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The control of specificity in guard cell signal transduction

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Stomatal guard cells have proven to be an attractive system for dissecting the mechanisms of stimulus–response coupling in plants. In this review we focus on the intracellular signal transduction pathways by which extracellular signals bring about closure and opening of the stomatal pore. It is proposed that guard cell signal transduction pathways may be organized into functional arrays or signalling cassettes that contain elements common to a number of converging signalling pathways. The purpose of these signalling cassettes may be to funnel extracellular signals down onto the ion transporters that control the fluxes of ions that underlie stomatal movements. Evidence is emerging that specificity in guard cell signalling may be, in part, encoded in complex spatio-temporal patterns of increases in the concentration of cytosolic-free calcium ($[Ca^{2+}]_{cyt}$). It is suggested that oscillations in $[Ca^{2+}]_{cyt}$ may generate calcium signatures that encode information concerning the stimulus type and strength. New evidence is presented that suggests that these calcium signatures may integrate information when many stimuli are present.

Keywords: stomata; guard cell; calcium; signal transduction; abscisic acid; signalling cassette

1. INTRODUCTION

Stomata are pores found on the aerial surfaces of plants that allow the uptake of CO_2 for photosynthesis and the loss of water which drives the transpiration stream. Alterations in stomatal pore diameter are brought about by turgor changes of the guard cells (Assmann 1993). Guard cells are a good model for the study of signal transduction in plants because they respond in an easily quantifiable manner to a range of important environmental stimuli such as light, CO_2 concentration, temperature, plant hormones (Mansfield *et al.* 1990; Assmann 1993) and the activity of the circadian clock (Webb 1998). As these cells are tractable to a range of single cell techniques we know a great deal about the control of the ion fluxes which underlie guard cell turgor alterations (MacRobbie 1997). This is important as it means that many of the final effectors in guard cell signalling have been identified, which in turn makes the delineation of signalling pathways easier (McAinsh *et al.* 1997; Leckie *et al.* 1998a).

In guard cells the signal transduction cascade by which abscisic acid (ABA) brings about stomatal closure has been most intensively investigated. Although there is as yet no consensus regarding the location of putative ABA receptor(s) (MacRobbie 1997) many other components of this signalling cascade have been directly identified. These include cytosolic-free calcium ($[Ca^{2+}]_{cyt}$), inositol (1,4,5) trisphosphate ($InsP_3$), protons and protein phosphatases (Leung & Giraudat 1998). In addition, many of the plasma membrane and tonoplast ion channels that are responsible for allowing the efflux of K^+ and anions that initiate stomatal closure have been well characterized and in some cases their genes have been cloned (MacRobbie 1997; Grabov & Blatt 1998). There is now direct evidence that an increase in $[Ca^{2+}]_{cyt}$ is a common component in the response of guard cells to the closure-inducing signals, ABA (McAinsh *et al.* 1990, 1992; Schroeder & Hagiwara 1990; Gilroy *et al.* 1991; Irving *et al.* 1992; Allan *et al.* 1994), elevated CO_2 (Webb *et al.* 1996), oxidative stress (McAinsh *et al.* 1996) and elevated extracellular Ca^{2+} ($[Ca^{2+}]_{ext}$) (Gilroy *et al.* 1991; McAinsh *et al.* 1995). Therefore, we shall review the evidence that common mechanisms underlie stomatal movements in response to different closure-inducing stimuli. To do this we shall compare the architecture of different signalling pathways with one another and suggest that closure-inducing signalling pathways in guard cells converge at, or near, some of the known common elements listed here.

