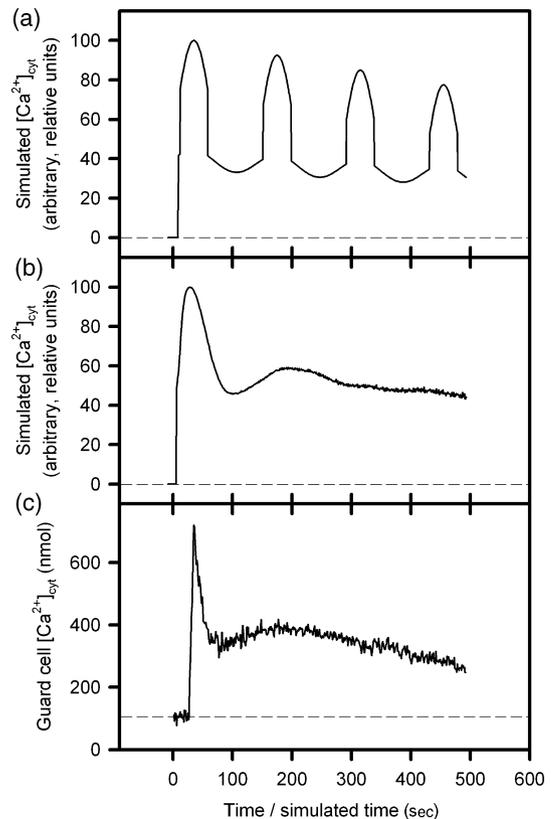
Guard cell resting  $[Ca^{2+}]_{cyt}$ , measured with guard cell-targeted aequorin (128 ± 9 nM), was similar to resting  $[Ca^{2+}]_{cyt}$  measured previously in detached epidermis in Arabidopsis (Allen *et al.*, 1999a, Fura-2-AM; Webb *et al.*, 2001; Fura-2), and *in planta* on *Vicia* (Levchenko *et al.*, 2005, Fura-2). The LT-induced increases in guard cell  $[Ca^{2+}]_{cyt}$  that we measured ranged from 250 to 1000 nM. These were similar to, and greater than, measurements in epidermal strips using yellowameleon 2.1 (YC2.1) (Allen *et al.*, 2000). However, we noticed differences between the extracellular-free  $Ca^{2+}$  ( $[Ca^{2+}]_{ext}$ )-induced  $[Ca^{2+}]_{cyt}$  signal that occurred in the guard cells of intact seedlings and detached epidermis (Table 1). In our experiments, 10 mM  $[Ca^{2+}]_{ext}$  caused  $[Ca^{2+}]_{cyt}$  to peak 100 nM above resting (Figure 4b), which is 10-fold lower than reported using epidermal strips (Allen *et al.*, 2000; Table 1). Application of  $[Ca^{2+}]_{ext}$  to epidermal strips causes rapid  $[Ca^{2+}]_{cyt}$  oscillations in guard cells (Allen *et al.*, 2000; McAinsh *et al.*, 1995; Webb *et al.*, 2001). At 1 mM  $[Ca^{2+}]_{ext}$  these Arabidopsis guard cell  $[Ca^{2+}]_{cyt}$  oscillations peak 160 nM (YC2.1; Allen *et al.*, 2000) or 1 μM ( $Ca^{2+}$  green-1 dextran and Fura-2; Webb *et al.*, 2001) above resting, and at 10 mM  $[Ca^{2+}]_{ext}$  guard cell  $[Ca^{2+}]_{cyt}$  oscillations peak at 1020 nM above resting (Allen *et al.*, 2000). In most cases, the  $[Ca^{2+}]_{ext}$ -induced  $[Ca^{2+}]_{cyt}$  increase is therefore greater in detached epidermis than intact seedlings. This could be because an intact and viable epidermis, or deeper cell layers, contribute to the accessibility of the  $[Ca^{2+}]_{ext}$  signal to the guard cell  $[Ca^{2+}]_{ext}$  sensor (Han *et al.*, 2003), or alternatively the epidermal cells contribute directly to modulation of  $[Ca^{2+}]_{ext}$ -induced  $[Ca^{2+}]_{cyt}$  increases. For example, the considerable  $Ca^{2+}$ -binding capacity of cell walls could produce a  $Ca^{2+}$  gradient between  $[Ca^{2+}]$  in the xylem or applied exogenously, and  $[Ca^{2+}]$  at the guard cell plasma membrane (de Silva *et al.*, 1996). Therefore, except for  $[Ca^{2+}]_{ext}$ -induced  $[Ca^{2+}]_{cyt}$  increases, the  $[Ca^{2+}]_{cyt}$  signals that occurred in intact seedlings were comparable with  $[Ca^{2+}]_{cyt}$  signals reported previously in detached epidermis.

