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# Imaging spatial and cellular characteristics of low temperature calcium signature after cold acclimation in *Arabidopsis*

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## Abstract

**Cooling-induced 'calcium signatures' were imaged in aequorin-expressing *Arabidopsis* plants after cold acclimation or growth at ambient temperature. In all tissues, signatures were altered after acclimation. Characterization of the components generating this response indicates that cold acclimation increases cold-induced vacuolar $Ca^{2+}$ release, but does not affect the influx of extracellular calcium.**

Key words: Calcium signature, cold acclimation, aequorin, vacuole, *Arabidopsis*.

## Introduction

Plants growing in a variety of environments experience low temperatures, including those below freezing point, at some point during their life cycle. Survival of sub-zero temperatures is to a large extent determined by the ability to cold acclimate. Species capable of cold acclimation become endowed with a greater than normal tolerance of freezing temperatures after undergoing previous exposure to low positive temperatures (Thomashow, 1994; Levitt, 1980). During cold acclimation, a number of changes occur including the expression of many genes, alterations in membrane lipid composition and accumulation of sugars, proline, soluble proteins, and organic acids (Hughes and Dunn, 1996; Thomashow, 1994).

One of the earliest events in a plant's response to low temperature is a transient elevation of the free cytosolic concentration of the intracellular second messenger calcium ($Ca^{2+}$). Such elevations have been demonstrated in *Arabidopsis* (Lewis *et al*., 1997; Knight *et al*., 1996; Polisensky and Braam, 1996), as well as in other species, including tobacco (Knight *et al*., 1991) and tomato (Sebastiani *et al*., 1999) and appear to be universal amongst higher plants. Elevations of $[Ca^{2+}]_{cyt}$ have also been detected in the moss *Physcomitrella* in response to cold (Russell *et al*., 1996). This elevation in cytosolic free $Ca^{2+}$ levels ($[Ca^{2+}]_{cyt}$) is due mainly to an influx of $Ca^{2+}$ from external sources (Monroy and Dhindsa, 1995; Knight *et al*., 1996), but there is also evidence for $IP_3$-mediated $Ca^{2+}$ release from the vacuole (Knight *et al*., 1996; De Nisi and Zocchi, 1996). As $[Ca^{2+}]_{cyt}$ increases occur not only in response to rapid cold shock (H Knight *et al*., 1996; MR Knight *et al*., 1991), but also to slower gradual reductions in temperature (Plieth *et al*., 1999), it is possible that such $[Ca^{2+}]_{cyt}$ changes occur as the temperature reduces during the acclimatory period. Indeed, evidence has emerged to support the role of $Ca^{2+}$ during cold acclimation to freezing temperatures (Tähtiharju *et al*., 1997; Monroy *et al*., 1993; Monroy and Dhindsa, 1995) and there are many reports of $Ca^{2+}$-regulation of processes occurring at low temperatures. These include the regulation of cold-inducible gene expression (Berberich and Kusano, 1997; Knight *et al*., 1996; Monroy *et al*., 1993; Polisensky and Braam, 1996; Tähtiharju *et al*., 1997).

There is evidence, therefore, of a requirement for $Ca^{2+}$ to mediate the cellular changes associated with cold acclimation. In this study, interest has been in the question of whether, having undergone cold acclimation, plants' $Ca^{2+}$ signalling in response to environmental stimuli is changed. Previous studies show that the ability to respond to mannitol treatment (simulated drought stress) with a $[Ca^{2+}]_{cyt}$ elevation is altered markedly after prior stress treatment (Knight *et al*., 1998). In the case of cold pretreatment of seedlings, data indicating that a short period of cold pretreatment can subtly alter the timing

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and magnitude of the response, or 'calcium signature', to cold shock has previously been shown.

The effect of cold acclimation treatment on the spatio-temporal features of subsequent cooling-induced calcium signatures is investigated here, in order to gain an insight into the possible signalling status of mature plants after prolonged acclimation. Preliminary data are presented from experiments with the $Ca^{2+}$ channel blocker lanthanum and measurements of $[Ca^{2+}]_{cyt}$ around the vacuolar membrane, aimed at determining a possible mechanism for this phenomenon.

## Materials and methods

*Arabidopsis* plants (ecotype RLD1) expressing a 35S-aequorin construct (Knight *et al*., 1991) were grown under a 16 h photoperiod at 20 °C in Petri dishes containing full strength Murashige and Skoog medium and 0.8% agar as described previously (Knight *et al*., 1996). Reconstitution of aequorin was performed *in vivo* essentially as described previously (Knight *et al*., 1991) by floating seedlings on water containing 2.5 μM coelenterazine in the dark, overnight at 20 °C.

