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Cell-type-specific calcium responses to drought, salt and cold in the *Arabidopsis* root

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Summary

Little is known about the signalling processes involved in the response of roots to abiotic stresses. The *Arabidopsis* root is a model system of root anatomy with a simple architecture and is amenable to genetic manipulation. Although it is known that the root responds to cold, drought and salt stress with increases in cytoplasmic free calcium, there is currently no information about the role(s) of the functionally diverse cell types that comprise the root. Transgenic *Arabidopsis* with enhancer-trapped GAL4 expression in specific cell types was used to target the calcium reporting protein, aequorin, fused to a modified yellow fluorescent protein (YFP). The luminescence output of targeted aequorin enabled *in vivo* measurement of changes in cytosolic free calcium concentrations ($[Ca^{2+}]_{cyt}$) in specific cell types during acute cold, osmotic and salt stresses. In response to an acute cold stress, all cell types tested as well as plants constitutively expressing aequorin displayed rapid $[Ca^{2+}]_{cyt}$ peaks. However, there were significant quantitative differences between different cell types in terms of their response to cold stress, osmotic stress (440 mM mannitol) and salt stress (220 mM NaCl), implying specific roles for certain cell types in the detection and/or response to these stimuli. In response to osmotic and salt stress, the endodermis and pericycle displayed prolonged oscillations in cytosolic calcium that were distinct from the responses of the other cell types tested. Targeted expression of aequorin circumvented the technical difficulties involved in fluorescent dye injection as well as the lack of cell specificity of constitutively expressed aequorin, and revealed a new level of complexity in root calcium signalling.

Keywords: calcium, GAL4, GFP, endodermis, stress, aequorin.

Introduction

The important role of calcium signalling in the transduction of environmental change into plant response has been documented for a wide range of stimuli (Bush, 1995; Kawano *et al.*, 1998; Knight *et al.*, 1996; Sedbrook *et al.*, 1996; Takahashi *et al.*, 1997). The induction of cytosolic calcium signalling results from hormonal action in some cases (Allen *et al.*, 1999b; Grabov and Blatt, 1998; McAinsh *et al.*, 1992), and as a direct response to abiotic stress in others (Knight *et al.*, 1996; Knight *et al.*, 1997). Changes in cytosolic free calcium ($[Ca^{2+}]_{cyt}$) are necessary for adaptative responses to stresses (Knight *et al.*, 1997; Tahtiharju *et al.*, 1997). The information encoded by calcium signals results in altered gene expression (Knight *et al.*, 1996; Trewavas, 1999) and changes in cellular metabolism (for example, changes in proline metabolism and transport) designed to prevent or minimize the deleterious effects of abiotic stress (Rentsch *et al.*, 1996).

Fluctuations in $[Ca^{2+}]_{cyt}$ may have a downstream effect upon regulatory components such as calmodulin (Hong *et al.*, 1999; van der Luit *et al.*, 1999), protein kinases (Allen *et al.*, 1999a) and ion channels (Bewell *et al.*, 1999; Li *et al.*, 1998). This provides a means for relatively rapid responses, such as the change in K^+ conductances in guard cells following increases in $[Ca^{2+}]_{cyt}$ (Lemtiri-Chlieh and MacRobbie, 1994), or may lead to specific changes in gene expression programmes (Knight *et al.*, 1997). However, understanding of the integration of cellular calcium responses into whole-tissue or whole-organ responses is lacking.

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There is an obvious functional difference between root and shoot, but there are few well-studied examples of tissue- and cell-type-specific functionality. The stomatal guard cell has been studied extensively as a model system of signal transduction and ion transport (MacRobbie, 1998), and our understanding of the root hair in a similar context has expanded rapidly in recent years (Bibikova *et al.*, 1999; Ehrhardt *et al.*, 1996). However, the roles of the root epidermis, cortex, endodermis and pericycle are less well understood, although the root is the primary sensor of drought and salt stress (Davies and Zhang, 1991; Schmidhalter *et al.*, 1998). Indirect evidence for the importance of root-specific adaptation mechanisms is provided by studies of stress-related genes highly expressed in roots. Examples of these include transcripts for an osmosensing histidine kinase (Urao *et al.*, 1999), NaCl-induced Ca^{2+} binding proteins (Jang *et al.*, 1998), Ca^{2+}/calmodulin (CaM) dependent glutamate decarboxylase (Zik *et al.*, 1998) and stress-induced calcineurin-B like proteins (Kudla *et al.*, 1999).

The endodermis and pericycle are central to the regulation of water and ion flow to the shoot (Clarkson, 1993). Their specialized role in controlling ion transport into the xylem places them in a crucial position to reduce water stress in the shoot while maintaining root turgor pressure during drought stress. Notably, rapid down-regulation by abscisic acid (ABA) of the activity of ion channels responsible for the loading of K^+ into the xylem (and the level of transcription of the genes encoding them) was observed to be specific to stelar parenchyma cells (Gaymard *et al.*, 1998; Roberts, 1998). This is consistent with a key role for these cells in the drought stress responses of plants (Tester, 1999).

Previous work has described the calcium response of whole plants to abiotic stresses (Knight *et al.*, 1997; Knight *et al.*, 1999), but it was impossible to determine the cell type(s) involved in the responses. In this report, we describe *in vivo* measurement of changes in $[Ca^{2+}]_{cyt}$ in specific populations of root cells in response to abiotic stresses, which allowed a spatial deconstruction of calcium responses. The data imply an important role for the endodermis and pericycle in the transduction of Ca^{2+}-mediated signalling in response to drought and NaCl stress.

Results

Targeted expression of aequorin

A YFP–aequorin gene fusion was expressed in specific cell types by GAL4 transactivation (Figure 1). GAL4 is a strong transcriptional activator from yeast which binds to an upstream promoter region, and has been shown to trigger ectopic gene expression in heterologous systems (Fischer *et al.*, 1988). The GAL4 protein has been modified by inclusion of an acidic VP16 transactivating domain, resulting in high levels of activation of genes harbouring the upstream activation sequence (UAS) to which GAL4 binds (Ptashne, 1988). This system has been used to create 'enhancer trap' lines in *Drosophila* (Brand and Perrimon, 1993) and in *Arabidopsis* by random insertion into the plant genome of a construct that contains a *GAL4* gene and a separate UAS-containing gene coding for ER-targeted GFP (mGFP5-ER) (J. Haseloff *et al.*, unpublished results; Figure 1a). Insertion of the construct in fortuitous locations in the plant genome placed GAL4 expression under the control of endogenous plant enhancers, resulting in cell-type-specific expression of GFP. Lines were selected from a library of such enhancer-trapped plants. In particular, lines were selected that demonstrated cell-type-specific expression in the epidermis (J0481), elongation zone cortex and epidermis (Q2393), endodermis (J3611) and pericycle (J2661) of the root (Figure 2b–e). Transformation with another construct containing the YFP–aequorin fusion under the control of five repeats of the GAL4 UAS (Figure 1b) resulted in expression of the YFP–aequorin fusion in cells labelled with mGFP5-ER.