We did not detect circadian oscillations of resting  $[Ca^{2+}]_{cyt}$  within the context of our experiments in guard cells or whole plants. Circadian  $[Ca^{2+}]_{cyt}$  oscillations have always been measured from seedlings growing on agar (Johnson *et al.*, 1995; Love *et al.*, 2004), whereas we transferred seedlings to luminometer tubes 24 h before cold shock, as this method allows quantification of  $[Ca^{2+}]_{cyt}$  alterations (Knight *et al.*, 1996). Possible explanations for the absence of circadian  $[Ca^{2+}]_{cyt}$  rhythms under our experimental conditions are that movement-induced  $[Ca^{2+}]_{cyt}$  signals (Haley *et al.*, 1995) might have masked or interfered with circadian  $[Ca^{2+}]_{cyt}$  oscillations, and/or circadian  $[Ca^{2+}]_{cyt}$  oscillations might not occur in guard cells. It has been hypothesized that circadian oscillations in resting  $[Ca^{2+}]_{cyt}$  might contribute to circadian rhythms of stomatal opening (Webb, 2003). However, the experimental approach used here was not designed, and indeed was not able, to detect circadian oscillations of resting  $[Ca^{2+}]_{cyt}$  in the guard cells or whole plants. Therefore it was not possible to test whether circadian  $[Ca^{2+}]_{cyt}$  oscillations regulate circadian rhythms of stomatal opening or *RD29A* transcript abundance.

#### $Ca^{2+}$ signatures within single cells and cell populations

The 'spike-shoulder' pattern of LT-induced increases in guard cell  $[Ca^{2+}]_{cyt}$  that we measured (Figure 5b) was typical of the  $[Ca^{2+}]_{cyt}$  increases that occur when aequorin is used to report  $[Ca^{2+}]_{cyt}$  from whole seedlings, roots or cell populations (Kiegle *et al.*, 2000). Our data suggest both the spike and the shoulder might encode information relating to time-of-day modulation of LT-induced  $[Ca^{2+}]_{cyt}$  signals. However, when cameleon was used to measure LT-induced increases in  $[Ca^{2+}]_{cyt}$  in single guard cells, repetitive  $[Ca^{2+}]_{cyt}$  oscillations occurred (Allen *et al.*, 2000). We have reconciled the difference in LT-induced guard cell  $[Ca^{2+}]_{cyt}$  dynamics that we measured from a guard cell population using aequorin, and those measured from a single guard cell with cameleon, using a computer simulation (Figure 7).

A possible explanation for the difference between single guard cell and guard cell-population  $[Ca^{2+}]_{cyt}$  dynamics is that the primary  $[Ca^{2+}]_{cyt}$  transient we measured from the guard cell population (Figures 4a and 5b) may represent the immediate synchronized increase in  $[Ca^{2+}]_{cyt}$  that occurs in all guard cells immediately after LT (Allen *et al.*, 2000). As stimulus-induced  $[Ca^{2+}]_{cyt}$  oscillations rapidly fall out of phase between guard cells, and there is variability in the period of  $[Ca^{2+}]_{cyt}$  oscillations, detection of synchronized  $[Ca^{2+}]_{cyt}$  oscillations from a large guard cell population is unlikely (Allen *et al.*, 1999b, 2000). The second sustained  $[Ca^{2+}]_{cyt}$  peak in the population guard cell  $Ca^{2+}$  signal may represent an amalgamation of asynchronous  $[Ca^{2+}]_{cyt}$  oscillations that occur in individual guard cells after the initial  $[Ca^{2+}]_{cyt}$  transient (Figure 5b, asterisk). Therefore summation of the LT-induced  $[Ca^{2+}]_{cyt}$  oscillations in single guard