For the cold acclimation experiments, 3-week-old plants growing on MS-agar plates were placed in a growth chamber for 3 d at 2 °C under normal light conditions (acclimated plants) or maintained for the 3 d period at 20 °C (non-acclimated control plants). Plates on which seedlings were grown were taken from the growth cabinet and placed on the bench for up to 1 h before being transferred to coelenterazine. After this time, plants were removed from Petri dishes and floated on water containing coelenterazine at ambient temperature overnight. Cooling-induced $[Ca^{2+}]_{cyt}$ elevations were measured on the following day. For the comparison of vacuolar microdomain $Ca^{2+}$ responses with overall cytosolic $Ca^{2+}$ responses, 7-d-old *Arabidopsis* plants expressing the HVA1 tonoplast membrane-targeted aequorin (Knight *et al*., 1996) were used alongside those expressing 35S-aequorin cytosolic aequorin (described above). In experiments comparing responses of plants expressing targeted and non-targeted aequorin, reconstitution was carried out with 10 μM coelenterazine in order to maximize the number of luminescence counts emitted. Lanthanum pretreatment was performed as described previously (Knight *et al*., 1997). Plants were floated in a solution of freshly made 10 mM lanthanum (III) chloride or water (control) for 30 min prior to measurement.

Aequorin imaging was performed using an intensified CCD camera (Campbell *et al*., 1996) (model EDC-02), with camera control unit (HRPCS-2) and image acquisition and processing software (IFS216), all from Photek (St Leonards-on-Sea, UK) as described previously (Knight *et al*., 1999). Plants were cooled from 20 °C to 0 °C using a Peltier cooling element (part of the camera assembly). Estimate of the total amount of reconstituted aequorin present in imaged seedlings was made by discharging aequorin from representative seedlings in a luminometer cuvette with 1 M calcium chloride and 10% ethanol as described previously (Knight *et al*., 1996). This was performed using a digital chemiluminometer consisting of a 9829A photomultiplier tube with a 1.5 kV potential from a FACT50 air-cooled thermoelectric housing and an AD2 amplifier/discriminator (all from Thorn EMI, Ruislip, UK) to produce a numerical output which was stored on a personal computer. Calculation of rate constants of luminescence was performed using this information as described previously (Knight *et al*., 1996). Statistical significance of differences in rate constants or peak ratios in each experiment was assessed by performing a standard Student *t*-test analysis assuming unequal variances (significance taken as $P < 0.05$).

## Results

In order to examine changes in calcium signature occurring after cold acclimation, 3-week-old plants were placed in growth cabinets under normal lighting conditions (see methods) at either 2 °C or 20 °C for 3 d. After this treatment, plants were removed from Petri dishes and reconstituted for 16 h overnight as described previously, (Knight *et al*., 1997, 1996) prior to making luminescence measurements. On the fourth day the plants were subjected to gradual cooling over a 6 min period, using a Peltier cooling element and $Ca^{2+}$-dependent luminescence responses were compared with those of non-acclimated plants using a sensitive photon-counting camera (Knight *et al*., 1999). Continuous cooling from 20 °C to 0 °C on the Peltier element consistently elicited a bimodal response consisting of two distinct phases (Fig. 1e) (Knight, 2000). It is important to note that this phenomenon is quite distinct from the two peaks described recently that are elicited by two temporally isolated cooling steps (Knight *et al*., 1999).

Figures 1a and b show a pseudocolour image of luminescence emitted from a cold-acclimated plant (RHS) and a non-acclimated plant (LHS) during the first and second phases, respectively, of the bimodal response. The periods over which luminescence was integrated to produce these images are represented in Fig. 1e and f and the temperature of the Peltier element at these times is shown in Fig. 1g by grey overlay bars. Figure 1c is a bright-field image showing the relative positions of the control and acclimated plants in the pseudocolour images. Figure 1d shows a pseudocolour image depicting spatially the ratio between the amounts of luminescence from the second integration relative to the first (i.e. a ratio of Fig. 1b : Fig. 1a luminescence). The ratio value for the non-acclimated plant is uniform throughout different tissues (Fig. 1d), showing that the bimodal response to cooling occurs throughout the plant equally. This ratio is clearly higher in the case of the acclimated plant, showing that the second phase of $Ca^{2+}$-induced luminescence was relatively larger in the acclimated plant. All parts of the plants displayed this property (Fig. 1d). The data from Fig. 1a and b are shown graphically in Fig. 1e and f, in which the bimodal responses to cooling are shown for the non acclimated control plant (Fig. 1e) and the acclimated plant (Fig. 1f), respectively.