The YFP–aequorin fusion was designed with the intention of using microscopy to screen for strong phenotypes, i.e. by rapidly monitoring the YFP fluorescence of the fusion. However, the low output of the YFP relative to the GFP proved prohibitory. Nonetheless, it was possible to verify YFP–aequorin expression in the cytosol of targeted cell types (Figure 2f,g). YFP fluorescence was not present in the dilated cisternae of the ER that characterize mGFP5-ER subcellular localization in *Arabidopsis* (Gunning, 1998). Whole plants were screened for aequorin luminescence by *in vitro* or *in vivo* reconstitution of aequorin (data not shown) (Knight *et al.*, 1991). The lines with the highest luminescence output were used for further study.

Measurement of targeted aequorin activity

Gradual cooling of plants (from 20 to 0.5°C over approximately 5 min) placed under a photon counting camera resulted in an increase in $[Ca^{2+}]_{cyt}$ in plants constitutively expressing aequorin (Knight *et al.*, 1999) and in plants with targeted YFP–aequorin gene expression. The total photon count during cooling was overlaid onto the bright field image of the seedlings to verify the location of aequorin in the regions correlated to GAL4 (and GFP) expression (Figure 3). The luminescence emission of the targeted aequorin agreed with the predicted location of expression driven by GAL4 in the enhancer trap lines. The root-specific location of aequorin luminescence in GAL4-targeted plants is in marked contrast to the whole-plant luminescence seen in plants constitutively expressing aequorin (compare Figure 3 with Knight *et al.*, 1999).

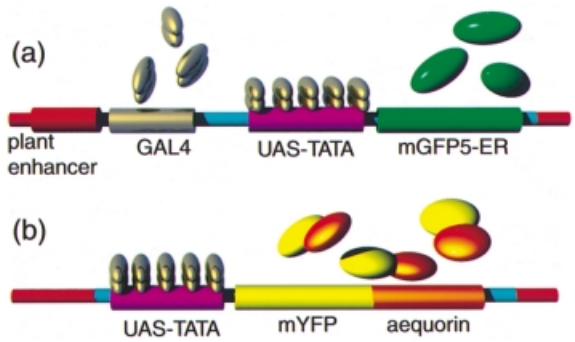

Figure 1. Diagrammatic representation of GAL4–UAS transactivation.
(a) An enhancer trap construct containing a *GAL4–VP16* domain that was randomly inserted into the plant genome. A linked mGFP5-ER driven by the GAL4–UAS allowed visualization of cell-specific patterns of expression driven by endogenous plant enhancers. (b) Transforming lines with specific cell types expressing GAL4 led to UAS-driven YFP–aequorin fusion gene expression only in GAL4–mGFP-ER-expressing cells (see Figures 2 and 3).

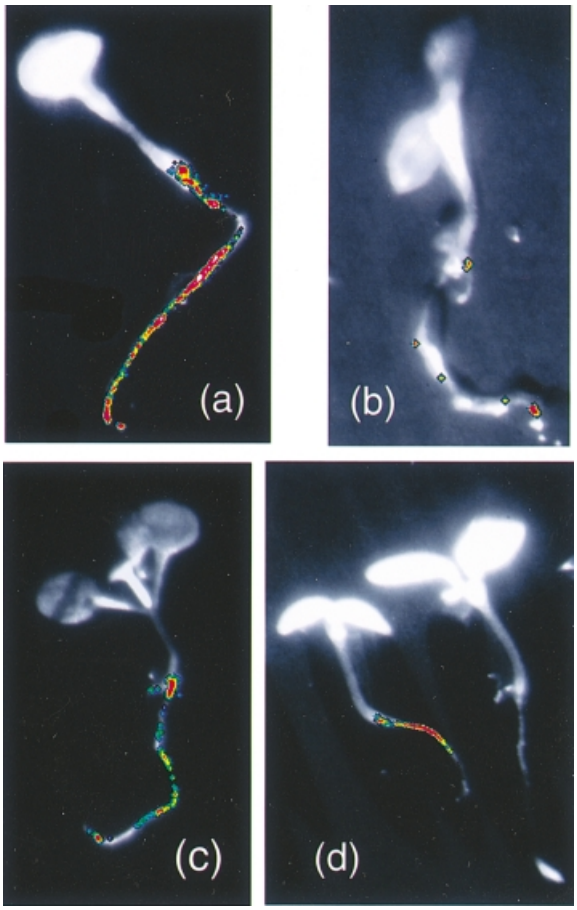

Figure 3. Ambient light images of seedlings overlaid with counts of total photons emitted by aequorin during a gradual cooling stress.
(a) Root epidermal aequorin expressor, (b) root elongation zone aequorin expressor (signal appears in primary and secondary root tips), (c) endodermis aequorin expressor, and (d) left, pericycle aequorin expressor; right, control (non-transformed C24) plant.

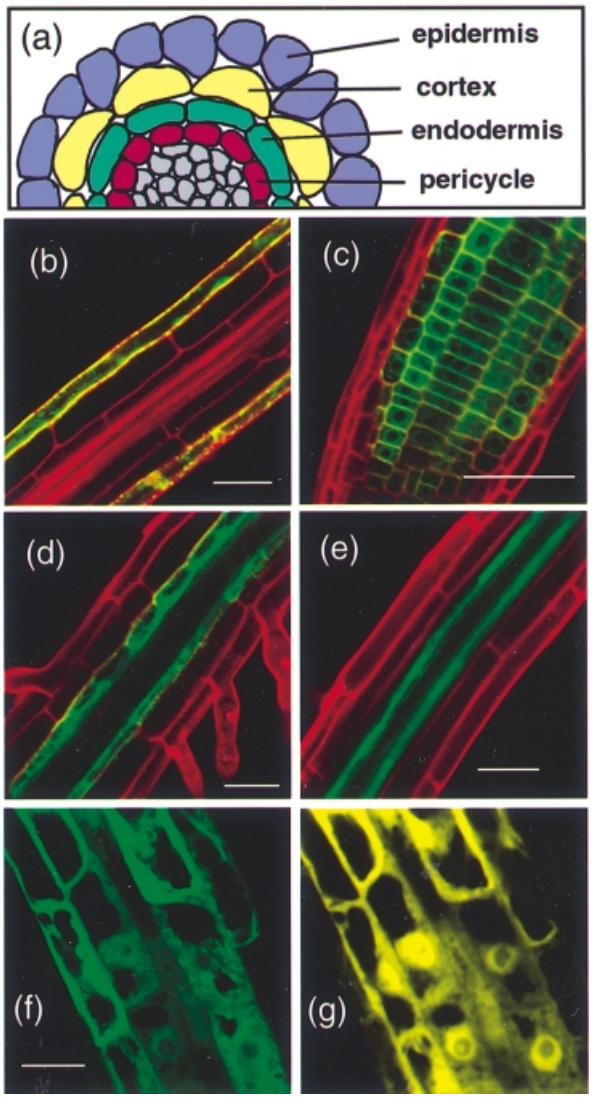

Figure 2. Location of GAL4–mGFP5-ER and YFP–aequorin.
(a) Cross-sectional diagram of mature *Arabidopsis* root showing the location of cell types used in this study. (b–e) Laser scanning confocal microscopic images of targeted aequorin lines. Cell walls were stained with propidium iodide (red fluorescence). GFP and YFP were localized to (b) epidermis, (c) elongation zone epidermis and cortex, (d) endodermis and (e) pericycle. (f,g) High-magnification localization of GFP and YFP in unstained epidermal cells of line J0481. The GFP (mGFP5-ER) is localized to the endoplasmic reticulum (f) and YFP–aequorin is present in the cytoplasm (excluded from the dilated cisternae of the ER containing mGFP5-ER) (g). Scale bars for b–e, 50 μm; scale bar for f and g, 25 μm.