**Figure 7.** Simulated and *in planta* cold-induced guard cell  $Ca^{2+}$  signatures. Simulated  $Ca^{2+}$  signatures with fundamental properties measured by Allen *et al.* (2000) from (a) one simulated cell; (b) 5000 simulated cells. (c) Representative cold-induced population guard cell  $Ca^{2+}$  signature, for comparison with simulated  $[Ca^{2+}]_{cyt}$ .

cells (Allen *et al.*, 2000) could yield a population  $Ca^{2+}$  signal defined by an initial  $Ca^{2+}$  peak followed by a secondary  $Ca^{2+}$  increase, assuming the initial  $Ca^{2+}$  transient of all guard cells was synchronized (Figure 4a). Cell population  $Ca^{2+}$  signals that exhibit a single sustained  $[Ca^{2+}]_{cyt}$  peak (Figure 4b) may arise when the initial stimulus-induced  $[Ca^{2+}]_{cyt}$  transients in individual guard cells are asynchronous.

To test the hypothesis that single-cell  $Ca^{2+}$  oscillations with variable periods give rise to the characteristic population  $Ca^{2+}$  signatures measured with aequorin, we used a computer simulation of idealized but representative single guard cell LT-induced  $Ca^{2+}$  oscillations (Figure 7a). The simulation was not designed to predict *in vivo*  $[Ca^{2+}]_{cyt}$ -based signalling events, but instead to compare  $Ca^{2+}$  signatures reported in single cells and populations using simplified, artificial oscillations.

Summation of 5000 simulated oscillations, with normally distributed period lengths, produced a simulated population  $Ca^{2+}$  signature that incorporated primary and secondary simulated  $Ca^{2+}$  peaks (Figure 7b), and had properties similar to LT-induced guard cell  $Ca^{2+}$  signatures measured from a population of guard cells using aequorin (Figure 7c). The

simulated guard cell  $\text{Ca}^{2+}$  oscillations are artificial and idealized, but the simulation demonstrates that the characteristics of population  $\text{Ca}^{2+}$  signatures have the potential to derive from populations of single-cell  $[\text{Ca}^{2+}]_{\text{cyt}}$  oscillations. When the number of simulated cells was reduced below 2000, the simulated population  $[\text{Ca}^{2+}]_{\text{cyt}}$  signature exhibited more random variation (noise, data not shown). Below 300 simulated cells, repetitive oscillations emerged in the population  $\text{Ca}^{2+}$  signal, which exhibited varying period lengths between successive oscillations (Figure S1 in Supplementary Material). Root cell type-specific expression of aequorin (Kiegle *et al.*, 2000) occurred in relatively small cell populations within individual seedlings, so overt  $[\text{Ca}^{2+}]_{\text{cyt}}$  oscillations in populations of specific root cell types may be explained by our simulation. Repetitive oscillations also occurred when large numbers of simulated cells (>2000) were combined with small standard deviations of period length (Figure S1). This explains why well defined circadian  $[\text{Ca}^{2+}]_{\text{cyt}}$  oscillations occur in large cell populations (Johnson *et al.*, 1995; Love *et al.*, 2004), because variability in circadian  $[\text{Ca}^{2+}]_{\text{cyt}}$  oscillation period length is relatively low (Love *et al.*, 2004).