Figure 1 shows data from two individual plants and is representative of all those observed. Figure 2a shows a comparison of the ratios of the two peaks of

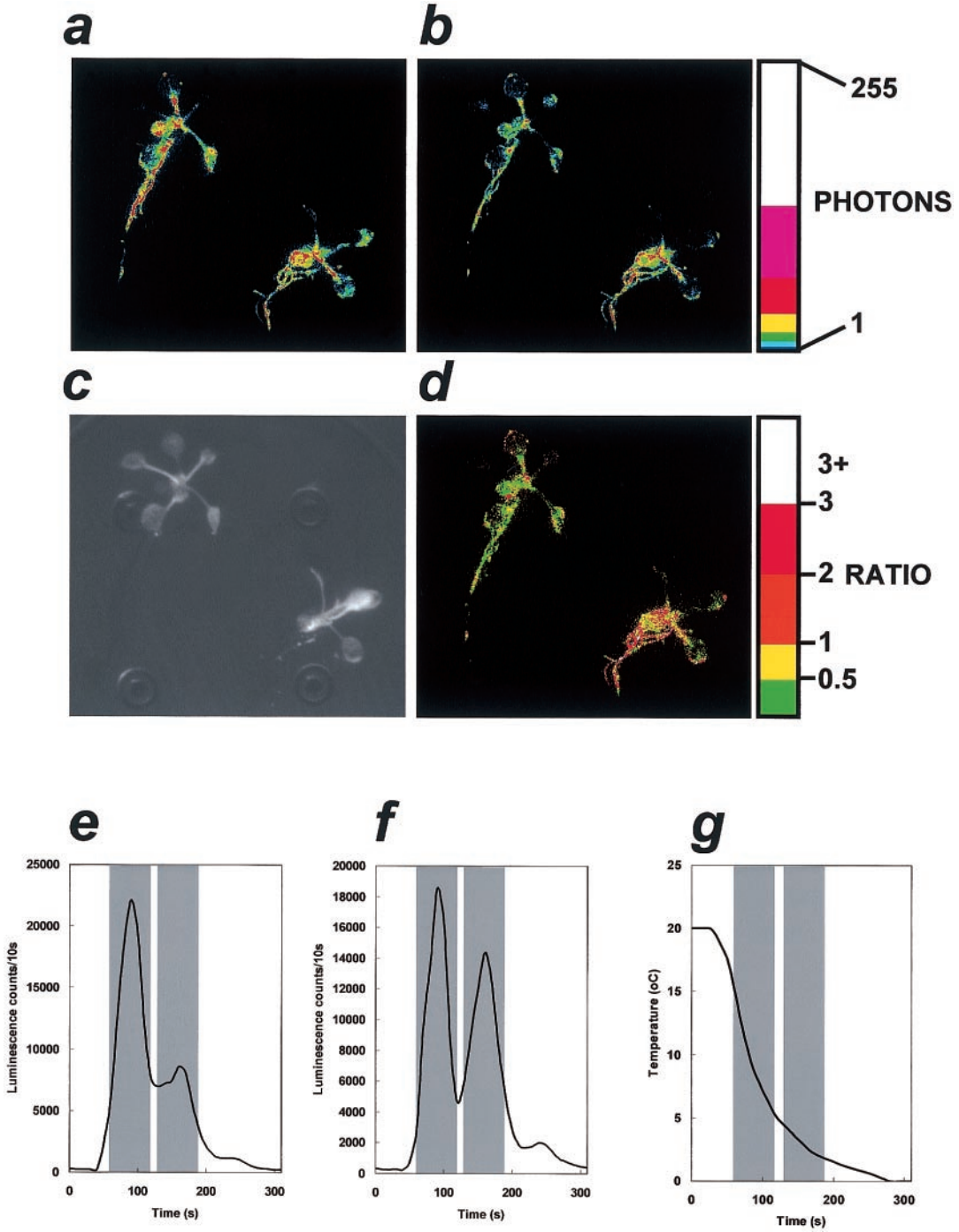


**Fig. 1.** Imaging of cytosolic free calcium responses to low temperature in cold acclimated and non-acclimated *Arabidopsis* plants. (a) Pseudocolour luminescence image of calcium-dependent photons emitted by aequorin in single cold acclimated (right) and control non-acclimated (left) plants accumulated over a 60 s integration period during which the seedlings were cooled from approximately 17 °C to 6 °C. (b) Pseudocolour luminescence image of calcium-dependent photons emitted by aequorin in single cold acclimated (right) and control (left) plants accumulated over a 60 s integration period during which the seedlings were cooled from approximately 5 °C to 2 °C. A colour scale indicating number of photons, represented in (a) and (b), is on right of this image. (c) Bright-field image of single cold acclimated (right) and control (left) plants to indicate position in luminescence images (a, b, d). (d) Pseudocolour luminescence image of ratio between calcium-dependent photons emitted by aequorin in single cold acclimated (right) and control (left) plants accumulated over the two 60 s integrations shown in (a) and (b). Colour scale, in this case indicating the ratio of photons obtained in (b) : (a) (second peak : first peak of luminescence), is on the right of this image. (e) Graph plotting photon counts per 10 s, generated by control plant shown in (a), (b), (c), and (d), against time. Grey bars indicate the integration period over which photons were accumulated to produce the images shown in (a) and (b). (f) Graph plotting photon counts per 10 s, generated by cold acclimated plant shown in (a), (b), (c), and (d), against time. Grey bars indicate the integration period over which photons were accumulated to produce the images shown in (a) and (b). (g) Graph plotting the temperature applied against time for the experiment shown in this figure. Again grey bars indicate the integration period over which photons were accumulated to produce the images shown in (a) and (b).

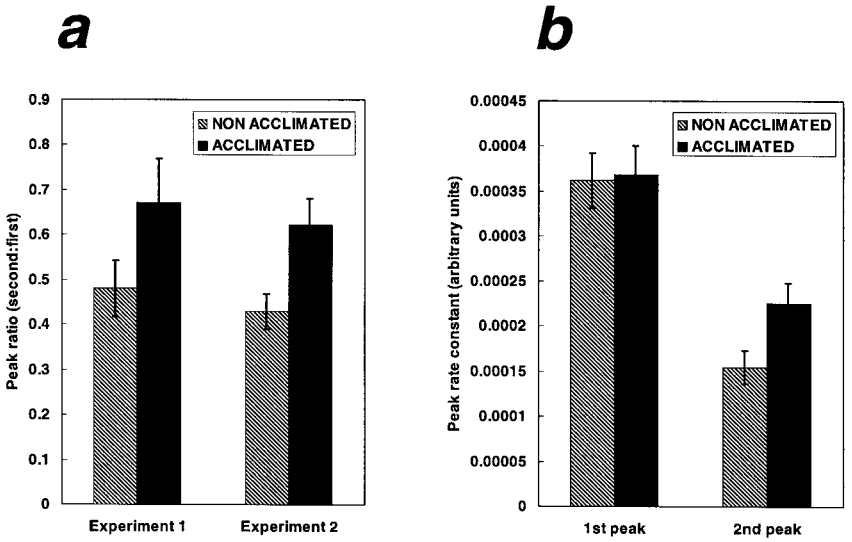