Although the output of the targeted aequorin was high enough to resolve with the photon-counting camera, luminometry was used for analysis of response to abiotic stresses as it is more sensitive and enables quantification of changes in cytosolic calcium concentration.

Luminometric measurements of changes in $[Ca^{2+}]_{cyt}$

Application of ice cold water over 5 sec resulted in an immediate and rapid rise in $[Ca^{2+}]_{cyt}$ (Figure 4). In plants

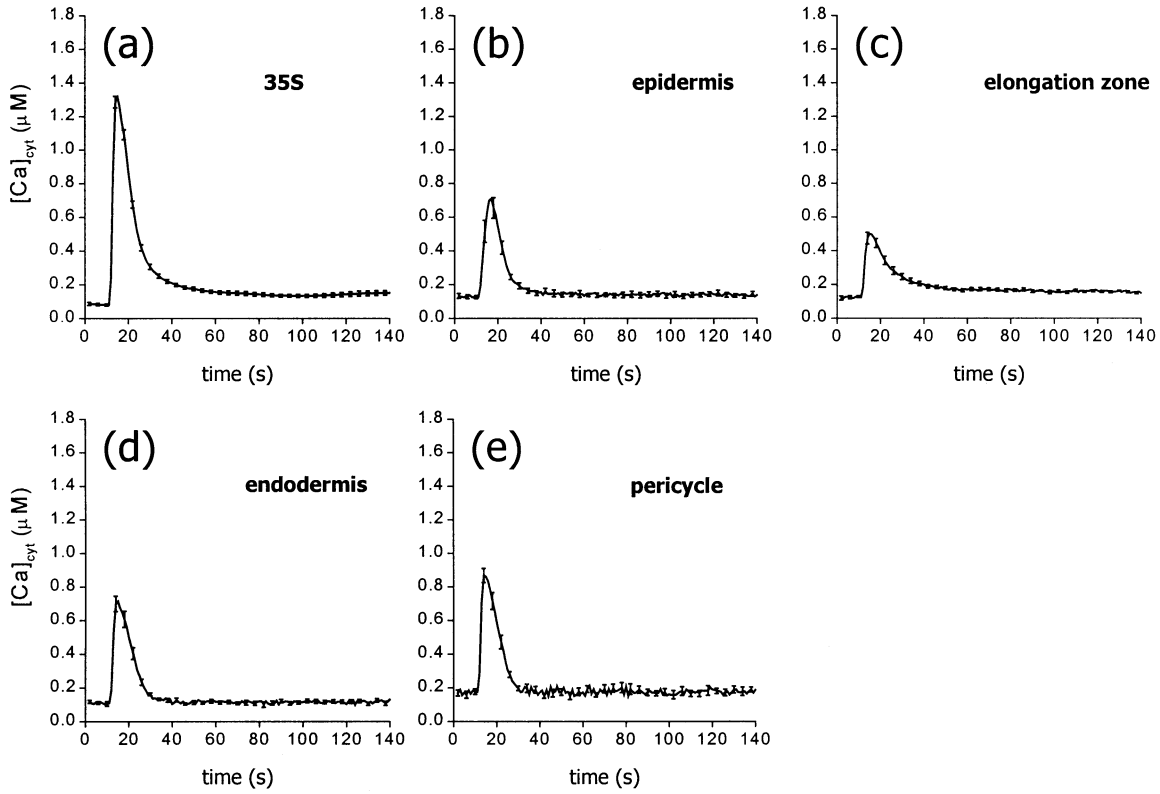

Figure 4. Average response to acute cold stress applied from $t = 10$ to $t = 15$ sec.
(a) Response of seedlings ($n = 5$) constitutively expressing aequorin. (b–e) Response of seedlings expressing aequorin in (b) root epidermis, (c) root elongation zone, (d) root endodermis and (e) root pericycle ($n = 10$ in each case). Data are presented as mean ± standard error of the mean.

constitutively expressing aequorin, $[Ca^{2+}]_{cyt}$ rose to $1.3 \pm 0.04\,\mu M$. In the epidermis, elongation zone, endodermis and pericycle, the rise in $[Ca^{2+}]_{cyt}$ was smaller. The lowest peak level reached was in the elongation zone ($0.5 \pm 0.03\,\mu M$), and the highest was in the pericycle ($0.9 \pm 0.04\,\mu M$). It is likely that the temperature change experienced by the root cell types was similar. This suggests that the magnitude of the perceived stimulus does not dictate the magnitude of the calcium response, and there is a strong cell-specific component. In any case, the deepest cell type (pericycle) showed the largest initial response to the cold stress (Figures 4a and 7a). Like the constitutively expressing plants, $[Ca^{2+}]_{cyt}$ in specific root cell types returned to a new resting level approximately 40 sec after the stimulus. The fact that the $[Ca^{2+}]_{cyt}$ peak values obtained with plants constitutively expressing aequorin were higher than for any of the four root cell types implies that $[Ca^{2+}]_{cyt}$ responses to cold in some (if not all) leaf/shoot cells are significantly higher than those in the root.

Compared to cold shock, application of an acute osmotic stress (440 mM mannitol) to constitutively expressing plants resulted in a lower and more prolonged initial peak increase in $[Ca^{2+}]_{cyt}$, and a longer time before reaching a new, higher resting $[Ca^{2+}]_{cyt}$ (Figure 5a). In contrast to cold shock, none of the targeted cell types regulated $[Ca^{2+}]_{cyt}$ back to the resting levels measured previous to the stress over the time course of measurements. The initial peak $[Ca^{2+}]_{cyt}$ in epidermis was significantly higher ($1.6 \pm 0.09\,\mu M$) than either the constitutively expressing plants ($0.99 \pm 0.08\,\mu M$), the elongation zone ($1.08 \pm 0.06\,\mu M$), endodermis ($1.2 \pm 0.06\,\mu M$) or pericycle ($0.78 \pm 0.04\,\mu M$) (Figures 5b–e and 7b). The fact that this peak was larger in the deeper endodermis than in the elongation zone suggests that the differences observed were not entirely due to differences in the degree of osmotic shock perceived by the target tissues. The epidermal response to mannitol was also greater than the epidermial response to cold (Figures 4a and 5a).

