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Calcium signaling in plants

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Abstract. Changes in the cytosolic concentration of calcium ions ($[Ca^{2+}]_i$) play a key second messenger role in signal transduction. These changes are visualized by making use of either Ca^{2+}-sensitive fluorescent dyes or the Ca^{2+}-sensitive photoprotein, aequorin. Here we describe the advances made over the last 10 years or so, which have conclusively demonstrated a second messenger role for $[Ca^{2+}]_i$ in a few model plant systems. Characteristic changes in $[Ca^{2+}]_i$ have been seen to precede the responses of plant cells and whole plants to physiological stimuli. This has had a major impact on our understanding of cell signaling in plants. The next challenge will be to establish how the Ca^{2+} signals are encrypted and decoded in order to provide specificity, and we discuss the current understanding of how this may be achieved.

Key words. Cytosolic calcium; signaling; Ca^{2+} imaging; angiosperms.

Signal transduction in plant cells has rapidly become a major topic of research that has emerged from the disciplines of genetics, molecular biology, physiology and biochemistry and now largely combines these disciplines in order to advance our knowledge of how plants sense, and respond to, the diversity of extracellular stimuli they are exposed to. These studies have often drawn comparisons with how a mammalian cell processes the information it receives in the form of extracellular signals, and how this information is then decoded to produce an appropriate response. The concept of signal perception followed by intracellular signal transduction, which precedes the cellular response, has proven a consistent formula adapted by many eukaryotic cells, including those of plants.

The exact mechanisms whereby this is achieved from start to finish is obviously beyond the scope of any single review article. We must, therefore, focus in on specific mechanisms of particular interest. This article focuses on what is now regarded as an extremely important 'second messenger' in plant cells, the Ca^{2+} ion. The focus of the discussion will be on the characteristics of changes in the cytoplasmic concentration of Ca^{2+} observed in intact, living cells that have been exposed to a physiological stimulus. We will briefly summarize how cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) is generally regulated in mammalian and plant cells. We will consider the major advances achieved in this field over the last 10 years or so, which have been made possible by the new generation of dyes which accurately report $[Ca^{2+}]_i$, together with the advent of use of aequorin in transformed plants. These new technologies, coupled with the availability of a handful of model systems which have clearly identifiable responses to defined physiologically relevant stimuli, have had a major impact on our current understanding of cell signaling in plants. Although lagging behind the mammalian field with respect to the detailed knowledge of calcium signaling, it has clearly been established that plants use similar methods of signaling to animal cells. Nevertheless, despite the advances made, there is much more to be learned about calcium signaling in plants.

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Calcium dynamics in animal and plant cells

The cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) in the cell is under strict biochemical and physiological control. Cells generally operate to keep $[Ca^{2+}]_i$ low ($\sim$ 100–200 nM) in the 'resting' or quiescent state (see fig. 1). Increases in $[Ca^{2+}]_i$ can be used as a 'second messenger' following cell stimulation, and in this way $[Ca^{2+}]_i$ can bring about