2. SIGNALLING PATHWAYS CONVERGE

Recent evidence suggests that increases in $[Ca^{2+}]_{cyt}$ (table 1; figure 1) are a common element in signalling

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Table 1. *Stimuli which have been shown to induce increases in* $[Ca^{2+}]_{cyt}$ *in guard cells*

(First reports are cited in brackets.)

closure	opening
ABA (McAinsh *et al.* 1990)	IAA (Irving *et al.* 1992)
high $[Ca^{2+}]_{ext}$ (Gilroy *et al.* 1991)	fusicoccin (Irving *et al.* 1992)
elevated CO_2 (Webb *et al.* 1996)	
H_2O_2 (McAinsh *et al.*1996)	
methyl viologen (McAinsh *et al.*1996)	
low $[K^+]_{ext}$ (Gilroy *et al.* 1991)	

Figure 1. Stimulus-induced increases in $[Ca^{2+}]_{cyt}$. The effect of (*a*) ABA (filled box); (*b*) CO_2 (filled box); (*c*) 1 mM $CaCl_2$ (filled box) and 0.1 mM $CaCl_2$ (hatched box); and (*d*) auxin (2,4-D) on $[Ca^{2+}]_{cyt}$ of guard cells of *C. communis* (*a–c*) and corn coleoptile cells (*d*). The auxin, IAA has also been reported to increase $[Ca^{2+}]_{cyt}$ of guard cells (Irving *et al.* 1992). Bars represent: (*a*) 1 min; (*b*) 5 min; (*c*) 10 min; and (*d*) 1 min. Data are reproduced with permission from: (*a*) McAinsh *et al.* (1990), copyright (1990) Macmillan Magazines; (*b*) Webb *et al.* (1996); (*c*) McAinsh *et al.* (1995), copyright ASPP; and (*d*) Gehring *et al.* (1990).

pathways leading to both increases and decreases in guard cell turgor. In addition to raising the question of how specificity is encoded in these signalling pathways (see §4) this adds weight to the suggestion that there is a degree of convergence in guard cell signalling pathways. Other common elements which have been identified are alterations in the cytosolic concentration of protons ($[H^+]_{cyt}$) (Grabov & Blatt 1998) and in the case of the three closure-inducing signals ABA, elevated $[CO_2]$, and $[Ca^{2+}]_{ext}$, the activity of the ABI1 and ABI2 proteins (Webb & Hetherington 1997). In this paper, we shall focus on the possibility that some of these components are arranged into functional arrays, which we have called cassettes (Webb & Hetherington 1997) that lead to a common response. In particular, we shall examine the possibility that the activity of ABI1 and ABI2 proteins and an increase in $[Ca^{2+}]_{cyt}$ form part of a signalling cassette that when activated initiates closure of the stomatal pore.

(a) *Increases in $[Ca^{2+}]_{cyt}$ are a common component in guard cell signal transduction pathways*