After the initial increase in $[Ca^{2+}]_{cyt}$, cells of the epidermis and elongation zone returned relatively rapidly (after approximately 35 sec) to values slightly above the resting level (Figure 5c). However, the endodermis and pericycle response contained a later, secondary peak 55–60 sec after the stress (Figure 5d,e). In the endodermis, the average secondary rise to approximately $0.44\,\mu M$ was followed by a gradual decrease over the 140 sec time course to $0.33\,\mu M$, well above the pre-stress resting level of

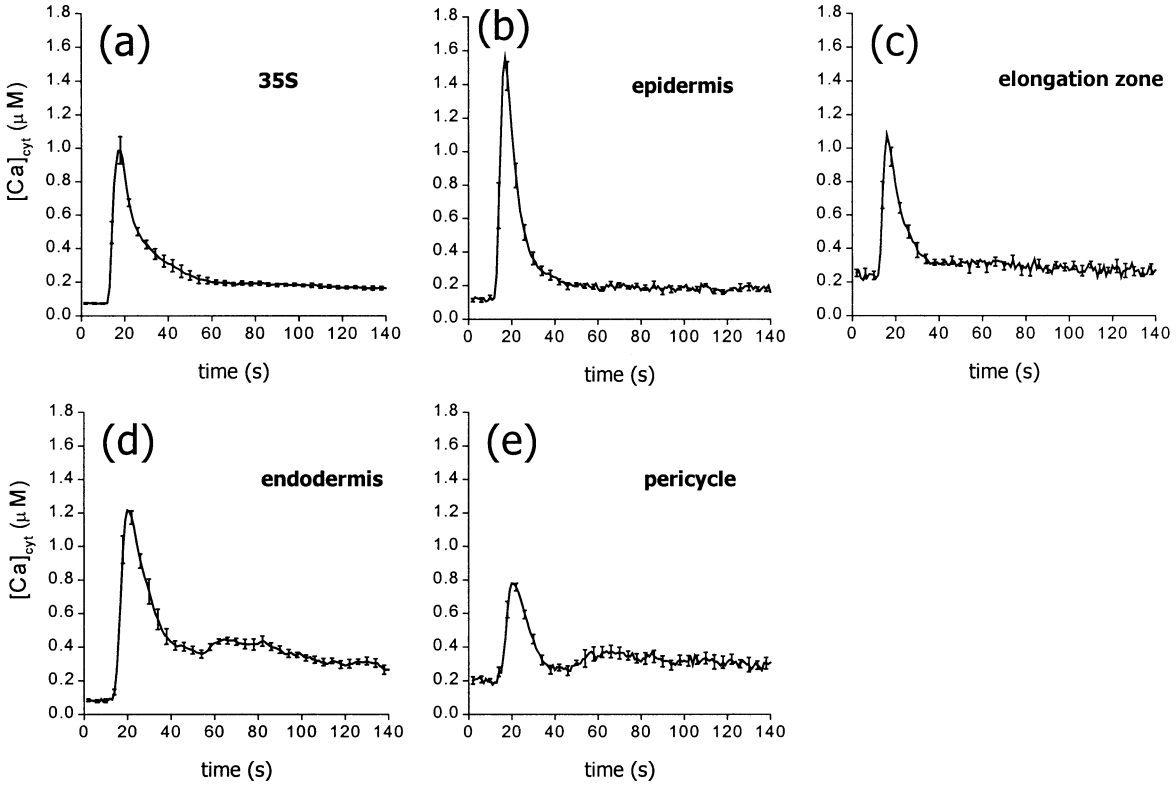

Figure 5. Average response to osmotic stress (440 mM mannitol applied from $t = 10$ to $t = 15$ sec).
(a) Response of seedlings ($n = 5$) constitutively expressing aequorin. (b–e) Response of seedlings expressing aequorin in (b) root epidermis, (c) root elongation zone, (d) root endodermis and (e) root pericycle ($n = 10$ in each case). Data are presented as mean ± standard error of the mean.

0.08 μM. Similarly, the average pericycle response included a second peak to 0.37 μM followed by a sustained increase at approximately 0.3 μM.

Upon application of salt stress (220 mM NaCl), plants consitutively expressing aequorin showed a more variable response (Figure 6a) than upon mannitol stress. The overall response differed primarily by the presence of a slower relaxation immediately after the primary increase in $[Ca^{2+}]_{cyt}$. Among the targeted cell types, there were differences in the magnitude of the primary increase in $[Ca^{2+}]_{cyt}$ in the elongation zone (Figure 6c) in response to salt (1.4 ± 0.05 μM) versus drought (1.1 ± 0.06 μM). The magnitude of the secondary rise in $[Ca^{2+}]_{cyt}$ in the endodermis and pericycle (Figure 6d,e) was greater during salt stress than during drought stress.

Interestingly, there was little temporal difference among the targeted cell types in the initial peak of calcium increase following NaCl application. The difference in the time of the initial peak response between the outermost cell type (epidermis) and the innermost cell type (pericycle) was small (5 versus 7 sec after addition of the solute; Figure 6). In response to drought stress, the epidermis and elongation zone responded 5–6 sec after application of the solute, whereas endodermis and cortex responded 9 sec after application of the stimulus (Figure 5).

The cell-type-specific differences in the peak responses to cold, osmotic and salt stresses are illustrated in Figure 7. The peaks of $[Ca^{2+}]_{cyt}$ for plants within each treatment (irrespective of the time of the peak) were tabulated to allow statistically relevant comparisons of peak magnitudes. Interestingly, the response to cold appears to be independent of cell depth (Figure 7a), while the response to osmotic (mannitol) stress decreases with cell depth (Figure 7b) and the response to salt drops sharply in the pericycle (Figure 7c). Summarizing these data, it seems that, in response to cold, the epidermal and endodermal $[Ca^{2+}]_{cyt}$ responses were insignificantly different (Student's *t* test, $P > 0.4$). The elongation zone $[Ca^{2+}]_{cyt}$ response was significantly lower ($P = 0.0022$) and the pericycle response was significantly higher ($P = 0.032$) than the epidermal or endodermal responses. In response to mannitol, the elongation zone and endodermal $[Ca^{2+}]_{cyt}$ responses were significantly different ($P = 0.047$), and the epidermal and pericycle $[Ca^{2+}]_{cyt}$ responses significantly higher and lower than the elongation zone or endodermal responses, respectively ($P < 0.0001$). In response to 220 mM NaCl, this pattern was actually quite different: there was no significant difference ($P = 0.16$) between the epidermal, elongation zone and endodermal $[Ca^{2+}]_{cyt}$ responses, the lower pericycle $[Ca^{2+}]_{cyt}$ response being the only difference