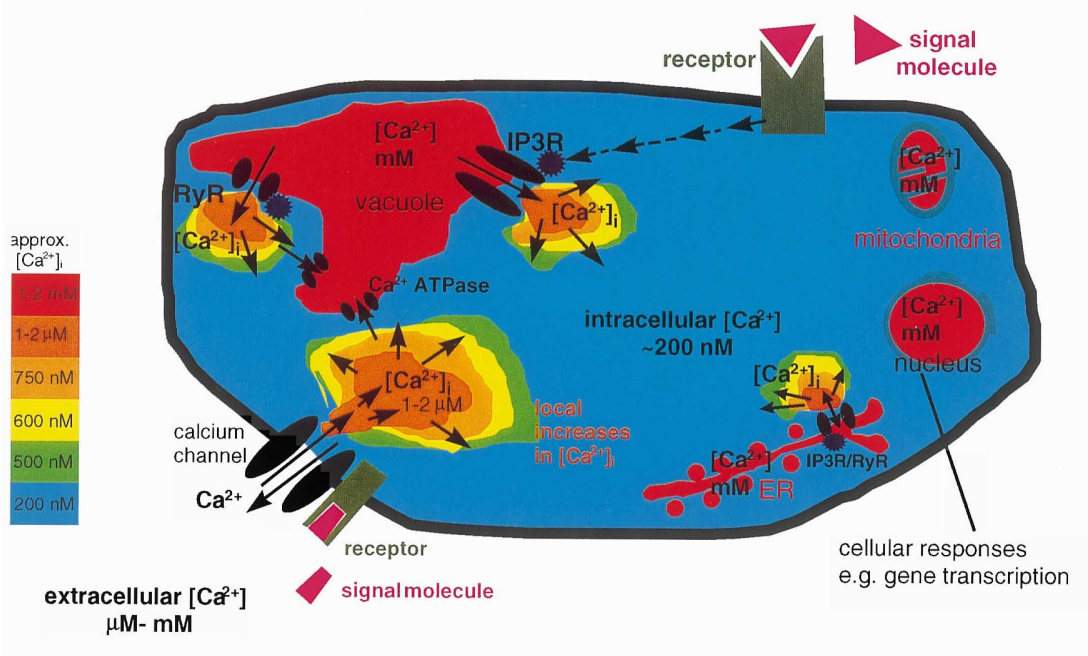


Figure 1. The localization and mobilization of Ca^{2+} during signal transduction in animal and plant cells. This cartoon of a generalized cell uses a pseudocolor scale (as indicated in the scale bar), in order to indicate the calcium concentration within the cytoplasm and organelles of this cell. It contains organelles that are known to store Ca^{2+} in the µM to mM range. These organelles are illustrated in red to indicate their high $[Ca^{2+}]$ (see scale bar). The intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$) of a quiescent, or unstimulated, cell is generally accepted to be ~100–200 nM (shown as blue; see scale bar). This low $[Ca^{2+}]_i$ is maintained, at least in part, by the action of Ca^{2+}-ATPases which act to pump Ca^{2+} into intracellular stores. The extracellular Ca^{2+} concentration ($[Ca^{2+}]_e$) is significantly higher than the $[Ca^{2+}]_i$ of the cytosol of a quiescent cell. Interaction between signal molecules and receptors (shown at the cell surface here) may result in Ca^{2+} influx. This is achieved by 'opening' of Ca^{2+} channels that are present in the peripheral plasma membrane, and this leads to local increases in $[Ca^{2+}]_i$, as illustrated by the color coding. Ca^{2+}-ATPases act to return $[Ca^{2+}]_i$ to basal levels. Signal molecules can also interact with receptors to result in the mobilization of Ca^{2+} from intracellular stores. The organelles identified as the predominant pool for mobilizable Ca^{2+} in animal and plant cells are thought to be different. Animal cells are thought to mainly release Ca^{2+} from the ER and/or the SR. Plant cells, however, appear to use the vacuole as the major mobilizable Ca^{2+} store, although there is some emerging evidence that they also use Ca^{2+} stored in the ER. Ca^{2+} in intracellular stores can be mobilized by the generation of IP_3 and cADPR, as indicated. These second messengers interact with specific receptors (IP3R and RyR) located on intracellular Ca^{2+} stores. These receptors form ion channels through which Ca^{2+} is released into the cytosol, resulting in spatially (and temporally) defined increases in $[Ca^{2+}]_i$ which are indicated in the diagram in pseudocolor. The dotted arrows linking the plasma membrane receptor to the IP_3R indicates that the pathway(s) are not well characterized in plants. These increases in $[Ca^{2+}]_i$ mediate the elicitation of specific cellular responses to the extracellular signals. The pseudocolors used in this figure may be broadly related to the images in figure 2, and in this way one can begin to envisage how some of the alterations in $[Ca^{2+}]_i$ may be brought about.

a cellular response to the stimulus. We attempt to explain some of the dynamics and controls involved in regulating Ca^{2+} in a 'generalized' cell, shown in figure 1, which encompasses features found in both animal and plant cells. The actual increases in $[Ca^{2+}]_i$ vary with cell types and the stimulus applied, but it is generally accepted that the $[Ca^{2+}]_i$ can increase up to 1–2 µM in stimulated cells. The nature of the downstream effectors are quite diverse and will not be described in any detail here, but interested readers should refer to [1] for a good general discussion.

The biochemical means by which $[Ca^{2+}]_i$ increases varies with the source of the mobilizable Ca^{2+} store. It may arise from outside the cell or from an internal pool. As indicated in figure 1, the $[Ca^{2+}]_i$ in both these locales is generally accepted to be in the micromolar to millimolar range, which is much higher than in the cytosol (100–200 nM). This is due, at least in part, to the activity of Ca^{2+}-ATPases (as shown in fig. 1) which actively pump the Ca^{2+} into these stores, thereby maintaining low $[Ca^{2+}]_i$. Figure 1 illustrates that following stimulation by receptor binding, this stored Ca^{2+} is permitted to move down its concentration gradient and into the cell cytoplasm, increasing $[Ca^{2+}]_i$. This can involve extracellular or intracellular Ca^{2+}, or both.

Ca^{2+} that originates from outside the cell often enters via selective ion channels located in the plasma membrane (as indicated in fig. 1). Intracellular Ca^{2+} can be

released in a number of ways. The first mechanism is the classical example of receptor-mediated increase in $[Ca^{2+}]_i$, resulting from the operation of the phosphoinositide-signaling pathway. This involves phosphoinositidase C (P_{IC})-mediated generation of inositol(1,4,5)-trisphosphate (IP_3) from the membrane phospholipid phosphatidylinositol(4,5)bisphosphate (PIP_2). IP_3 interacts with specific IP_3 receptors (IP_3R) that form Ca^{2+} channels in the membranes of intracellular Ca^{2+} stores (see fig. 1). Once bound to its receptor, IP_3 induces channel opening, allowing the efflux of Ca^{2+} into the cytosol, through IP_3-induced Ca^{2+}-release (IICR) [2, 3]. A further mechanism for release of Ca^{2+} from intracellular stores involves Ca^{2+} itself. This process, Ca^{2+}-induced Ca^{2+} release (CICR), involves a second distinct Ca^{2+} channel receptor, the ryanodine receptor (RyR), which is located on internal stores, as indicated in figure 1. RyrR is also responsive to cyclic adenosine diphosphate (ADP) ribose (cADPR), another second messenger [4].

Examples of production of IP_3 and cADPR in response to physiological stimuli in plant cells remain sparse. Of the few reported examples, IP_3 production has been observed for the response of *Medicago sativa* to fungal elicitors [5] and for *Brassica napus* in response to freezing [6], and cADPR has been seen to be produced in the etiolated hypocotyls of tomato as a result of treatment with abscisic acid (ABA) [7]. The detailed operation of the phosphoinositide pathway has, consequently, not been conclusively demonstrated in plant cells.

A major difference exists between plant and animal cells with respect to the identity of the major intracellular mobilizable Ca^{2+} store. In mammals this is believed to be the endoplasmic reticulum (ER) or sarcoplasmic reticulum (SR) (see fig. 1). However, the experimental evidence acquired so far points to the vacuole being the major mobilizable intracellular Ca^{2+} store in plant cells (as shown in fig. 1). Good evidence exists for the presence of receptors for both IP_3 and cADPR on plant cell intracellular Ca^{2+} stores [8, 9], as indicated in figure 1. There is little doubt that the second messengers, IP_3 and cADPR, are able to mobilize Ca^{2+} sequestered in the vacuole of plant cells. In vitro studies using isolated plant vacuoles demonstrated the ability of both IP_3 and cADPR to induce the release of Ca^{2+} from this store [10, 11]. Moreover, it has become clear that both agents can work independently to release Ca^{2+} from the same population of vacuoles. This suggests the presence of the respective receptor/ion channels for both agents on the plant vacuole [11]. Although studies of pollen tubes have suggested that the ER is likely to be a potential Ca^{2+} store in plant cells [12, 13], it is only very recently that evidence that plant cells can mobilize Ca^{2+} stored in the ER has been obtained. By using cells from cauliflower inflorescences that were not highly vacuolated, it has been demonstrated that IP_3-induced Ca^{2+} release may take place from nonvacuolar membranes [14]. This suggests that plant cells also have the ability to mobilize Ca^{2+} stored in the ER as well as the vacuole, for use in cell signaling.