**Fig. 2.** Comparison of the two phases of cytosolic free calcium response to drops in temperature in cold acclimated and non-acclimated *Arabidopsis* plants. (a) Bar chart indicating ratios of peaks of luminescence obtained in response to cooling in cold acclimated and control non-acclimated plants. Ratios presented represent peak 2 : peak 1 values obtained when cooling from 20 °C to 0 °C (as shown in Fig. 1g). Results from two separate experiments are shown (In experiment 1 $n=7$ and $n=5$ for cold acclimated and control plants, respectively; in experiment 2 $n=14$ for both cold acclimated and control plants). (b) Bar chart showing peak rate constants (proportional to $[Ca^{2+}]_{cyt}$) for cold acclimated and control plants responding to cooling from 20 °C to 0 °C (as shown in Fig. 1g). Rate constants are shown for both first and second peak obtained in the biphasic $[Ca^{2+}]_{cyt}$ response to cooling ($n=14$ for both cold acclimated and control plants). Error bars represent ± SEM.

luminescence obtained from full datasets for two separate experiments. The data from these two experiments show that the peak ratio is significantly higher in cold-acclimated plants ($n=7$ and $n=14$) than wild type ($n=5$ and $n=14$). To ascertain whether this change in peak ratio was due to an increase in the size of the second peak or a decrease in the size of the first, rate constants for luminescence were calculated, using the values for total number of counts per plant obtained from luminometry of aequorin discharge (Knight *et al.*, 1996). The rate constant equals the rate of luminescence divided by maximum possible rate, and it is directly proportional to the calcium concentration reported by aequorin, and independent of the amount of reconstituted aequorin (Campbell, 1983). There was no significant difference ($P=0.87$) between the average rate constant for the first peaks (and hence cold-induced $[Ca^{2+}]_{cyt}$ elevation) of non-acclimated control and cold-acclimated plants ($n=14$ in both cases; Fig. 2b). However, the average rate constant (and hence cold-induced $[Ca^{2+}]_{cyt}$) for the second peak was significantly greater ($P=0.02$) in acclimated than non-acclimated plants ($n=14$ in both cases; Fig. 2b).