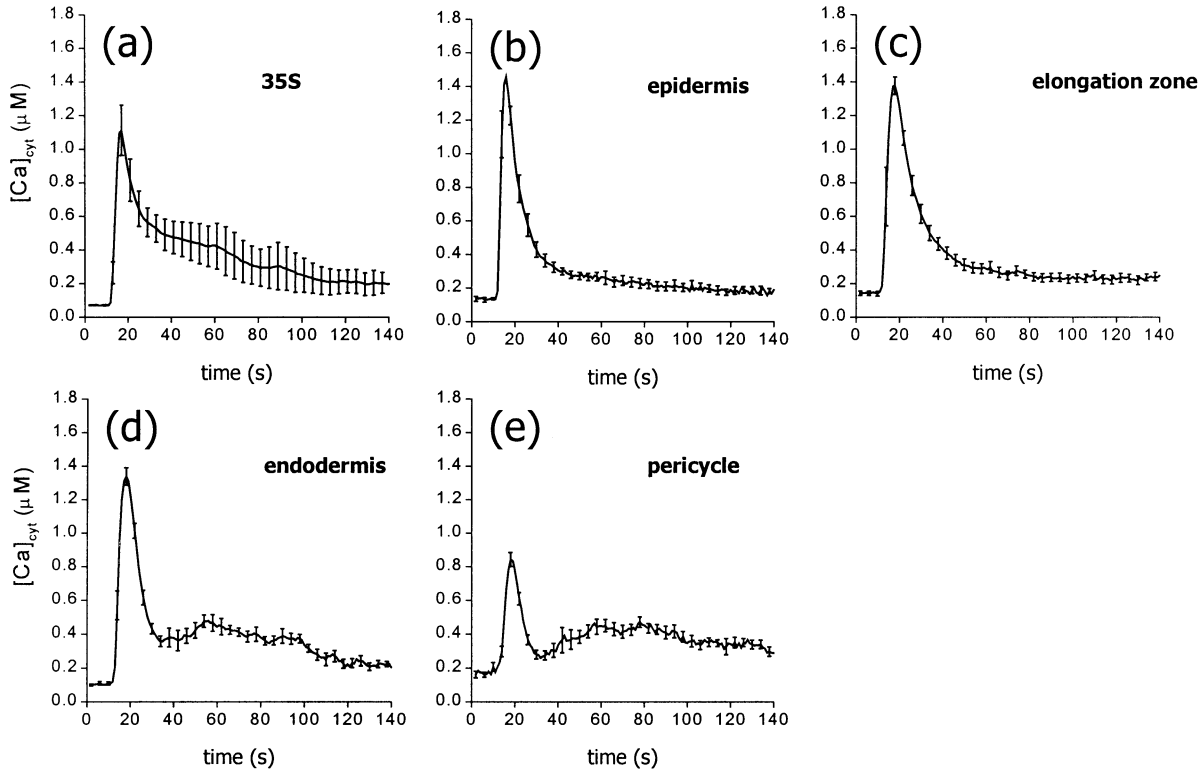

Figure 6. Average response to salt stress (220 mM NaCl applied from $t = 10$ to $t = 15$ sec).
(a) Response of seedlings ($n = 5$) constitutively expressing aequorin. (b–e) Response of seedlings expressing aequorin in (b) root epidermis ($n = 10$), (c) root elongation zone ($n = 10$), (d) root endodermis ($n = 16$) and (e) root pericycle ($n = 10$). Data are presented as mean ± standard error of the mean.

($P < 0.0001$). It is interesting to note the contrasting sensitivity/specificity of the different cell types to different stimuli, as represented by their $[Ca^{2+}]_{cyt}$ responses. For instance, the pericycle produces the largest $[Ca^{2+}]_{cyt}$ response to cold of the four cell types, but the lowest $[Ca^{2+}]_{cyt}$ response to both salt and mannitol.

The average response of endodermis- or pericycle-expressing plants veiled the complex oscillatory behaviour that is present in these cell types during drought or salinity stress (Figure 8). Neither the constitutively expressing plants, epidermis expressors nor the elongation zone expressors responded to cold, drought or NaCl with oscillations in $[Ca^{2+}]_{cyt}$ (Figures 5 and 6). However, in response to drought ($n = 10$) and salt ($n = 10$), but not cold ($n = 10$), the endodermis consistently responded with sustained oscillations in $[Ca^{2+}]_{cyt}$ (Figure 8c,d). The pericycle response to drought stress was less pronounced, two out of 10 plants showed few oscillations of low amplitude (e.g. Figure 8e). In response to salt stress, marked oscillations in $[Ca^{2+}]_{cyt}$ in pericycle were observed in nine of 10 pericycle expressor plants tested (Figure 8f). As can be seen in Figure 8, the $[Ca^{2+}]_{cyt}$ oscillations observed in some cases dampened down with time after stimulation. This is not due to consumption of aequorin, as the calibration procedure takes this into account, and represents *bona fide* dampening of $[Ca^{2+}]_{cyt}$ oscillations *in vivo*. Aequorin has been used to measure $[Ca^{2+}]_{cyt}$ oscillations in many other species of plants, e.g. tobacco (Johnson *et al.*, 1995) and animals, e.g. Zebrafish (Gilland *et al.*, 1999) and mouse (Lawrence and Cuthbertson, 1995). With aequorin, oscillations with durations of seconds (Lawrence and Cuthbertson, 1995), minutes (Gilland *et al.*, 1999) and days (Johnson *et al.*, 1995) can easily be measured.

When the response of endodermis to salt stress was measured over a longer time course (325 sec), three components of the $[Ca^{2+}]_{cyt}$ response were discernable: (1) a rapid, large initial peak for approximately 25 sec, (2) a second, broader rise between 40 and 100 sec (sometimes comprising more than one peak), and (3) a series of 5–12 sec oscillations for approximately 200 sec (Figure 8g, $n = 6$).

Discussion

Calcium is involved in many plant responses to environmental stimuli (e.g. cold, oxidative stress, touch, salt, anoxia) (Kawano *et al.*, 1998; Knight *et al.*, 1996; Knight *et al.*, 1997; Sedbrook *et al.*, 1996; Takahashi *et al.*, 1997).

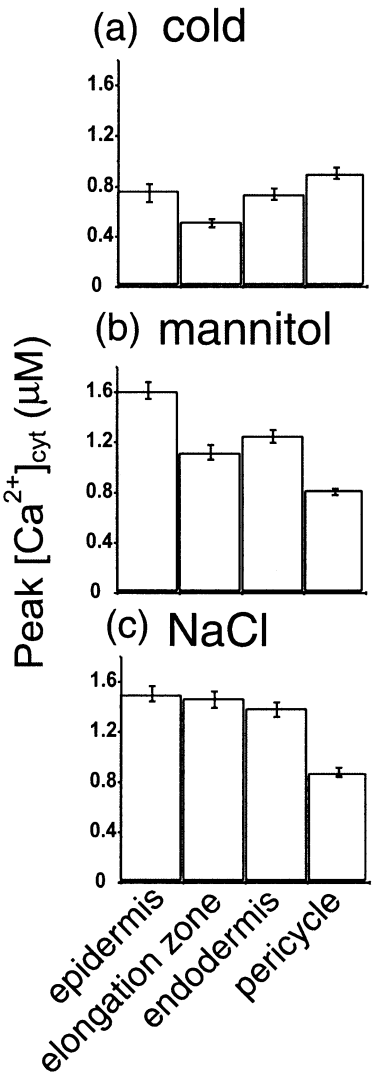

Figure 7. Peak $[Ca^{2+}]_{cyt}$ responses of targeted cell types to cold, osmotic and salt stresses.
The peak value of each plant for each treatment was tabulated regardless of peak time. (a) Cold stress, (b) osmotic stress (440 mM mannitol) and (c) salt stress (220 mM NaCl). In each case, $n = 10$. Data are presented as mean ± standard error of the mean.