Although the population  $\text{Ca}^{2+}$  signature may be diagnostic for underlying single-cell  $[\text{Ca}^{2+}]_{\text{cyt}}$  oscillations, it is unlikely to be representative of single-cell  $\text{Ca}^{2+}$  signatures. Alteration of the shape of simulated single-cell  $\text{Ca}^{2+}$  oscillations had very little effect on the shape of simulated population  $\text{Ca}^{2+}$  signatures (Figure S2).  $\text{Ca}^{2+}$  may therefore encode specific signals under circumstances of similar population  $\text{Ca}^{2+}$  signatures, because encoding of specific information occurs at the scale of  $[\text{Ca}^{2+}]_{\text{cyt}}$  oscillations within single cells, rather than cell populations. This could explain why dissimilar stimuli sometimes cause identical population  $[\text{Ca}^{2+}]_{\text{cyt}}$  signatures, despite leading to distinct cellular responses (Scrusefield and Knight, 2003), because measurement of  $[\text{Ca}^{2+}]_{\text{cyt}}$  signals at the cell-population level can obscure the encoding of stimulus-specific information at the single-cell scale.

Interpreting our guard cell population  $[\text{Ca}^{2+}]_{\text{cyt}}$  signatures, within the context of this simulation, suggests that there was time-of-day modulation of the acute LT-induced  $[\text{Ca}^{2+}]_{\text{cyt}}$  increase, and subsequent LT-induced  $[\text{Ca}^{2+}]_{\text{cyt}}$  oscillations. However, there was no significant relationship between these parameters and the degree of LT-induced stomatal closure. It is possible that LT-induced  $[\text{Ca}^{2+}]_{\text{cyt}}$  signals have multiple functions in the guard cell, one being stomatal closure, while other unidentified responses are graded to the time of day.

Our study highlights the value of both single-cell and whole-plant measurements. Single-cell measurements reveal mechanisms that potentially encode information within  $\text{Ca}^{2+}$  oscillations. In comparison, seedling-level measurements confirm that single-cell measurements are representative of signalling events that occur in whole plants, rather than artefacts arising from cell or tissue isolation.

We have demonstrated that, in whole plants,  $\text{Ca}^{2+}$ -based signalling events are modulated by the time of day, and that the circadian clock may gate  $\text{Ca}^{2+}$  signalling in response to cold. Our data suggest that population  $[\text{Ca}^{2+}]_{\text{cyt}}$  dynamics represent, but do not describe, the  $[\text{Ca}^{2+}]_{\text{cyt}}$  dynamics of individual cells in the population.

## Experimental procedures

### Plant material and growth conditions

*Arabidopsis thaliana* (Columbia-0 ecotype for all lines) was grown on 0.5 × Murashige and Skoog nutrient mixture dissolved in 0.8 % (w/v) agar, pH 6.8–KOH, 12 h light/12 h dark. Seeds were surface sterilized and grown at 19°C as described by Love *et al.* (2004), and harvested for experimentation 14 days after germination. Experiments were performed with plants remaining under conditions identical to those used for growth. For selection of the E1728 guard cell-specific enhancer trap line, fluorescence of GFP and YFP was imaged and recorded as described by Kiegle *et al.* (2000).

### Analysis of T-DNA copy number in E1728

One microgram of genomic DNA purified using the DNeasy Plant DNA extraction kit (Qiagen, Hilden, Germany) was digested with *Bgl*II and *Spe*I restriction endonucleases (NEB, Hitchin, UK). *Spe*I did not cut the T-DNA, enabling single- and multiple-copy T-DNA insertion loci to be distinguished. Genomic DNA was also digested with *Bst*XI, *Not*I, *Nru*I and *Stu*I (NEB). *Bst*XI, *Not*I and *Nru*I do not cut the T-DNA; *Stu*I cuts between the right border and *GAL4-VP16* sequence. The digests were separated by 0.8% (w/v) agarose gel electrophoresis, and transferred to Hybond-N nylon membrane (Amersham Pharmacia, Little Chalfont, UK) using standard Southern blotting techniques (Ausubel *et al.*, 1999).