These data revealed therefore that in plants that have been acclimated, the bimodal $Ca^{2+}$ response to cooling is characterized by a larger second peak than is seen in non-acclimated plants (Figs 1, 2). To investigate the possible mechanism by which this altered bimodal kinetic was generated, the likely origins of the two peaks were considered, and the possibility that they correspond to different subcellular stores of $Ca^{2+}$. The contribution to the cold response of $Ca^{2+}$ influx from the external medium (Knight *et al.*, 1996) and release from internal sources (Knight *et al.*, 1996) were examined. Plants were treated with 10 mM lanthanum chloride 30 min prior to cooling, in order to block, predominantly, plasma membrane $Ca^{2+}$ channels. It has been shown previously that this treatment causes a marked lowering of the $[Ca^{2+}]_{cyt}$ response to cold shock (Knight *et al.*, 1996) and it has been demonstrated that lanthanum causes a large reduction in cold-induced $^{45}Ca^{2+}$ uptake in alfalfa cells (Monroy and Dhindsa, 1995). Plants cooled after this treatment showed a significant reduction ($P=0.0004$) in the peak 1 : peak 2 ratio ($n=12$) when compared with their control water-treated counterparts ($n=13$) (Fig. 3a). Figure 3b shows the bimodal cooling response of plants that were not pretreated with lanthanum and Fig. 3c the altered response seen in lanthanum-treated plants, exhibiting a relatively lower first peak. The fact that the first of the two cooling-induced peaks was more sensitive to lanthanum inhibition than was the second peak, suggested that the first peak includes a significant contribution from extracellular $Ca^{2+}$ sources and that a significant proportion of the second peak might be attributable to release of $Ca^{2+}$ from an internal, source.

Using plants expressing aequorin targeted to the cytosolic face of the vacuole (the 'vacuolar microdomain' (Knight *et al.*, 1996)) the question of whether the second of the two cooling-induced $Ca^{2+}$ peaks corresponds to $Ca^{2+}$ released from the vacuole was addressed. Previously it has been shown that rapid cold shock-induced $[Ca^{2+}]_{cyt}$ elevations in the vacuolar microdomain were

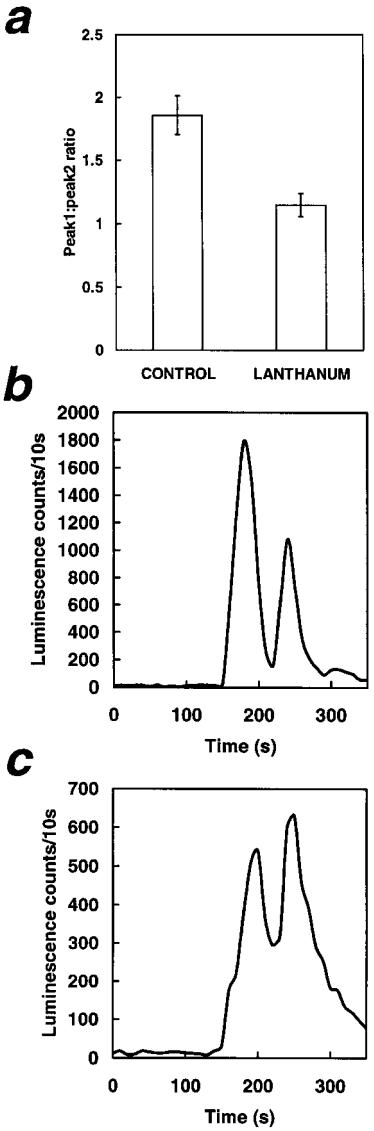


**Fig. 3.** Effect of lanthanum pretreatment on the relative magnitudes of the two cooling-induced $Ca^{2+}$-dependent luminescence peaks. (a) Ratio of peak luminescence counts from peak 1 : peak 2 in response to continuous cooling from 20 °C to 0 °C, in plants pretreated for 30 min in water (Control) or 10 mM $LaCl_3$ (Lanthanum). Error bars show ± SEM (control $n=13$, lanthanum $n=12$). (b) Typical luminescence response of a control plant (pretreated for 30 minutes in water) subjected to continuous cooling from 20 °C to 0 °C. (c) Typical luminescence response of a plant pretreated for 30 min in 10 mM $LaCl_3$, subjected to continuous cooling from 20 °C to 0 °C.