Figure 1 illustrates how these mechanisms may operate in a cell to produce spatially localized high $[Ca^{2+}]_i$ as a result of the activity of both intracellular Ca^{2+} ion channels and those that are distributed on the peripheral plasma membrane. It is thought that messages carried by $[Ca^{2+}]_i$ are decoded to give an appropriate characteristic response in the stimulated cell. This can only be achieved if the signaling inputs that control Ca^{2+} dynamics via ion channels and second messenger generation corroborate to give spatially and temporally distinct Ca^{2+} signals. Changes in $[Ca^{2+}]_i$ are thought to provide signaling information that is 'encoded' by their magnitude, duration and spatial patterning. This information appears to be in the form of quarks, blips, sparks, puffs, spikes, repetitive spikes (oscillations) and waves of $[Ca^{2+}]_i$ [15]. The first four of these are regarded as fundamental and elementary changes in $[Ca^{2+}]_i$ and have been observed in small 'microdomains' of cells, where they result from the localized release of Ca^{2+} from individual or small numbers of intracellular ion channels [16]. Oscillations and waves of $[Ca^{2+}]_i$ are believed to result from the recruitment of these elementary release events, which have been visualized in single cells [17, 18]. Since at least some of these patterns of Ca^{2+} increases have been observed in plant cells, this phenomenon also appears to be a feature of Ca^{2+} based signaling in plants and will be discussed later. We will first consider how changes in $[Ca^{2+}]_i$ may be observed/measured in living cells, together with some examples.

Measuring cytosolic free calcium in living plant cells

Measuring cytosolic calcium ($[Ca^{2+}]_i$) in living cells requires a nondestructive means of incorporating a calcium sensor into the cytoplasm. Assuming that the sensor does not affect the normal functioning of the cell, a stimulus may be applied, and both the physiological and calcium response may be monitored. In this way, direct evidence of signal-response coupling is possible. Broadly speaking, there are two major forms of calcium sensors that have been introduced into plant cells in order to report $[Ca^{2+}]_i$. These are calcium-sensitive fluorescent dyes and the calcium-binding photoprotein, aequorin. We will briefly describe some of the methodology below, but readers requiring more detail of the technology involved are referred to [19].

Measuring $[Ca^{2+}]_i$ using calcium-sensitive fluorescent dyes

The use of Ca^{2+}-sensitive fluorescent dyes is an approach which has been widely used to investigate the quantitative, spatial and temporal distribution of intracellular calcium in living plant cells. Figure 2A–E illustrates some examples of plant cells where Ca_i^{2+} has been imaged using these dyes. Techniques routinely used for introducing these dyes into mammalian cells have not, in general, been widely used in plants. This is primarily due to the permeability problems posed by the plant cell wall. The most successful, and therefore most widely used, method of introducing dyes into plant cells is to microinject the dye, using either pressure injection or iontophoretic injection (using an electrical current), directly into the cytoplasm of plant cells. Protoplasts have also been used to study plant cell calcium, and are more amenable to noninvasive techniques such as ester and acid loading. However, as protoplasts have lost their cell wall and polarity, their responses may not always be strictly physiological.

The nature of the dye that is introduced, and the method of detecting calcium-induced changes in fluorescence, dramatically affects the nature of the information obtained. Two major classes of fluorescent dyes are generally used: the single-wavelength dyes such as Fluo-3 and Calcium Green, and the dual wavelength 'ratiometric' dyes, such as Indo-1 and Fura-2. The single-wavelength dyes have the advantage that, to date, they are compatible with laser confocal scanning microscopes equipped with an argon laser. When calcium is bound, there is an increase in fluorescence, but as the fluorescence is only measured at one wavelength, calibration of the increase in $[Ca^{2+}]_i$ is notoriously difficult, because fluorescence is proportional to the amount of dye present. Furthermore, there is a potential problem with possible artefacts, as redistribution of the dye could potentially give the impression of increases in $[Ca^{2+}]_i$. However, the evidence suggests that single-wavelength dyes are generally reliable, and are liable to be 'missing' detail, rather than creating artefacts. For example, the apical gradient of high $[Ca^{2+}]_i$ known to be present in pollen tube tips is usually not detected using single-wavelength dyes (see fig. 2B). Nevertheless, single-wavelength dyes in conjunction with detection mechanisms such as laser confocal scanning microscopy have provided significant information with respect to alterations in the spatial distribution of calcium in living plant cells following application of stimuli (see later). One particular advantage these dyes have over currently available ratiometric dyes is that they may be used in conjunction with caged probes, which require a flash of ultraviolet (UV) light to release the probe (see later, and [20–23]).