A 411-bp DNA probe was synthesized with nested PCR from pET15 (Haseloff, 1999), using mPPR1-5 [5'-CGCAAGCTTGGATC CAACAATG-3'; forward PCR primer (FP)] and mPPR1-3 [5'-CCCGGAGCTCGTCCCCAGGCTG-3'; reverse PCR primer (RP)] primers, followed by 5'-GACATCTGCCGCTCAAG-3' (FP), and 5'-GCTCGAGACGGTCAACTG-3' (RP). 50 ng DNA was  $\alpha$ - $^{32}\text{P}$ -labelled using the Prime-It RmT random primer labelling kit (Stratagene, La Jolla, CA, USA), using 5  $\mu\text{l}$  [ $\alpha$ - $^{32}\text{P}$ ] dCTP (3000 Ci mmol<sup>-1</sup>). Membranes were probed (Ausubel *et al.*, 1999; Stratagene) and imaged using a Typhoon 8600 (Molecular Dynamics, La Jolla, CA, USA).

### Construction and selection of the UAS:APOAEQUORIN reporter

*APOAEQUORIN* was bordered with *Bgl*II and *Eco*R1 restriction sites by PCR amplification of the AEQ1 coding sequence (Prasher *et al.*, 1987) using 5'-GAAGATCTATCGTCCCAACGGCAACAGGC-3' (FP) and 5'-GGAATTCGTTTCTTAGGGGACAGCTCCACCG-3' (RP), and cloned into pGEM-T Easy (Promega, Southampton, UK). *APOAEQUORIN* was ligated downstream of the GAL4 upstream activation sequence (UAS) in pBIBUASKNAT1 (J.H., unpublished data) using *T*<sub>4</sub> DNA ligase following digestion of both *APOAEQUORIN* in pGEM-T Easy and pBIBUASKNAT1 with *Bgl*II and *Eco*R1. This replaced *KNAT1* with *APOAEQUORIN* to form pBIBUASAEQ. E1728 was transformed with pBINYPFAEQ (Kiegle *et al.*, 2000, *GAL4UAS:YFPAPOAEQUORIN*) or pBIBUASAEQ (*GAL4UAS:APOAEQUORIN*), and homozygous transformants selected using 40  $\mu\text{g ml}^{-1}$  hygromycin. *T*<sub>1</sub>, *T*<sub>2</sub> and *T*<sub>3</sub> lines were selected for those with maximum

aequorin activity by discharging all available aequorin activity in the presence of 1 M CaCl<sub>2</sub> dissolved in 10% (v/v) ethanol, and measuring aequorin bioluminescence.

#### Detached-leaf assay for stomatal function

Five leaves were removed from 4-week-old Col-0 and E1728 growing on soil in a glasshouse (20°C) at ZT0. Leaves were weighed, placed in a Sanyo MLR-350 growth cabinet maintained at 19°C (70 μmol photons m<sup>-2</sup> sec<sup>-1</sup>), and weighed at regular intervals over the subsequent 3 h.

#### Quantification of guard cell and whole plant [Ca<sup>2+</sup>]<sub>cyt</sub>

Guard cell [Ca<sup>2+</sup>]<sub>cyt</sub> was quantified using E1728 transformed with *GAL4UAS:APOAEQUORIN* in homozygous transformants of T<sub>3</sub> or T<sub>4</sub> as described (Kiegle *et al.*, 2000), while *GAL4UAS:YFPAEQUORIN* was used to confirm guard cell-specificity of aequorin, but not used in the LT studies. Single intact seedlings were incubated overnight in 1 ml of 10 μM coelenterazine free base (Prolume, Pinetop, AZ, USA) to reconstitute activate aequorin, within a luminometer tube. Coelenterazine was dissolved in MES buffer (10 mM 2-[*n*-morpholino]ethanesulphonic acid, pH 6.15–KOH). Whole-plant [Ca<sup>2+</sup>]<sub>cyt</sub> was measured using plants expressing *35S:APOAEQUORIN* (Johnson *et al.*, 1995), following aequorin reconstitution as described for lines with guard cell-targeted aequorin.