Stimulus-induced increases in $[Ca^{2+}]_{cyt}$ in guard cells have been observed in response to stimuli that cause both stomatal closure and opening (table 1; figure 1). This apparently paradoxical result raises the important question of how specificity is encoded in calcium-based systems (McAinsh & Hetherington 1998), which we discuss next. It also highlights the fact that increases in $[Ca^{2+}]_{cyt}$ appear to act as a point of convergence or a common component in guard cell signalling pathways. The central importance of increases in $[Ca^{2+}]_{cyt}$ to guard cell signal transduction is illustrated by data which demonstrate that directly interfering with the stimulus-induced increase in $[Ca^{2+}]_{cyt}$ prevents alterations in guard cell turgor. For example, Webb *et al.* (1996) showed the presence of the divalent cation chelator ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetra-acetic acid (EGTA) in the extracellular medium prevented CO_2-induced increases in $[Ca^{2+}]_{cyt}$ and prevented the associated stomatal closure. Similarly, we have shown that buffering $[Ca^{2+}]_{cyt}$ to, or near to, resting levels by injecting 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetra-acetic acid tetrapotassium salt (BAPTA) directly into the cytosol prevents the full stomatal response to ABA (A. A. R. Webb and A. M. Hetherington, unpublished observations). In addition to these experiments, a host of data from investigations based on pharmacological
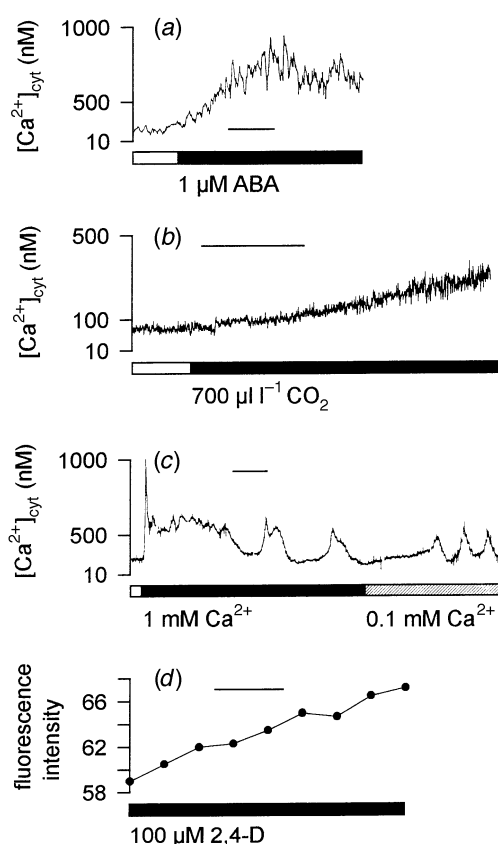
intervention (reviewed by Mansfield *et al.* (1990)) demonstrates that inhibiting components of calcium-based signal transduction pathways results in impaired stomatal movements in response to a variety of signals. Finally, artificial elevation of guard cell $[Ca^{2+}]_{cyt}$ either by the release of caged calcium (Gilroy *et al.* 1990; McAinsh *et al.* 1995), the addition of $InsP_3$ (Gilroy *et al.* 1990) or of cyclic ADP-ribose (cADPR) (Leckie *et al.* 1998*b*) invariably results in reductions in guard cell turgor. Taken together these three lines of evidence establish an increase in $[Ca^{2+}]_{cyt}$ as being central to the guard cell turgor response and as such are at or near the point of convergence for signalling pathways. Having stated this, there are data which suggest that when *Commelina communis* is grown at low temperature, the guard cell ABA response becomes independent of a detectable increase in $[Ca^{2+}]_{cyt}$ (Allan *et al.* 1994). These are interesting data as they seem to suggest that growth history can have profound effects on signalling pathways. However, having made the point that an increase in $[Ca^{2+}]_{cyt}$ represents a common component of the transduction cascades activated by signals which promote both stomatal opening and stomatal closure, what is not yet clear is how these elevations in $[Ca^{2+}]_{cyt}$ are coupled to the correct response.

(b) *ABI proteins are common components in the ABA, CO_2 and $[Ca^{2+}]_{ext}$ signalling pathways*

Another point of convergence in the transduction cascades by which ABA, CO_2 and $[Ca^{2+}]_{ext}$ cause stomatal closure are the products of the *ABI1* and *ABI2* genes (Webb & Hetherington 1997). The abi1-1 and abi2-1 loci were identified by a genetic screen which identified mutants that are able to germinate at prohibitively high levels of ABA (Koorneef *et al.* 1984). The *ABI1* and *ABI2* genes have been cloned and shown to encode proteins which exhibit type 2C phosphoprotein phosphatase activity [PP2C] (Leung & Giraudat 1998; Himmelbach *et al.*, this issue). *Arabidopsis* carrying either the *abi1-1* or *abi2-1* mutations are defective in the guard cell ABA stimulus–response coupling pathway (Roelfsema & Prins 1995). Furthermore, the stomata of the *abi1-1* and *abi2-1* mutants also failed to close in response to CO_2 and $[Ca^{2+}]_{ext}$ (Webb & Hetherington 1997). These data identify the products of the *ABI1* and *ABI2* loci as components of the transduction cascades by which ABA, CO_2 and $[Ca^{2+}]_{ext}$ cause stomatal closure, and suggest that the signalling cascades activated by these three signals converge on or close to these proteins.