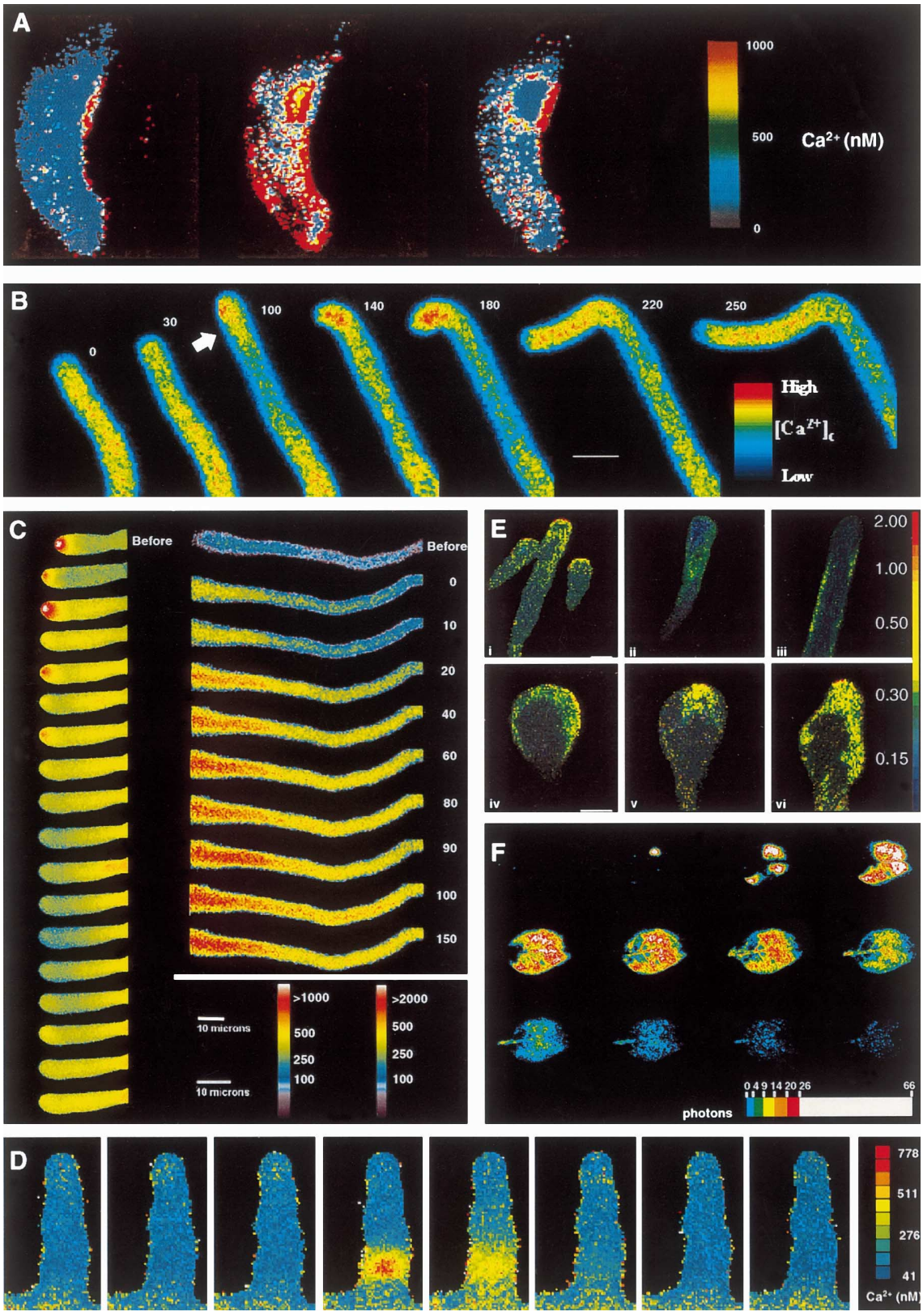
Single-wavelength calcium-sensitive dyes, however, suffer from the drawback that they are unable to provide accurate information with respect to $[Ca^{2+}]_i$. This problem is overcome by using dual-wavelength ratio dyes. These dyes have two characteristic excitation/emission wavelengths, one of which is Ca^{2+}-dependent and monitors changes in $[Ca^{2+}]_i$, the other Ca^{2+}-independent and used as a correction factor for differences in dye distribution. A 'ratio' may be calculated from fluorescent images collected in pairs at the Ca^{2+}-dependent and Ca^{2+}-independent wavelengths in order to obtain accurate, quantitative information with respect to spatial and temporal patterns of $[Ca^{2+}]_i$. Figure 2A, C, D, E illustrates the spatial and temporal diversity of alterations in $[Ca^{2+}]_i$ observed in different cells following specific stimulation, which have been detected using ratiometric techniques; these examples are discussed later in detail. Readers interested in further technical detail about these dyes are referred to [24] and [19].

Measuring $[Ca^{2+}]_i$ using aequorin

In contrast, the photoprotein aequorin is suited to the study of calcium-mediated responses to stimuli imposed upon an entire plant, rather than individual cells. In brief, aequorin is more correctly referred to as a calcium-sensitive luminescent protein. The biochemical mechanism of sensing changes in $[Ca^{2+}]_i$ by this method relies on the dissociation of the apo-aequorin polypeptide and the luminophore coelenterazine upon binding Ca^{2+}. The luminophore then emits blue light. Emitted light can be detected by a luminometer, or can be imaged by using an intensified CCD photon-counting imaging camera. The use of luminometry provides an excellent method for determining the temporal characteristics of changes in $[Ca^{2+}]_i$, but it is unable to provide spatial information, for which imaging is necessary.

Aequorin was first used to report $[Ca^{2+}]_i$ in the alga *Chara* [25], following microinjection directly into the cytosol. Use of transformation to introduce these probes into whole plants has revolutionized this type of study [26]. The growth or form of transgenic *Nicotiana plumbaginifolia* plants that constitutively expressed apo-aequorin in the cytoplasm of each cell was not affected when compared with wild type [26]. Furthermore, incubation with coelenterazine resulted in the production of reconstituted aequorin that was able to report changes in $[Ca^{2+}]_i$ not only in cells and tissues, but also in whole seedlings.

This technology has enabled the study of Ca^{2+}-based signal-response coupling in whole plants and tissues, rather than generally being limited to studying Ca^{2+} responses in individual cells. Figure 2E demonstrates an example of the use of imaging for visualization of the response of a leaf to a 'cold shock' stimulus. It is also possible to use this approach to observe 'long-range' signaling responses whereby $[Ca^{2+}]_i$ can be monitored in


A
1000
500
0
Ca2+ (nM)
B
0
30
100
140
180
220
250
High
[Ca2+]c
Low
C
Before
Before
0
10
20
40
60
80
90
100
150
10 microns
10 microns
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500
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100
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E
i
ii
iii
2.00
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iv
v
vi
0.30
0.15
F
0 4 9 14 20 26
66
photons
D
778
511
276
41
Ca2+ (nM)

cells and tissues distant to the site where a stimulus is applied. Examples of this will be discussed later. The aequorin technology has been developed to increase sensitivity, using aequorin in conjunction with a semisynthetic, *h*-form coelenterazine, which has increased sensitivity to Ca^{2+} [27–30]. This has allowed small, yet potentially significant, changes in $[Ca^{2+}]_i$ to be detected. It has been suggested for some time that the major drawback of the use of transgenic plants expressing aequorin as a means of reporting $[Ca^{2+}]_i$ is not knowing the proportion of the apo-aequorin that is reconstituted in each cell. This presents problems with respect to determining the quantitative changes in $[Ca^{2+}]_i$. However, the development of the semisynthetic aequorins has overcome this problem as they now possess the ability to report $[Ca^{2+}]_i$ due to an ability to luminesce at distinct wavelengths. Therefore, as previously described for the use of ratiometric dyes, the ratio of luminescence can used to quantify $[Ca^{2+}]_i$.