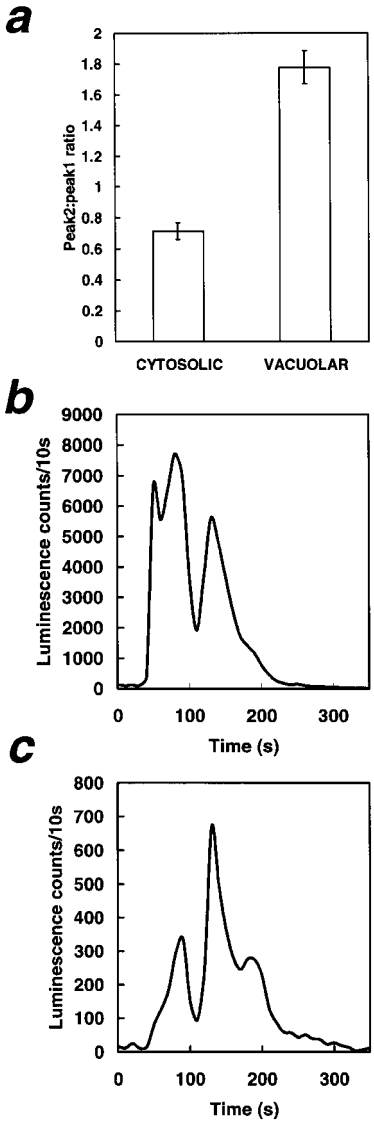


**Fig. 4.** Comparison of the relative magnitudes of the two cooling-induced $Ca^{2+}$-dependent luminescence peaks measured throughout the cytosol or in the 'vacuolar microdomain'. (a) Ratio of peak luminescence counts of peak 2 : peak 1 measured in response to continuous cooling from 20 °C to 0 °C in plants expressing aequorin throughout the cytosol (Cytosolic) or in the 'vacuolar microdomain' (Vacuolar). Error bars show ± SEM (cytosolic $n=15$, vacuolar $n=16$). (b) Typical luminescence response of a cytosolic plant subjected to continuous cooling from 20 °C to 0 °C. (c) Typical luminescence response of a vacuolar microdomain plant subjected to continuous cooling from 20 °C to 0 °C.

prolonged in comparison with those occurring throughout the cytosol, suggesting that vacuolar $Ca^{2+}$ release occurs during cold stimulation and contributes to the global elevation of $[Ca^{2+}]_{cyt}$ in response to cold (Knight *et al.*, 1996). When considering the data from the present study, therefore, it seemed possible in response to a slower rate of cooling, that the initial influx of $Ca^{2+}$ across the plasma membrane would be seen as a first $Ca^{2+}$ peak, temporally separated from a second peak, representing a subsequent release of $Ca^{2+}$ from the vacuole.

The $Ca^{2+}$ responses of plants expressing aequorin either throughout the cytosol (using 'cytosolic plants') or in the area of the cytosol adjacent to the vacuolar membrane (in 'vacuolar microdomain' plants) were imaged (Fig. 4). The ratio of peak 2 : peak 1 magnitude was calculated for microdomain ($n=16$) and cytosolic plants ($n=15$; Fig. 4a). This qualitative comparison shows that the proportion of the whole $Ca^{2+}$ response that makes up the second peak, is relatively and significantly higher ($P < 0.0001$) in the vacuolar microdomain

plants, suggesting that the vacuolar release is at least contributing to the second peak, and possibly represents the whole of the second peak (Fig. 4a). Figure 4b shows the bimodal cooling response from a cytosolic plant and Fig. 4c the response of a vacuolar microdomain plant, with a relatively larger second peak.

## Discussion

It has been demonstrated previously that rapid cold-shock immediately elevates $[Ca^{2+}]_{cyt}$ in *Arabidopsis* and tobacco (H Knight *et al*., 1996; MR Knight *et al*., 1991). A slower cooling of *Arabidopsis* from 20 °C to 0 °C was made here using a water-cooled Peltier element, which elicits a bimodal $Ca^{2+}$ response in mature *Arabidopsis* plants (Fig. 1; (Knight, 2000)). Under the cooling regime used (Fig. 1g) the first peak was generated very soon after cooling was started (between 19 °C and 18 °C) and the second when the temperature reached approximately 6 °C (Fig. 1). This observation is distinct from previous measurements showing that two successive, temporally separated cooling events cause two $Ca^{2+}$ response peaks (Knight *et al*., 1999). Biphasic $[Ca^{2+}]_{cyt}$ elevations of this type have been observed before, for instance in response to ozone (Clayton *et al*., 1999), hypo-osmotic stress (Cessna *et al*., 1998) and anoxia (Sedbrook *et al*., 1996) and may be attributable to an elevation of $[Ca^{2+}]_{cyt}$ occurring either in more than one tissue or cell type, or originating from more than one different subcellular store. In the case of the response to cooling, the bimodal kinetic is seen within each organ type and is not likely to be due to particular cell types signalling differently (Fig. 1d). It seems possible, therefore, that the bimodal response occurs, in this case, due to the use of more than one subcellular store of $Ca^{2+}$.