(c) *Protons*

There is very good evidence that alterations in $[H^+]_{cyt}$, are a common component of the transduction cascades by which ABA and the auxin, indole acetic acid (IAA), regulate stomatal movements (see figure 2). For example, Irving *et al.* (1992) demonstrated that ABA induces a cytoplasmic alkalinization, whereas IAA induces cytoplasmic acidification. In addition, artificial increases in cytoplasmic pH alter channel activity in a manner which would be expected to result in decreases in guard cell turgor (Blatt & Grabov 1997). Taken together these data would seem to suggest that increases in pH are associated with decreases in stomatal turgor, while acidification occurs during increases in guard cell turgor. This is, of course, of considerable significance to the issue of how specificity is encoded in signalling pathways. More recent data have called into question whether the relation between the direction of the pH shift and the final response is quite so simple. In a recent study, Brearly *et al.* (1997) measured $[H^+]_{cyt}$ using the ratiometric pH indicator BCECF and showed that during CO_2-induced reductions in stomatal aperture $[H^+]_{cyt}$ does not alter (see figure 2). Therefore, although the ABA, CO_2, and auxin transduction cascades all converge at, or near $[Ca^{2+}]_{cyt}$, on the basis of the evidence currently available, alterations in $[H^+]_{cyt}$ represent a point of convergence between the ABA and auxin signal transduction cascades but not a point of convergence with the CO_2 transduction cascade. The functional significance of these important observations awaits further experimentation.

3. SIGNALLING CASSETTES

The current data demonstrate that a number of signalling components appear to be common to the transduction cascades in guard cells activated by a range of environmental and physiological signals. This suggests an economy in signal transduction pathways. Webb & Hetherington (1997) have proposed that in the guard cell
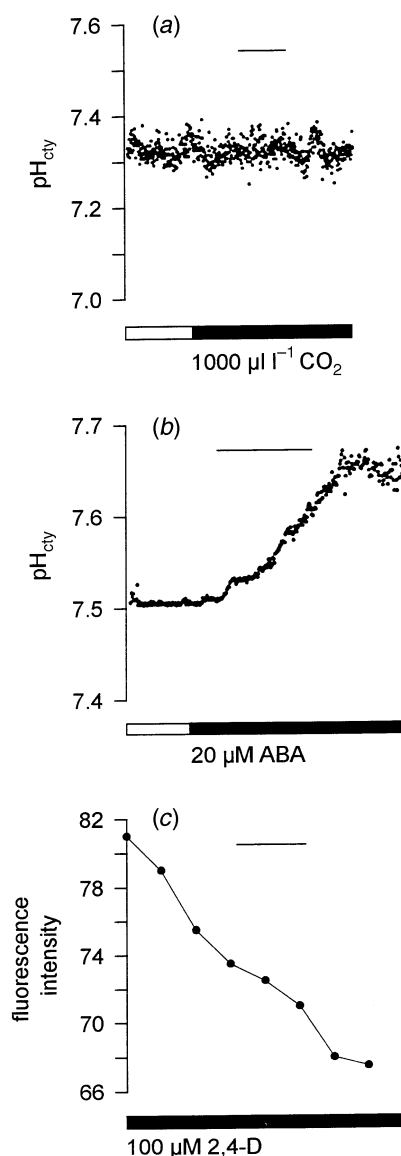

Figure 2. Stimulus-induced changes in cytosolic pH. The effect of (*a*) CO_2, (*b*) ABA and (*c*) auxin (2,4-D) on cytosolic pH of guard cells of *Vicia faba* (*a*); guard cells of *abi1-1* transgenic tobacco (*b*); and corn coleoptile cells (*c*); auxin (IAA) also has been reported to decrease pH of guard cells (Irving *et al.* 1992). The filled boxes under the traces indicate challenge with the appropriate stimuli. Bars represent: (*a*) 2 min; (*b*) 2 min; and (*c*) 1 min. Data are reproduced with permission from: (*a*) Brearly *et al.* (1997), copyright Springer-Verlag; (*b*) Blatt & Grabov (1997); and (*c*) Gehring *et al.* (1990).