Another significant adaptation to the standard recombinant aequorin technology has arisen from the ability to target the photoprotein to distinct subcellular locations, where it can act to report fluctuations in $[Ca^{2+}]_i$ either within organelles or in the cytosol immediately adjacent to the membranes of these organelles, in regions referred to as 'microdomains'. For instance, the introduction of an H^+-pyrophosphatase-apo-aequorin construct into *Arabidopsis thaliana* seedlings has enabled visualization of changes in $[Ca^{2+}]_i$ in regions adjacent to the vacuole [31].

All of the methods described for reporting plant cell calcium have advantages and drawbacks, some of which we have touched on here. These will be highlighted in the following discussion that will focus on the few key

Figure 2. Imaging dynamic changes in cytosolic Ca^{2+} $[Ca^{2+}]_i$ in living plant cells. Figure 2A–E illustrates some examples of the use of Ca^{2+}-sensitive fluorescent dyes in single cells. (*F*) Example of the use of the photoprotein aequorin to report changes in $[Ca^{2+}]_i$ (in photons of light emitted) for an entire leaf. The images are presented in pseudocolor, which indicates alterations in $[Ca^{2+}]_i$ clearly, and each has a color calibration scale indicating $[Ca^{2+}]_i$ for that particular set of images. (*A*) The response of a *Commelina* guard cell to treatment with ABA. The Ca^{2+}-sensitive dual-wavelength dye Indo-1 was microinjected into the cytosol of a guard cell on an epidermal strip, and fluorescence ratio imaging was used to visualize $[Ca^{2+}]_i$. The left-hand image shows the resting $[Ca^{2+}]_i$ in an unstimulated guard cell. The middle and right-hand images display $[Ca^{2+}]_i$ in the same guard cell at 15 s and 2 min after the addition of 100 nM ABA. Note that the response to ABA induces punctate, transient elevations in the $[Ca^{2+}]_i$, which are spatially localized spikes. © American Society of Plant Physiologists. Reproduced, with permission, from McAinsh et al. (1992). (*B*) Reorientation of an *Agapanthus* pollen tube. The Ca^{2+}-sensitive single-wavelength dye Calcium Green-1 together with caged Ca^{2+} (Nitr-5) was microinjected into the cytosol of a pollen tube growing in vitro, and Ca_i^{2+} was imaged using laser confocal scanning microscopy. The first two images (at 0 and 30 s) show the pollen tube before treatment. At 100 s, the caged Ca^{2+} was photoactivated using a flash of UV light in a region to the left of the pollen tube tip (as indicated by the arrow). The images at subsequent time points show further localized increases in $[Ca^{2+}]_i$ in the tip region, and reorientation of the direction of growth of the pollen tube. © American Society of Plant Physiologists. Reproduced, with permission, from Malhó and Trewavas (1996). (*C*) The response of a *Papaver rhoeas* pollen tube to stigmatic self-incompatibility (S) proteins. The Ca^{2+}-sensitive dual-wavelength dye fura-2 dextran was microinjected into two pollen tubes in order to visualize alterations in $[Ca^{2+}]_i$ in response to addition of incompatible stigmatic S proteins, using fluorescent ratio imaging. The left-hand series illustrates alterations (at 10-s intervals) in the apical Ca^{2+} gradient in a pollen tube. This gradient of Ca_i^{2+} is clearly lost within 60 s of receiving the stimulus. The left-hand series shows changes in the $[Ca^{2+}]_i$ in the shank of a pollen tube, in a region ~ 100 μm behind the tip. Alterations in $[Ca^{2+}]_i$ (indicated in seconds after application of the stimulus) were visualized, and appear to originate in the shank of the tube. The spatiotemporal appearance of these changes appears to have the form of a wave, which moves forward, towards the tip of the pollen tube. © American Society of Plant Physiologists. Reproduced, with permission, from Franklin-Tong et al. (1997). (*D*) The response of *Medicago sativa* root hairs to treatment with *Rhizobium meliloti* Nod factors. The Ca^{2+}-sensitive dual-wavelength dye fura-2-dextran was microinjected into a root hair, and $[Ca^{2+}]_i$ was visualized using fluorescent ratio imaging. The images shown are a series taken ~ 50 min after the application of a *Rhizobium* Nod-factor, with 5-s intervals between each image, reading from left to right. A transient increase in $[Ca^{2+}]_i$, that is localized to the nuclear region of the cell, is visualized. These transient increases in $[Ca^{2+}]_i$ appear to oscillate approximately every 60 s (data not shown). © Cell Press. Reproduced, with permission, from Ehrhardt et al. (1996). (*E*) The deformation of a *Vicia sativa* root hair following application of a *Rhizobium* lipochito-oligosaccharides (Nod factors) The Ca^{2+}-sensitive dual-wavelength dye Indo-1 was introduced into root hairs using acid loading, and $[Ca^{2+}]_i$ was determined using fluorescence ratio imaging. Image (i) illustrates the apical high $[Ca^{2+}]_i$ gradient of growing zone I root hairs. Images (ii) and (iii) show nongrowing zone II and III root hairs that lack the apical high $[Ca^{2+}]_i$ gradient. Images (iv); (v) and (vi) show $[Ca^{2+}]_i$ in a zone II root hair, 70, 100 and 130 min, respectively, after the addition of lipochitooligosaccharides. Reestablishment of a high apical $[Ca^{2+}]_i$ gradient is accompanied by reinitiation of growth in (vi). © Blackwell Science Ltd. Reproduced, with permission, from de Ruijter et al. (1998). (*F*) The response of a *Nicotiana* leaf to cold-shock. The gene encoding the Ca^{2+}-sensitive photoprotein apo-aequorin was introduced into *Nicotiana plumbaginifolia* plants, using transformation. The active luminophore was reconstituted by incubation with coelenterazine. Luminescence, which reports $[Ca^{2+}]_i$, was imaged using an intensified CCD photon-counting imaging camera (courtesy of Prof. Anthony Campbell, University of Wales College of Medicine). The images are at 10-s intervals (reading from the top left-hand corner to the bottom right-hand image) and illustrate the response of a leaf to chilling (a reduction in temperature from 25 °C to 2 °C). The images show that the cold shock stimulus induces transient, spatially localized increases in $[Ca^{2+}]_i$ of the leaf. Note that although a small number of cells initially respond, the increases in $[Ca^{2+}]_i$ spread through the leaf before returning to basal levels. Reproduced with permission, © Marc Knight, University of Oxford.