Seedlings were cold-shocked by injection of 500 μl MES buffer at 0°C onto the seedling, and aequorin luminescence was measured using a photon-counting luminometer (Electron Tubes, Ruislip, UK). Experiments were performed under green physiological safelight when stimuli were applied during the dark period. Growth-chamber temperature remained constant through the experiment (19°C), and chambers were switched to constant light at the onset of circadian time courses. External Ca<sup>2+</sup> was applied by injection of 1 ml 20 mM CaCl<sub>2</sub> to achieve [Ca<sup>2+</sup>]<sub>ext</sub> 10 mM CaCl<sub>2</sub>. [Ca<sup>2+</sup>]<sub>cyt</sub> was calculated from aequorin luminescence by total aequorin discharge (Fricker *et al.*, 1999).

#### Epidermal bioassay for LT-induced stomatal closure

LT-induced stomatal closure was measured using the E1728 enhancer trap line transformed with pBIBUASAEQ. Two 14-day-old seedlings were homogenized at room temperature in 20 ml MES buffer, using three 30-sec blending pulses (Waring Laboratories, Torrington, CT, USA), and the homogenate filtered through 100 μm nylon mesh (Northern Mesh & Technical Fabrics, Oldham, UK). Mesophyll cells destroyed by blending passed through the nylon mesh, whereas epidermal fragments containing live guard cells remained on the filter (Pei *et al.*, 1997). The residue was immediately mounted on a microscope slide, in MES. Stomatal apertures were measured on a monitor using calibrated acetate sheets, by coupling a digital camera to the microscope.

Three fully developed stomatal pores were chosen on a suitable epidermal fragment, and pore widths were measured. The slide was cold-shocked at 0°C for 1 min, then stomatal pore widths were measured at 1-min intervals for 10 min. Identical experiments were performed over 10 days to achieve sufficient replication (*n* = 60) for each time point.

#### Determination of RD29A induction by LT treatment

For each time point, four to six seedlings were removed from agar plates to luminometer tubes that contained 1-ml water at 19°C,

12 h prior to the LT shock. LT was administered by adding 2.5-ml water at 0°C (LT shock), or at 19°C (control), before placing tubes on ice (LT shock) or leaving at 19°C (controls). After 20 min, samples were moved to 19°C for 1 h to allow gene induction prior to snap-freezing in liquid N<sub>2</sub>. The initial LT-induced [Ca<sup>2+</sup>]<sub>cyt</sub> increase, which occurs within 5 min of LT treatment, is sufficient to promote *RD29A* induction (Henriksson and Trewavas, 2003). However, extended periods of cold increase the magnitude of *RD29A* induction. Therefore the 20-min exposure to 0°C increased the dynamic range of *RD29A* detection relative to shorter periods of LT exposure.

RNA was purified using the RNeasy Plant Mini-Kit (Qiagen) according to the manufacturer's instructions. SuperScriptIII RNase H<sup>-</sup> (Invitrogen, Paisley, UK) was used for reverse transcription using 400 ng of RNA as template and a poly (dT)<sub>15</sub> primer (Roche Diagnostics, Basle, Switzerland). *ACTIN2* and *RD29A* template was amplified using gene-specific primers (*ACTIN2* forward: 5'-CTCCAGCTATGTATGTTGCCATTC-3'; *ACTIN2* reverse: 5'-CATACTGTGCTTAGAGATCCAC-3'; *RD29A* forward: 5'-GAACCTGAAGTGATCGATGCA-3'; *RD29A* reverse: 5'-GGTACTCCTCCAGTTTCTTCT-3') and 30 cycles (*ACTIN2* and *RD29A* LT-induced samples) or 32 cycles (*RD29A* control samples) of PCR (HotStarTaq; Qiagen). A smaller number of PCR cycles were used to amplify *RD29A* from LT-treated plants, than from unstressed plants, because *RD29A* mRNAs were substantially more abundant in LT-treated plants than in controls. Therefore amplicon intensities are not compared directly between the two treatments (Figure 6c,d).