this may be achieved by a group of signalling components including ABI1, ABI2 and possibly Ca^{2+} acting together to constitute part of a 'closure signalling cassette' (see figure 3). The function of this cassette would be to funnel signals, which promote stomatal closure and inhibit stomatal opening, down onto the ion transporters responsible for controlling stomatal aperture. This funnelling of signals may be possible because stomatal movements in response to a range of stimuli appear to be brought about by the same subset of ion transporters and by the interconversion of malate and starch. In animals, examples of signalling cassettes, such as the groupings found downstream of receptor tyrosine kinases, are encountered in developmental situations including pattern formation in
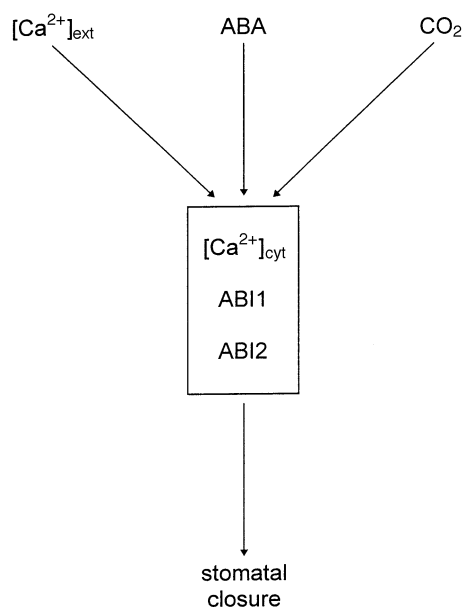
Figure 3. A simplified possible model of signal transduction via a 'closure signalling cassette' in guard cells. In this model it is proposed that ABA, CO_2 and $[Ca^{2+}]_{ext}$ bring about stomatal closure via a calcium-dependent transduction cascade and via the regulation of the PP2C-like activity of the *ABI1* and *AB12* gene products. These elements, along with other, as yet unidentified components may act together as a functional array or signalling cassette to regulate the activity of the K^+ and anion efflux channels that facilitate stomatal closure. The relative positions of alterations in $[Ca^{2+}]_{cyt}$, ABI1 and ABI2 in the putative closure signalling cassette are unknown. In addition, it is not known if the ABA, CO_2 and $[Ca^{2+}]_{ext}$ transduction pathways converge at the putative closure signalling cassette or upstream. $InsP_3$ and cADPR are known to act upstream of $[Ca^{2+}]_{cyt}$ in guard cell signalling, but detailed investigations will be required to determine if these molecules represent a point of convergence in the ABA, CO_2 and $[Ca^{2+}]_{ext}$ transduction cascades. Protons also are important signalling molecules in guard cells, but are not a point of convergence between the CO_2 and ABA signalling cascades, and therefore are unlikely to be a component of the putative closure signalling cassette.

Drosophila (Diazbenjumea & Haffen 1994; Duffy & Perrimon 1994; MacDougall & Waterfield 1996; Perkins *et al.* 1996) and neuronal development (Rusanescu *et al.* 1995). Although further experimental work will be required before it is possible to establish whether the signalling cassette is an appropriate concept to apply to the field of signal transduction in plants, the considerable similarities among pathogenesis, symbiosis, and wounding signal transduction pathways have been noted (Baron & Zambryski 1995). More specifically, there are interesting data which support the idea that there is convergence of plant growth regulator-activated signalling pathways. The pathways by which lipochito-oligosaccharide and auxin induce cell division appear to be separate, but may converge on or before the transcription of the *AXII* gene, possibly with cytokinin acting as an intermediate (Röhrig *et al.* (1995) but see also Schell *et al.* (1998)). There also is evidence that disease resistance-signalling pathways make use of a common signalling component, providing further examples of convergence in plant cell signal transduction pathways. This has been proposed by Century *et al.* (1995),

who identified in *Arabidopsis* a locus (*NDRI*) that is required for disease resistance to both a fungal and a bacterial pathogen.

The convergence of guard cell signalling cascades and the presence of common elements in a number of signalling cascades presents the opportunity for 'cross-talk' between these transduction pathways. Evidence that there may be 'cross-talk' between different guard cell signal transduction cascades is provided by observations that the sensitivity of stomata to a signal can be altered by other stimuli. For example, it has been demonstrated that ABA can sensitize stomata to CO_2 (see Raschke (1987) for a review). Conversely, auxins antagonize the effects of ABA and CO_2 on stomata such that high concentrations of IAA reduce stomatal sensitivity to both ABA and CO_2 (Snaith & Mansfield 1982). Although these data suggest that there is 'cross-talk' between the signalling cascades activated by ABA, CO_2, and auxins, it is not known whether differential regulation of the points of convergence outlined earlier underlie the interactive effects of these signals. Alternatively, other, as yet unidentified, components may be responsible for allowing 'cross-talk' between the guard cell signal transduction cascades.