Table 1. Examples of physiological stimuli that have been shown to induce changes in $[Ca^{2+}]_i$ in plants.

Physiological stimulus	Cell/tissue type	Response	Reference
Abscisic acid (ABA)	stomatal guard cells	stomatal closure	[20, 32–34]
High CO_2	stomatal guard cells	stomatal closure	[19]
Auxin (IAA)	stomatal guard cells	stomatal opening	[32]
Directional growth signals	pollen tubes	reorientation	[23, 52, 60]
Self-incompatibility (S) proteins	pollen tubes	growth inhibition	[12, 13, 56]
Sperm cell fusion	egg cells	fertilization	[74]
Physical obstruction	root hairs	reorientation	[83]
Nodulation (Nod) factors	root hairs	deformation, curling and nodule formation	[84, 88, 89]
Touch	seedlings	morphological changes	[26, 27, 92]
Wind	seedlings	morphological changes	[26, 92, 95]
Cold	seedlings	chill resistance	[26, 27, 31, 92]
Fungal elicitors	seedlings	defense response	[26]

We list examples of plant systems in which responses to physiologically relevant stimuli have been studied and shown to involve Ca^{2+} as a second messenger. We indicate the stimulus, the cell tissue and type, and the response. In all of these examples, measurements of $[Ca^{2+}]_i$ were made using either Ca^{2+}-sensitive fluorescent dyes or the Ca^{2+}-sensitive photoprotein aequorin, and increases in $[Ca^{2+}]_i$ were shown to precede the response.

model experimental systems that have been used to investigate signal transduction via changes in $[Ca^{2+}]_i$ in higher plants. These studies (which are summarized in table 1) have collectively highlighted the different roles Ca^{2+} appears to play in the mediation of many different biological responses, and will now be described in some detail.

Plant systems for studying stimulus-response coupling via alterations in $[Ca^{2+}]_i$

Several contrasting model systems have been used to measure changes in $[Ca^{2+}]_i$ in plant cells that have been exposed to a physiological stimulus. By this we mean a stimulus that a plant or a plant cell would expect to encounter in its natural environment. We will attempt to concentrate our discussion mainly on responses to physiological stimuli in order to provide greater depth with respect to the systems that have been studied in this manner. Table 1 lists some of the best-characterized systems where responses to physiological stimuli have been studied with respect to Ca_i^{2+} acting as a second messenger. Figure 2 illustrates some examples of imaging $[Ca^{2+}]_i$ in some of these systems.

Changes in $[Ca^{2+}]_i$ are associated with stomatal opening/closure

Stomatal guard cells are the key cells in the epidermis of the leaf which allow it to control gaseous exchange: entry of CO_2 and exit of H_2O as vapor. Although some of the water is used to cool the plant in high temperatures, most water is lost unnecessarily, and the plant generally has to attempt to conserve water, using the stomata. The stomata, therefore, have to balance maximizing photosynthesis with minimizing water loss. Responsiveness to environmental factors enables this.

Two major environmental factors, light and CO_2, influence the behavior of stomata. In addition, other factors, including air humidity, temperature and wind movement also interact to modulate changes in stomatal aperture. Stomata generally operate under a circadian rhythm, and most plants open their stomata in the day and close them at night. This enables them to take up CO_2 during the day for photosynthesis in the tissues of the plant. In addition to light as a stimulus, stomata also respond to CO_2. Generally stomata respond rapidly to changes in CO_2, closing as CO_2 rises and opening as CO_2 decreases. The guard cells play a key role in the regulation of the water relations of the plant, and act to control water loss by closing under conditions of drought in order to prevent loss of water through transpiration. Osmotic changes in the environment, therefore, play a major role in regulating guard cell aperture. In addition to environmental factors, stomata also respond to hormonal stimuli. Perhaps the best-characterized is the response to the hormone ABA. It should be remembered that the effects of environmental factors on stomata may be mediated by hormones. For example, water stress and salt stress can result in elevated ABA levels, resulting in subsequent stomatal closure.

Arguably the best-characterized plant system with respect to signaling via changes in $[Ca^{2+}]_i$ is that of the stomatal guard cell. As a model system, the stomatal guard cells are ideal, as many stimuli are known to elicit opening and closing, as described above. The movements resulting from changes in guard cell turgor are easily identifiable and measurable. Furthermore, as the two guard cells act independently, one of the pair can act as an integral control. A well-established assay using epidermal strips containing stomata makes this a model system

which is amenable to both the perfusion of extracellular agents, and the imaging and photometry of individual guard cells microinjected with Ca^{2+}-sensitive fluorescent dyes.