The major outstanding question is not what stimuli trigger calcium signal transduction but how the necessary specificity for adaptive response is encoded. The ability to transduce a calcium-mediated signal into a specific response must occur at the cellular level (Trewavas, 1999). The hypothesis that there is coordination of stress perception and/or response within populations of functionally distinct root cell types implies the existence of cell-type-specific encoding mechanisms. Neither the injection of single cells with calcium-sensitive fluorescent dyes nor the constitutive expression of aequorin can adequately describe the calcium dynamics in specific populations of differentiated cell types. We have used a GAL4 transactivation strategy to target aequorin to specific cell types in the root to monitor the responses of epidermis, elongation zone epidermis and cortex, endodermis and pericycle to cold, osmotic and salt stress.

The targeting of an *in vivo* calcium indicator to specific root tissues and cell types was possible using GAL4-mediated transactivation of the aequorin gene (Figure 1). GAL4 transactivation resulted in levels of aequorin in small numbers of cells that were discernable by a photon-counting camera and enabled quantified measurement of $[Ca^{2+}]_{cyt}$. The signal strength was strong enough to allow localization of aequorin output even in the elongation zone of root tips of whole seedlings during cold stress (Figure 3b). The ability to target the indicator in this way allowed tissue-specific measurement of changes in $[Ca^{2+}]_{cyt}$ for the first time. These data may be compared with changes in $[Ca^{2+}]_{cyt}$ in plants constitutively expressing aequorin to deduce cell types important in the transduction of Ca^{2+}-mediated stress signals.

All cell types reacted in a similar way to cold stress (Figure 4), but there was cell-type specific variation in the magnitude of the response (Figure 4b–e). Plieth *et al.* (1999) showed that the magnitude of the Ca^{2+} peak during cold-induced increase in $[Ca^{2+}]_{cyt}$ was a function of the cooling rate, with a greater response during faster cooling regimes. Interestingly, in the targeted cell types, the variation in the magnitude of the response to an identical, rapid cold stress varied independently of the depth of the cells, suggesting that the variations in peak response were due to *bona fide* differences in Ca^{2+} homeostasis in different cell types (Figures 4 and 7a).

The quantitative differences between constitutively expressing plants and plants expressing aequorin in specific cell types may be due to the significant contribution of the shoot during cold response. Constitutively expressing plants showed increases in $[Ca^{2+}]_{cyt}$ in cells of the entire plant when visualized under a photon-counting camera (Knight *et al.*, 1999). However, the root plays a key role in osmotic response: when constitutively expressing plants were placed under a photon-counting camera and subjected to mannitol or salt stress, only the root responded (Davies and Zhang, 1991; Schmidhalter *et al.*, 1998; S. Scrase-Field and M.R. Knight, unpublished results). Evidence of a role for particular root cell types in these responses is reported here.

The endodermis and pericycle are specialized tissues in the subcortical region of the root (the stele) involved in the transfer of water and ions to the xylem. Stelar cells respond to drought or ABA application with changes in ion transport that are not seen in the root cortex (Cram and Pitman, 1972; Roberts, 1998). A decrease in the loading of ions from these cells into the xylem could retain the osmotic pressure necessary to maintain root growth during osmotic stress while minimizing solute transfer (and thus potential osmotic stress) to the shoot (Tester,

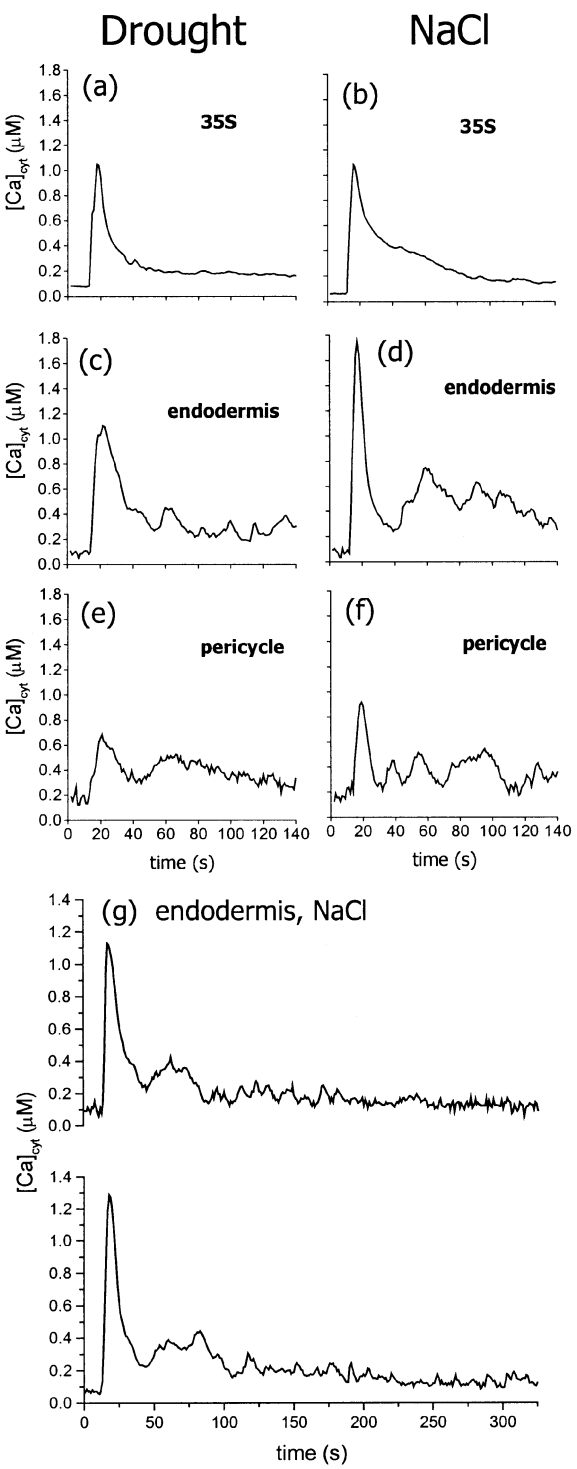

Figure 8. Response to osmotic and salt stress: representative individual measurements.
(a,b) Constitutively expressing plants, (c, d) response of the endodermis, and (e, f) response of the pericycle to osmotic stress (440 mM mannitol; a,c,e) or salt stress (220 mM NaCl; b,d,e). (g) Representative measurements of the response of endodermis to salt stress over a longer time course ($n = 6$).