4. SPECIFICITY

As discussed here, the convergence of a number of signal transduction pathways on common elements in guard cells raises the problem of specificity. This is particularly obvious in the case of $[Ca^{2+}]_{cyt}$ where it is clear that both stimuli which result in opening and closure use this second messenger. Recently, we have discussed a number of mechanisms by which specificity may be encoded into calcium-based signalling systems (McAinsh & Hetherington 1998). One possible mechanism for ensuring that the correct outcome is produced would be through the generation of a stimulus-specific calcium signal or calcium signature. It is clear from combined imaging and photometric studies (Gilroy *et al.* 1991; McAinsh *et al.* 1992, 1995) that guard cells are capable of generating complex spatiotemporal patterns of increases in $[Ca^{2+}]_{cyt}$. This suggests that plant cells have the capacity to encode specificity in the form of localized increases in $[Ca^{2+}]_{cyt}$. Perhaps the best examples of stimulus-specific calcium signals or calcium signatures (McAinsh & Hetherington 1998) in guard cells are produced in response to $[Ca^{2+}]_{ext}$ (McAinsh *et al.* 1995) and ABA (Staxén *et al.* 1998). Using $[Ca^{2+}]_{ext}$ stimulus, McAinsh *et al.* (1995) demonstrated that it is possible to correlate the strength of the external stimulus with both the magnitude of the final response and the form of the stimulus-induced oscillations in increases in $[Ca^{2+}]_{cyt}$. Other data from our recent experiments show that oscillations in $[Ca^{2+}]_{cyt}$ can be modified by the introduction of a second stimulus. For example, the data in figure 4 show that guard cells that are exhibiting a particular calcium signature in response to $[Ca^{2+}]_{ext}$ generate a completely new calcium signature when simultaneously challenged with a second stimulus (M. R. McAinsh and A. A. R. Webb, unpublished observations). Importantly, the change in the pattern of the oscillations is dependent both on the type (figure 4*a*,*b*) and strength of the stimuli (figure 4*c*). These data demonstrate that oscillations in guard cell $[Ca^{2+}]_{cyt}$ have the potential to integrate
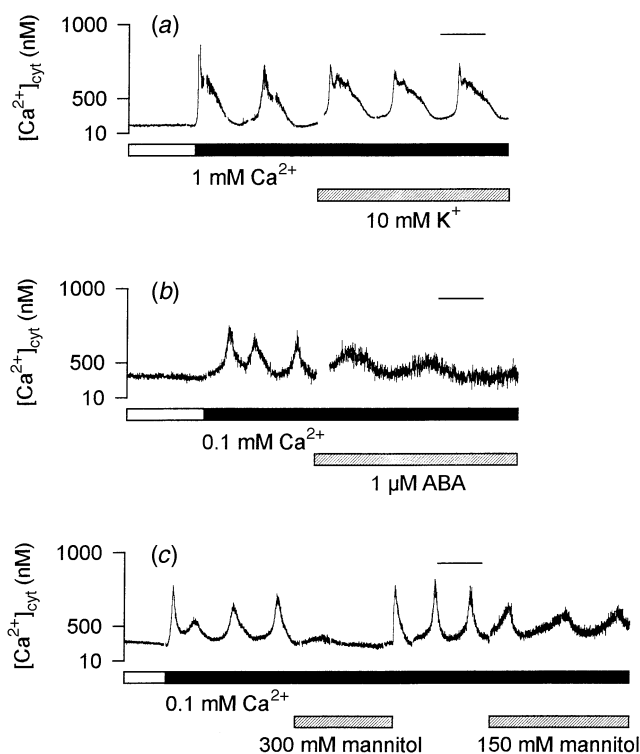
Figure 4. The effect of (*a*) external K$^+$ concentration; (*b*) ABA; and (*c*) osmotic shock induced by mannitol on oscillations in [Ca^{2+}]$_{cyt}$ induced by either (*a*) 1 mM or (*b*, *c*) 0.1 mM CaCl$_2$ (M. R. McAinsh and A. A. R. Webb, unpublished observations).