Convincing evidence, using these approaches, has established that Ca^{2+} acts as a second messenger in the process of both stomatal closure and opening [32]. ABA-induced increases in $[Ca^{2+}]_i$ were first reported in guard cells prepared as epidermal strips that had been treated with exogenous ABA [33]. Figure 2A illustrates this response for guard cells of *Commelina communis*. Ca^{2+}-imaging of a guard cell prior to the addition of ABA is shown in the left-hand cell of figure 2A, and indicates the $[Ca^{2+}]_i$ of the resting cell, which is ~ 200 nM (comparable with resting levels in cells in general, as indicated in fig. 1). However, ~ 15 s following the addition of ABA, increases in the $[Ca^{2+}]_i$ were observed (see the middle cell of fig. 2A). These peak levels of $[Ca^{2+}]_i$ are ~ 1 μM, and comparable to concentrations in stimulated cells (again, indicated in fig. 1). The right-hand cell displays the $[Ca^{2+}]_i$ ~ 2 min after the addition of ABA, and shows $[Ca^{2+}]_i$ decreasing. It is therefore clear that ABA induces transient and localized increases in the $[Ca^{2+}]_i$ of the stomatal guard cell. The distribution and duration of these increases will be discussed later when we consider specificity in Ca^{2+}-based signaling responses of plant cells.

A curious observation that has arisen from studies of this type is that ABA-induced increases in $[Ca^{2+}]_i$ do not precede stomatal closure in all of the test groups. Although elevations in $[Ca^{2+}]_i$ were observed in some of the stomata exposed to ABA, all of them subsequently closed, regardless of whether $[Ca^{2+}]_i$ increased [20]. This type of response, whereby ABA-induced stomatal closure occurs independent of a rise in $[Ca^{2+}]_i$, has prompted the suggestion that there may exist another, Ca^{2+}-independent, pathway for ABA-induced stomatal closure [34]. Further data suggest that $[Ca^{2+}]_i$ is also involved in mediating stomatal closure in response to high CO_2 and oxidative stress [35, 36]. Again, a small proportion of the cells responded in the absence of any detectable increase in $[Ca^{2+}]_i$. These 'anomalies' are discussed later, when we consider the way in which changes in $[Ca^{2+}]_i$ can elicit specific responses in plant cells.

Research has focused on elucidating how the information encrypted in these changes in $[Ca^{2+}]_i$ is decoded by the cell. It is also important to establish the source of the $[Ca^{2+}]_i$, and how it affects other components. Guard cell turgor is dependent upon several important physiological processes, the most significant of which is the influx and efflux of K^+, which is associated with stomatal opening and closure (see the chapter on ion channels). Increases in $[Ca^{2+}]_i$ have been shown to be involved in the regulation of stomatal aperture [32]. A key question, therefore, is whether increases in $[Ca^{2+}]_i$ are also involved in changes in K^+ channel activity. Use of caged IP_3 in guard cells of *Vicia faba* suggested that IP_3-induced release of $[Ca^{2+}]_i$ influenced K^+ fluxes associated with stomatal closure [37], and direct evidence for a role for $[Ca^{2+}]_i$ was provided by imaging the effect of release of caged IP_3 and caged Ca^{2+} on $[Ca^{2+}]_i$ [38]. Both of these treatments resulted in a reduction in stomatal aperture, thereby clearly demonstrating that guard cells possess the ability to respond to extracellular stimuli that may induce increases in IP_3 and the subsequent mobilization of Ca^{2+} from internal stores. Exactly how these changes in $[Ca^{2+}]_i$ affect ion channels is suggested to involve changes in the phosphorylation state of what are presumably regulatory proteins [39].

A further interesting feature of the study of $[Ca^{2+}]_i$ in guard cells again relates to the mobilizable calcium pool and more specifically to the intracellular distribution of the elevated $[Ca^{2+}]_i$. Due to the immobility of Ca^{2+} with respect to diffusion in the cytosol, the spatial identification of the source of the increase is of significant interest. For instance, a difference in the subcellular distribution of $[Ca^{2+}]_i$ during oscillatory increases that were induced by high extracellular Ca^{2+} ($[Ca^{2+}]_e$) has been observed [40]. Specifically, it was noted that the transient increases in $[Ca^{2+}]_i$ that preceded the closing response to 1.0 mM external Ca^{2+} was localized to the peripheral regions of the guard cell in proximity to the plasma membrane. This was then followed by an increased $[Ca^{2+}]_i$ surrounding the vacuole of the cell. This illustrates the tight control, and the possible utilization of several Ca^{2+} stores, in calcium signaling in these systems. Increases in $[Ca^{2+}]_i$ in regions surrounding the vacuole and nucleus of guard cells have also been reported to occur when the external $[K^+]$ is lowered [20].

The importance of Ca^{2+} in plant reproduction

Another model system that has been the subject of extensive Ca^{2+} imaging is the pollen tube. An important feature of these studies has, again, been the availability of a simple experimental technique by which pollen can be grown in vitro. This facilitates investigations into the biochemical control of pollen tube growth in vitro which would otherwise be practically impossible in vivo using the technology presently available. To date, fluorescent dyes have been used to monitor $[Ca^{2+}]_i$. However, although technical problems preclude the current use of aequorin in this system at present, in the near future it may be possible to use this technique to study truly in vivo events.

How might the pollen tube, in its natural environment, be signaled? Although it is clear that pollen can grow without the presence of a stigma or style, there is considerable evidence for communication between pollen tubes and the pistil. Let us consider pollination. First, pollen grain hydration upon contact with the

stigma is a situation that may involve signaling. Establishment of polarity and initiation of tube growth may also require signals.

The pollen tube must require mechanism(s) for ensuring correct directional growth. This is a situation for which some sort of guidance system, which is likely to involve signaling between the pollen tube and the stylar transmitting tract, is required. An extracellular matrix in the tract contains components which are thought to be important in adhesion, nutrition and directional guidance involved in pollen tube growth [41, 42]. Although this interaction is poorly understood at present, there is evidence that the stylar exudate contains several hydroxyproline-rich glycoproteins, including heavily glycosylated arabinogalactans (AGPs) [43], some of which may be taken up and incorporated into pollen tubes growing in vivo [44]. Some AGPs, such as tobacco-transmitting tissue glycoprotein (TTS), can stimulate pollen tube growth and may play a role in pollen tube guidance [45–47], though this topic is controversial [48]. By whatever guidance mechanism they use, pollen tubes reaching the bottom of the pistil must undergo further reorientation, because the pollen tube has to find the micropyle through which it enters the ovary. Ultimately, there is the meeting of the pollen sperm cells with the egg cell, between which one would expect signaling in order to effect fertilization.