1999). Thus, processes specific to the stelar cells are likely to be central in the adaptive response of whole plants to drought or salt stress.

Increases in $[Ca^{2+}]_{cyt}$ have been measured in response to both hypo-osmotic and hyper-osmotic shock (Knight *et al.*, 1997; Takahashi *et al.*, 1997). Hypo-osmotic shock of aequorin-transformed tobacco suspension cells resulted in a biphasic increase in $[Ca^{2+}]_{cyt}$ that comprised an initial trans-plasma membrane uptake and a second, intracellular release (Cessna *et al.*, 1998). Hyper-osmotic shock of *Arabidopsis* seedlings constitutively expressing aequorin results in a single peak, but includes release from the vacuole (Figure 5a; Knight *et al.*, 1997). The epidermis and elongation zone also displayed a single increase in $[Ca^{2+}]_{cyt}$ in response to drought stress (Figure 5a,b).

However, several features of the response of the endodermis and pericycle are unique. The endodermis and pericycle responded with an initial peak, and a lower, secondary rise in $[Ca^{2+}]_{cyt}$ (Figure 5c,d). In the endodermis, drought stress induced oscillations in $[Ca^{2+}]_{cyt}$ in nine out of 10 seedlings (Figure 8c), which is reflected in the average as a secondary rise in $[Ca^{2+}]_{cyt}$ (Figure 5d). In pericycle, this secondary rise was primarily the result of a second, sustained increase in $[Ca^{2+}]_{cyt}$, although two out of 10 seedlings exhibited pulsatile increases in $[Ca^{2+}]_{cyt}$ (Figure 8e). Furthermore, the responses to osmotic stress and salt stress were much reduced in the pericycle relative to other cell types (Figure 7b,c), a result consistent with a role of the endodermis in limiting the flow of water and ions to the xylem (Clarkson, 1993).

In terms of signal specificity, cells of the elongation zone, endodermis and pericycle showed significant differences (based on Student's *t* test: $P < 0.001$, $P = 0.045$ and $P = 0.053$, respectively) between their mannitol and salt $[Ca^{2+}]_{cyt}$ responses (Figures 5 and 6). This is very interesting, as analysis of constitutively expressing plants (Figures 5 and 6) showed no significant differences, as observed previously (Knight *et al.*, 1997). This led us to conclude that $[Ca^{2+}]_{cyt}$ was unable to encode specificity between salt and drought in *Arabidopsis* (Knight *et al.*, 1997). However, it now appears that $[Ca^{2+}]_{cyt}$ specificity was undetectable in plants constitutively expressing aequorin as the $[Ca^{2+}]_{cyt}$ signal is an aggregate one, and the data presented in this paper show that at the cell-type level there is scope for signal specificity to be encoded by differences in $[Ca^{2+}]_{cyt}$ response. It is interesting to note that, although there are subtle differences in the $[Ca^{2+}]_{cyt}$ responses of the different root cell types to all the signals we tested, none of the cell types are non-responsive to any signal. This would suggest that all cells in the root that we have tested are capable of calcium signalling to drought, salt and cold.

The oscillatory behaviour of $[Ca^{2+}]_{cyt}$ in pericycle, and more distinctly in endodermis, following salt stress, resembles that observed in stomatal guard cells in

response to ABA (Grabov and Blatt, 1998), and, more closely, the response of alfalfa root hairs to 1 nM nod factor (Ehrhardt *et al.*, 1996). In general, the oscillatory $[Ca^{2+}]_{cyt}$ patterns in guard cells are slower (with a period of approximately 5–10 min; Allen *et al.*, 1999b; McAinsh *et al.*, 1995; Staxen *et al.*, 1999) than those observed in roots (period of 60 sec in alfalfa root hairs; Ehrhardt *et al.*, 1996). However, it is important to bear in mind the caveat that caution should be exercised when comparing $[Ca^{2+}]_{cyt}$ oscillations measured by different reporters as the properties of the reporters themselves may affect the oscillations. Analysis of the response of endodermis and pericycle to salt stress did not always reveal a regular period, rather pulsatile rises of the order of tens of seconds (Figure 8). In the earliest stages of nod factor response, the $[Ca^{2+}]_{cyt}$ 'spikes' occurred with a frequency similar to the changes in $[Ca^{2+}]_{cyt}$ observed in the endodermis during drought or salt stress. The unique behaviour of the endodermis in response to drought and salt stress suggests that there may be a signalling system in endodermal cells that is of comparable complexity or flexibility to that found in guard cells or leguminous root hairs. It is also interesting to note that there must be some degree of synchrony in the approximately 1500 endodermal cells expressing aequorin (assuming cells 40 μm long and 1 cm of root) during the response, or cells within the population with markedly higher concentration changes (Figure 8c,d,g).

The similarities in the response of epidermis, elongation zone, endodermis and pericycle to cold, drought and salt suggest that there are mechanisms for $[Ca^{2+}]_{cyt}$ signal transduction that are common to all root cell types. This is reflected in the rapid spike in calcium that follows the application of all three stimuli. However, the markedly different responses of the pericycle and endodermis to drought or salt stress suggest that these cell types have a specialized role in the detection of and/or response to osmotic stress. The fact that the complex oscillatory response seen in the endodermis in response to drought and salt stress was seen in the pericycle only after salt stress further implies that understanding the response of the root to osmotic and salt stress requires a tissue-specific level of analysis.

To date, correlative analysis of the accumulation of stress-induced transcripts is limited to an organ-specific level. Nonetheless, these data suggest that components of calcium signal transduction are not ubiquitously expressed or regulated in the plant. There is evidence for enhanced or root-specific expression of osmosensing histidine kinase (Urao *et al.*, 1999), NaCl-induced Ca^{2+} binding proteins (Jang *et al.*, 1998), Ca^{2+}/CaM-dependent glutamate decarboxylase and stress-induced calcineurin B-like proteins (Kudla *et al.*, 1999). The ability to mis-express such genes in a tissue- and cell-specific manner using enhancer-trapped transactivation promises to elucidate the unique roles of the pericycle and endodermis in the response to osmotic stress.

The ability to target gene expression using GAL4 has provided insights into *Drosophila* and plant development (Brand and Perrimon, 1993; J. Haseloff, unpublished results). Crossing UAS-driven genes into lines that contain cell-type- or developmental-stage-specific GAL4-driven gene expression is particularly useful for the ablation of cells in developmental studies, as the UAS-bearing construct is silent until crossed into a GAL4-expressing line. Therefore, potentially lethal transgenes can be maintained via genetic control of the transcriptional activator. The GAL4 system in *Drosophila* has been used to target aequorin to renal tubules, and it was shown that response to a cardioactive neuropeptide was limited to a specific set of 77 cells (Rosay *et al.*, 1997).