Microinjection of fura-2 and ratio photometry were done according to McAinsh *et al.* (1995). Resting [Ca^{2+}]$_{cyt}$ was determined at the start of each experiment during perfusion with 0.01 mM CaCl$_2$ (open boxes); this concentration of [Ca^{2+}]$_{ext}$ does not cause oscillations in [Ca^{2+}]$_{cyt}$ (McAinsh *et al.* 1995). All experiments were done in CO$_2$-free 10 mM 2-[N-morpholino]ethane sulphonic acid (MES) pH 6.2 + 50 mM KCl, unless otherwise stated. Bars represent 10 min.

information from a number of stimuli that are being perceived simultaneously by the guard cell by generating novel calcium signatures. Thus, oscillations in guard cell [Ca^{2+}]$_{cyt}$ appear to represent a dynamic signalling mechanism capable of encoding information concerning stimulus type and concentration.

Oscillations in [Ca^{2+}]$_{cyt}$ in guard cells are generated by complex mechanisms involving the activity of phospholipase C (Staxén *et al.* 1998), the influx of Ca^{2+} from extracellular stores, and the release of Ca^{2+} from intracellular stores (McAinsh *et al.* 1995), possibly through the action of InsP$_3$ (Gilroy *et al.* 1990) and cADPR (Leckie *et al.* 1998*b*). This raises the possibility that different Ca^{2+} stores or pools could be activated by different environmental and physiological signals. This, and the generation of stimulus-specific Ca^{2+}-signatures (McAinsh & Hetherington 1997), may contribute to encoding specificity in the converging Ca^{2+}-based transduction cascades that operate in guard cells.

5. CONCLUSIONS

In this paper we have discussed data obtained from guard cells which addresses the question of how specificity is encoded in calcium-based signalling systems. The

calcium ion appears to act as a ubiquitous intracellular messenger in plants and therefore issues concerning the encoding of specificity are likely to be encountered in many plant systems. Indeed there is evidence that other plant cell types have the potential to encode information in the calcium signal. Temporal oscillations in [Ca^{2+}]$_{cyt}$ (Ehrhardt *et al.* 1996) and spatial gradients and 'waves' of [Ca^{2+}]$_{cyt}$ (Franklin-Tong *et al.* 1997) have been observed in root hairs and pollen tubes. Studies of the mechanisms of encoding specificity are perhaps further advanced in guard cells not because of any special intrinsic property but because guard cells have been the subject of intensive investigation. Here, we have presented evidence that information specifying outcome may be encoded in the calcium signature, and have suggested mechanisms which can be used to generate these unique spatial and temporal patterns.

The next research objective is to identify downstream elements of the guard cell signalling pathway(s) because it is these which will be responsible for decoding the calcium signature. In doing these experiments it will be very important to identify where these signalling components reside within the cell. Differential localization or compartmentalization of downstream signalling components is one obvious mechanism for controlling specificity. There is, as yet, little evidence for this in plants although recent evidence from guard cells points towards a role for cytoskeletal reorganization during ABA signalling (Hwang *et al.* 1997). In addition, a number of signalling components including phosphatidylinositol 3-hydroxykinase (Dove *et al.* 1994) and GTP-binding proteins (Drøbak *et al.* 1995) are associated with the cytoskeleton, and the actin-binding protein profilin inhibits the plant plasma membrane phosphoinositide phospholipase C (Drøbak *et al.* 1994). Related to this issue is the question of grouping components together in functional arrays or cassettes which we have discussed here. There is already good evidence that groups of signalling components are held together on scaffold proteins in animals and yeast (Pawson & Scott 1997). In these cases signalling specificity will be dictated by the protein–protein interactions which govern the recruitment of individual components into the complex. In this context it is, at this moment, not yet possible to speculate on whether similar complexes exist in plants. For this reason it will be important to make efforts to identify further points of convergence and divergence in plant signalling pathways. It is also difficult to predict how many cassettes might exist in a given cell, although at present we predict on the basis of the limited number of active signalling roles of a particular cell that the number may be low. Considerably more work is obviously required before these questions can be resolved.

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