It has also been shown previously that young *Arabidopsis* seedlings exhibited an alteration in their cold shock-induced $Ca^{2+}$ signature after a brief pretreatment with low temperatures (3 h in the cold and dark on three consecutive days prior to the experiment) (Knight *et al*., 1996). Cold-pretreated plants exhibited a lower but more prolonged peak $[Ca^{2+}]_{cyt}$ value than those seen in untreated seedlings. In the present study, natural cold acclimation conditions were chosen as far as possible, providing normal light conditions during the day cycle and providing these conditions for 3 d at 2 °C constantly. Mature 3-week-old plants were used in the present study as they provide a more realistic model for cold acclimation. After the acclimation period, plants were challenged with gradual cooling rather than rapid cold shock, allowing clear temporal resolution of the kinetics of the $Ca^{2+}$ response.

Imaging of the cooling response in plants that had, and had not, undergone the acclimation treatment revealed a higher ratio of peak 2 : peak 1 heights after acclimation, indicating a change in the nature of the $[Ca^{2+}]_{cyt}$ elevation after acclimation (Fig. 1). Although it is not possible to calibrate luminescence images in terms of absolute $[Ca^{2+}]_{cyt}$ concentrations, calculation of luminescence rate constants (directly proportional to $[Ca^{2+}]_{cyt}$ concentration) (Fig. 2b) shows that the higher peak ratio seen in acclimated plants (Fig. 2a) is due specifically to an increased $[Ca^{2+}]_{cyt}$ response in the second peak. These data show that this phenomenon is ubiquitous to all tissues. This is shown by ratio imaging the two peaks (Fig. 1d). Although it is a formal possibility that heating plants from 2 °C, prior to reconstitution in coelenterazine to 20 °C may have caused the changes in the calcium responses seen after acclimation, this seems unlikely as in previous studies (Plieth *et al*., 1999) it was found that calcium responses to cooling, after heating from 4 °C to 20 °C were never greater, and in fact both the first and second peaks were actually reduced due to attenuation. Thus it seems that the enhanced second peak of calcium response seen here is specific for cold acclimation.

As both $[Ca^{2+}]_{cyt}$ peaks seem to occur in the same proportions in all organs of the plants (Fig. 1), the possibility was discounted that the two peaks represent $[Ca^{2+}]_{cyt}$ elevations occurring in different tissues. It appeared possible that the biphasic response may be a result of $Ca^{2+}$ entering the cytosol from external and internal stores (Knight *et al*., 1996). The possible sources of $Ca^{2+}$ for the two cold-induced peaks have been considered individually and the relative contributions of $Ca^{2+}$ influx and vacuolar $Ca^{2+}$ release to the generation of the two peaks in plants that have undergone a period of cold acclimation have been examined.

Plants were pretreated with the plasma membrane channel blocker lanthanum (Knight *et al*., 1996), and after 30 min their cold-induced $[Ca^{2+}]_{cyt}$ responses imaged and compared with those of water-pretreated plants. This treatment is known to reduce significantly the magnitude of the $[Ca^{2+}]_{cyt}$ response to cold shock (Knight *et al*., 1996). Moreover, it has been shown that lanthanum specifically inhibits uptake of extracellular $^{45}Ca^{2+}$ in response to low temperature treatment (Monroy and Dhindsa, 1995). Therefore, the reduction in the size of the peak 1 : peak 2 ratio observed after pretreatment in lanthanum (Fig. 3) was interpreted to be due to a selective inhibition of the first peak rather than augmentation of the second peak. The data indicate that the first peak is more sensitive to inhibition by lanthanum than is the second. As lanthanum acts primarily to block $Ca^{2+}$ influx, it was deduced from this that the first peak is likely to correspond, in the main, to $Ca^{2+}$ moving into the cell from the external medium. It is known that lanthanum can enter plant cells (Quiquampoix *et al*., 1990) and intracellular channels are inhibited by $La^{3+}$ (Allen and Sanders, 1994; Gelli and Blumwald, 1993). However, the

treatment used in this study is likely to exert most of its effect on the more easily accessible plasma membrane channels.