The capacity to target aequorin in *Arabidopsis* is limited only by the range of GFP-decorated cell types and developmental stages of the enhancer trap libraries. Crossing wild-type plants transformed with the (silent) UAS–YFP–aequorin construct with GAL4–GFP-expressing plants enables targeting to a wide range of cell types. Shoot cell types, including the apical meristem and guard cells, will be interesting targets for an *in vivo* calcium reporter. Application of this technology to the root has provided novel insights about the cellular specificity of calcium signalling in root. An understanding of the cell types and signal transduction pathways involved in adaptive responses will eventually allow the generation of targeted transgenes designed to modify tolerance in a less disruptive way than constitutive transformation.

Experimental procedures

Construction of the aequorin reporter

A binary vector based on pBI121 (Jefferson *et al.*, 1987) was previously modified to include five repeats of a modified GAL4–VP16 binding site upstream of the sequence for a KNAT3–YFP fusion protein (pBINKNAT3-YFP) (K.R. Siemering and J. Haseloff, unpublished results; Haseloff, 1999). The *KNAT3–YFP* fusion gene was bordered with a 5′ *Bam*HI site and a 3′ *Sac*I site. *YFP* from this vector was amplified by polymerase chain reaction to introduce a 5′ *Bam*HI site and a 3′ *Eco*RI site. The apoaequorin gene from pAEQ1 (Prasher *et al.*, 1987) was amplified by polymerase chain reaction to add a 5′ *Eco*RI site and a 3′ *Sac*I site. The modified *YFP* and modified aequorin gene sequences were ligated and subjected to polymerase chain reaction to generate a YFP–aequorin fusion. The resulting DNA was sequenced, restricted with *Bam*HI and *Sac*I (NEB), and subcloned upstream of five repeats of the GAL4 upstream activation sequence into *Eco*RI/*Sac*I-digested pBINKNAT3-YFP to form pBINYFPAEQ.

Plant material

Experiments were performed using enhancer-trapped lines of *Arabidopsis thaliana* (ecotype C24) expressing GAL4-transacti-

vated mGFP5-ER (Haseloff *et al.*, 1997; Siemering *et al.*, 1996). Plants with interesting root cell-type-specific expression patterns were selected from an enhancer trap library of GAL4–GFP-expressing plants and screened using epifluorescent and confocal microscopy. A catalogue of enhancer trap lines is available at http://www.plantsci.cam.ac.uk/Haseloff/Home.html. Homozygous enhancer trap lines with cell-type-specific expression of GAL4–mGFP5-ER were transformed with *Agrobacterium tumifaciens* strain AgII harbouring pBINYFPAEQ either by vacuum infiltration or by the 'floral dip' method (Bechtold *et al.*, 1993; Clough and Bent, 1998). Plants were selected on agar plates for antibiotic resistance using 20 mg l^{-1} hygromycin (Sigma). Wild-type C24 was also transformed to enable crosses with other GAL4–mGFP5-ER lines in the future. *Arabidopsis thaliana* ecotype Columbia constitutively expressing aequorin under the control of a 35S promoter (Knight *et al.*, 1999) were grown under identical conditions for control experiments. Plants were grown on MS medium supplemented with 1% sucrose and 0.8% agar under full-spectrum fluorescent tubes on a 16 h light regime at 22°C.

Microscopy

Plants were routinely examined using a Nikon Diaphot 200 fitted with epifluorescence and an FITC filter set suitable for visualization of mGFP5 fluorescence (Nikon, Tokyo, Japan). Laser scanning confocal microscopy of GFP and YFP expression patterns was performed with a Leica TCS-SP microscope (Leica, Milton Keynes, UK). For imaging of mGFP5-ER expression patterns in enhancer trap lines, roots were treated with 10 μg ml^{-1} propidium iodide (Molecular Probes, Eugene, OR, USA) in water for 5–20 min to stain cell walls, and mounted in water on glass slides with cover slips. Dual channel imaging was performed using an excitation wavelength of 476 nm and emission window of 510–525 nm for GFP, and an excitation wavelength of 488 nm and emission window of 490–650 nm for propidium iodide. For comparative localization of GFP and YFP, unstained roots were mounted on slides and YFP was imaged using an excitation wavelength of 514 nm and emission window of 527–563 nm (GFP was imaged as above). Images were merged or analysed using Adobe Photoshop 5.0 software (Adobe Systems, San Jose, CA, USA).

$[Ca^{2+}]_{cyt}$ measurements

Five- to seven-day-old YFP–aequorin-expressing plants were incubated overnight in the presence of 10 μM coelenterazine (Prolume, Pittsburgh, PA, USA) to reconstitute the active photoprotein. For control experiments, *Arabidopsis* seedlings (ecotype Columbia) constitutively expressing aequorin under the control of a 35S promoter (Knight *et al.*, 1999) were incubated under identical conditions.

Luminometry was performed essentially as described elsewhere (Knight *et al.*, 1997) using a purpose-built luminometer sample housing and 1 sec integration time. Individual seedlings were placed in a luminometer cuvette containing 200 μl of water, and after 10 sec, stimuli were injected from a light-tight syringe in a 400 μl volume to reach a final concentration of 440 mM mannitol or 220 mM NaCl (Knight *et al.*, 1997). Cold stress was applied by injection of 1 ml of ice cold water as previously described (Knight *et al.*, 1996), with the exception that injection of the water was performed over a 5 sec interval to minimize touch response and allow comparison with mannitol and NaCl injections.

A double-logarithmic relationship exists between the concentration of free calcium present in cells containing reconstituted aequorin, and the proportion of total remaining aequorin present in the cell that is discharged at any point in time (Blinks *et al.*, 1978). Calibrations were performed by estimating the amount of aequorin remaining at the end of experiment, by discharging all remaining aequorin in 1 M $CaCl_2$, 10% ethanol. The calibration equation was derived empirically from measurements made with the aequorin sequence encoded by the specific aequorin coding sequence PCR product we have used to produce the chimeric constructs for plant transformations (data not shown) and the temperature at which our experiments were carried out. The calibration equation is $pCa = 0.332588(-\log k) + 5.5593$ where k is a rate constant equal to luminescence counts per second divided by the total remaining counts (Knight *et al.*, 1996). Calibration thus normalizes differences in the amounts of aequorin and potential attenuation of light travelling through different numbers of cell layers.

For luminometric experiments, five individual seedlings of 35S–aequorin-expressing plants or 10 individual seedlings of UAS–YFP–aequorin-expressing plants were used for each experiment. Data are presented as mean ± standard error of the mean. The error is reported every 4 sec for clarity.

Imaging of cold response over time in intact plants was performed using a Photek photon-counting camera to integrate photon emissions during a gradual cold stress from 20 to 0.5°C over 5–10 min. Overlays of white light images of plants and photon counts (Knight *et al.*, 1999) were performed using Photek software (Photek Ltd, St. Leonards-on-Sea, UK).

No luminescence was seen in wild-type C24 transformed with the UAS–YFP–aequorin under any experimental conditions (data not shown).

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