#### Simulation of LT-induced guard cell [Ca<sup>2+</sup>]<sub>cyt</sub> oscillations

Simple wave forms with properties comparable with LT-induced guard cell [Ca<sup>2+</sup>]<sub>cyt</sub> oscillations (Allen *et al.*, 2000) were simulated using a VISUAL BASIC FOR APPLICATIONS (VBA; Microsoft, Redmond, WA, USA) program (Program P1 in Supplementary Material). A sine function generated an oscillation of user-specified period (Equation 1, where *y*<sub>1</sub> is the oscillation, *t* is time from stimulus application and *p* is period length).

$$y_1 = 1 + \sin[(2\pi/p)t] \quad (1)$$

The oscillation *y*<sub>1</sub> was conditionally modified to an idealized shape that was comparable with LT-induced guard cell [Ca<sup>2+</sup>]<sub>cyt</sub> oscillations, which exhibit rapid increases to peak [Ca<sup>2+</sup>]<sub>cyt</sub> that decrease in amplitude with successive oscillations, and a baseline elevated above resting [Ca<sup>2+</sup>]<sub>cyt</sub> (Allen *et al.*, 2000). When *y*<sub>1</sub> ≥ 1.5, [Ca<sup>2+</sup>]<sub>cyt</sub> was simulated as being near to maximum values within the oscillation by Equation 2, where *k*<sub>d</sub> is a constant that simulates the decay in amplitude of successive [Ca<sup>2+</sup>]<sub>cyt</sub> oscillations in single guard cells (Allen *et al.*, 2000), and *y*<sub>2</sub> is the simulated [Ca<sup>2+</sup>]<sub>cyt</sub> for an individual guard cell.

$$y_2 = y_1 - \left( \frac{t}{p} \right) \cdot k_d \quad (2)$$

[Correction added after online publication 17 November 2006: Equation (2) corrected from  $y_2 = y_1 - \left( \frac{t}{p} \right) - k_d$ .]

When *y*<sub>1</sub> < 1.5, [Ca<sup>2+</sup>]<sub>cyt</sub> was simulated to be in a trough between oscillations, at a baseline elevated above resting [Ca<sup>2+</sup>]<sub>cyt</sub>. Equation 3 simulated these troughs, where *k*<sub>t</sub> is a constant that simulates the decrease in the elevated [Ca<sup>2+</sup>]<sub>cyt</sub> baseline that gradually occurs following LT stimulus (Allen *et al.*, 2000). The first term of the equation simulates the elevated baseline, and the second term simulates baseline decay over time.

$$y_2 = [(y_1/10) + 0.7] - [(t/p)k_t] \quad (3)$$

Simultaneous stimulus-induced  $[Ca^{2+}]_{\text{cyt}}$  oscillations in many cells were simulated by summing multiple iterations of the simulation. This was performed for a normal distribution of period lengths around the mean guard cell LT-induced  $[Ca^{2+}]_{\text{cyt}}$  oscillation period of  $154 \pm 11$  sec ( $\pm$ SE;  $n = 20$ ; SD = 49.2 sec; Allen *et al.*, 2000).  $k_d$  was set to 0.15, to simulate the approx. 15% decrease in amplitude of successive LT-induced  $[Ca^{2+}]_{\text{cyt}}$  oscillations that occurs in single guard cells (Allen *et al.*, 2000), and  $k_i$  was adjusted empirically to 0.05, to yield an elevated baseline that decayed in a manner similar to (Allen *et al.* 2000). The outputs were plotted as a proportion of maximum simulated  $[Ca^{2+}]_{\text{cyt}}$ .

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### Supplementary Material

The following supplementary material is available for this article online:

**Figure S1.** Simulated cell-population  $Ca^{2+}$  oscillations with fundamental properties measured by Allen *et al.* (2000): (a) in a relatively small population of 100 cells, (b) with relatively low standard deviation (variability) of oscillation periods between 5000 simulated cells.

**Figure S2.** Simulated single-cell  $Ca^{2+}$  oscillations with differing shapes (a–c) do not lead to simulated cell-population  $Ca^{2+}$  signatures with substantially different properties. Simulated  $Ca^{2+}$  oscillation shape was altered using variations in Program S1 and Equations 1–3.

**Program S1.** VBA code for simulation of stimulus-induced  $[Ca^{2+}]_{\text{cyt}}$  oscillations in varying numbers of guard cells.

This material is available as part of the online article from <http://www.blackwell-synergy.com>

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