To test whether the second peak might be representing $Ca^{2+}$ release from the vacuole, the responses of microdomain plants to cooling were imaged and were compared with those of cytosolic plants. It was observed here that the magnitude of the second peak was proportionately larger in the vacuolar plants (Fig. 4), indicating that vacuolar $Ca^{2+}$ release occurs during, and makes up at least part of, the second peak. Taken together, these data indicated to us that in plants that had been cold-acclimated, the contribution of vacuolar calcium to the whole response may be higher than in non-acclimated plants (Fig. 4). The altered second peak after acclimation could be due to altered properties of the calcium transporters in the tonoplast, altered calcium gradient across the tonoplast or due to differing numbers or sizes of the vacuoles. It is possible that $Ca^{2+}$ channel activities in the vacuolar membrane may be altered in previously acclimated plants either indirectly or directly. Such altered properties of vacuolar membrane changes might result indirectly from changes in membrane composition occurring after acclimation (Uemura *et al*., 1995) or may occur as part of a feedback loop after sustained cold-treatment.

An alternative explanation for the change in signature in plants that had been cold acclimated is that the second peak represents both vacuolar release of $Ca^{2+}$ and accumulation of $Ca^{2+}$ in the cytosol due to reduced activity of $Ca^{2+}$-ATPases at low temperatures. If this were the case, the increased magnitude of the second peak in acclimated plants could be attributable to a general decrease in $Ca^{2+}$-ATPase activity after acclimation. This could be seen as an energy-saving measure used by plants during survival of low temperatures. However, recent work using winter rye indicated that cold acclimation increases the ability of the $Ca^{2+}$ATPase to operate during exposure to low temperatures (Puhakainen *et al*., 1999). The $Ca^{2+}$-ATPase activity of winter wheat appears to be similarly altered after acclimation (Jian *et al*., 1999). It remains a formal possibility that $Ca^{2+}$-ATPase activity may be reduced in this system after acclimation, but in the light of data such as the studies mentioned above, this seems unlikely and a change in vacuolar $Ca^{2+}$ release appears the most likely reason for the acclimation-altered signature. Although the second peak was relatively larger in the vacuolar plants, analysis of the kinetics (initiation, peak and period) showed that there were no differences (data not shown). This suggests either that vacuolar release and diffusion is happening too rapidly to be detected as a change in kinetics, or the fact that the vacuolar plants contain cytosolic aequorin which may be masking kinetic differences.

Upon transfer of acclimated plants to ambient temperatures, the process of deacclimation begins whereby the acquired freezing tolerance is progressively lost over time (Thomashow *et al*., 1990). These authors found that deacclimation took 3 days to reach non-acclimated levels of freezing tolerance. Their data shows that after 16 h (the interval after acclimation at which we measured the calcium response) the plants were still 78% freezing tolerant compared to fully acclimated plants (Thomashow *et al*., 1990). Therefore although it is possible that the 16 h dark exposure at 20 °C resulted in some deacclimation of the plants, it is expected to be no more than 1–2 °C (P Steponkus, personal communication). Therefore, it would be expected that the plants used would still be at least partially acclimated after only 16 h deacclimation after which time the changes in calcium homeostasis caused by cold acclimation were still evident. The change in cooling-induced $Ca^{2+}$ signature seen after acclimation may indicate that low temperature signalling is altered as a consequence of acclimation and that the increased vacuolar contribution has some specific beneficial effect on the control of $Ca^{2+}$-regulated functions during prolonged low temperature stress.

Other instances of stress-induced $Ca^{2+}$ signatures altering after stress acclimation or stress pre-treatment have been observed (Knight *et al*., 1998). Interestingly, altered $Ca^{2+}$ signatures persist for hours, even days, after the end of the stress pretreatment period. In some cases, the degree to which $Ca^{2+}$ signatures are altered increases with increasing time elapsed between stress pretreatment and subsequent challenge with the stress (Knight *et al*., 1998). In some cases, these alterations are associated with corresponding changes in the level of expression of $Ca^{2+}$-regulated stress genes (Knight *et al*., 1998; Knight, 2000). However, no change has been seen in the ability to induce expression of the $Ca^{2+}$-regulated cold-inducible gene *KIN1* gene in plants that were cold-acclimated as described in this study (data not shown). Clearly, as the increased second calcium peak does not lead to greater *KIN1* expression in cold, this would suggest several possibilities. Firstly, the second peak may not be the part of the calcium signature that encodes information leading to *KIN1* expression. It may be that the first peak encodes information leading to *KIN1* expression, and that the second peak encodes other, as yet unidentified, information relating to cold acclimation. Secondly, it may be that *KIN1* expression is already maximal and so an increased second peak has no effect.

This preliminary dissection of bimodal cooling-induced $Ca^{2+}$ signature leads to the conclusion that after the cold acclimation treatment used here, cooling-induced $Ca^{2+}$ signatures are altered in *Arabidopsis*. The influx of $Ca^{2+}$ appears to remain unchanged whereas internal release, probably from the vacuole, increases after acclimation. Future work should be aimed at clarifying

whether or not this change in signature has any effects on the downstream signalling events in response to cold.

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