All this occurs when the original pollen grain is perceived as compatible by the recipient pistil, and is allowed to grow normally! A situation which frequently occurs is an interaction between pistil and pollen that is termed 'incompatibility'. The most obvious example of this is the interaction of a pollen grain and pistil from different species, where interspecific incompatibility exists to prevent fertilization. Although the mechanisms involved have not yet been determined, it is reasonable to suggest that these involve signal perception and transduction. Incompatibility also occurs within species. This intraspecific incompatibility is often known as self-incompatibility (SI). These are often genetically determined mechanisms whereby an individual plant prevents self-fertilization by selectively inhibiting pollen of identical self-incompatibility genotype (*S*-genotype) to that of its pistil. This obviously includes its own pollen. We will discuss some of the evidence for Ca^{2+} signaling in normal pollen tube growth before Ca^{2+} signaling in incompatible interactions.

The role of Ca^{2+} in mediating pollen tube growth. A crucial requirement for Ca^{2+} in pollen tube growth has been appreciated for many years. However, it was not until the availability of reliable Ca^{2+}-sensitive fluorescent imaging techniques that information about the spatial and dynamic changes in $[Ca^{2+}]_i$ in pollen tubes became available. It is therefore relatively recently that we have begun to obtain information about $[Ca^{2+}]_i$ and its distribution in the pollen tube. Over the last 10 years good evidence for a positive correlation between high apical $[Ca^{2+}]_i$ in pollen tubes and growth has accumulated. This tip-focused gradient in $[Ca^{2+}]_i$ is a consistent feature of growing pollen tubes, and is common to other tip-growing cells, including neurites of nerve cells, fungal hyphae and root hairs (see later discussion).

A number of independent studies on pollen tubes, using ratiometric Ca^{2+} imaging, have established that tip-focused apical Ca_i^{2+} gradients (see fig. 2C), estimated to be between 3 and 10 μM at its maximum [49–57], are likely to be a general phenomenon common to all growing pollen tubes. It is worth noting that the $[Ca^{2+}]_i$ in the tip region is consistent with the generally accepted values of $[Ca^{2+}]_i$ in stimulated cells, whereas the $[Ca^{2+}]_i$ in the 'shank' region behind this area is within the levels of $[Ca^{2+}]_i$ in resting or quiescent cells (see fig. 1). Evidence from a variety of sources supports the idea that the apical Ca_i^{2+} gradient results from Ca^{2+} influx restricted to the extreme apex of the pollen tube [52–55, 57–60]. It is generally thought that when growth ceases, the apical gradient is dissipated due to the closing of these channels. The nature of these Ca^{2+} channels is currently not known.

Good evidence that apical Ca^{2+} has an oscillatory behavior in growing pollen tubes has recently been obtained [54–57, 61]. Detailed measurements have established that oscillating $[Ca^{2+}]_i$ and growth rate are closely associated and have the same periodicity [54, 55, 57]. This has resulted in the suggestion that there is a direct causal link. The exact relationship between influx and growth needs further clarification, as does the exact function of the Ca^{2+} gradient. As these processes are not the result of any obvious, identifiable physiological 'stimulus' in the strict sense of the word, we will not consider $[Ca^{2+}]_i$ with respect to 'normal' pollen tube growth in any further detail here. Several recent reviews have discussed the evidence that Ca^{2+} plays a key role in the regulation of the pollen tube growth in detail [62–65].

A role for Ins(1,4,5)P_3 in the regulation of pollen tube growth. Although there is good evidence for the involvement of extracellular Ca^{2+} in pollen tube growth, increases in intracellular Ca^{2+} and the calcium stores involved are largely uncharacterized in normally growing pollen tubes. However, there are data which provide good evidence that a functional phosphoinositide signal-transducing system, involving IP_3-stimulated increases in $[Ca^{2+}]_i$, plays a role in the regulation of pollen tube growth in *Papaver rhoeas* [22]. Measurable increases in $[IP_3]_i$ could be stimulated in pollen tubes, and these increased levels of IP_3 could result in increases in $[Ca^{2+}]_i$ in growing pollen tubes. Subsequent modulation of pollen tube growth (altered tip morphology, temporary or permanent inhibition of growth, and re-

orientation) correlated well with these events [22]. Data suggest that low-level phosphoinositide turnover and IICR are also required for normal pollen tube growth [22]. This prompts one to envisage a two-tier level of control involving phosphoinositide signaling modulating pollen tube growth. Evidence suggests that low-level turnover of IP_3 is required for normal pollen tube growth, whereas levels consistent with those in 'stimulated' cells result in modulation of growth, including inhibition.

Confocal imaging of Ins(1,4,5)P_3-stimulated increases in $[Ca^{2+}]_i$ in *P. rhoeas* pollen tubes has provided evidence for Ca^{2+} waves which travel towards the pollen tube tip. We will discuss the possible physiological role of these waves later. Data suggest that heparin-sensitive IP_3 receptors and IICR play a role in Ca^{2+} wave propagation in the pollen tube. A model has been suggested whereby the wave is propagated by Ca^{2+}-dependent positive feedback loop on pollen PIC, resulting in a cascade of increasing IP_3 generation and Ca^{2+} mobilization [22]. The nature of these Ins(1,4,5)P_3-sensitive Ca^{2+} stores remains to be established, but there is evidence, in contrast to plant systems characterized in detail to date, that suggests that rather than the vacuole, the ER may be the major Ca^{2+} store implicated in this response [12, 13, 22].

Ca^{2+} is involved in the reorientation of pollen tube growth. How the perception of extracellular signals leads to modulation of growth patterns and directionality of plant cells is currently a key question in the plant-signaling field. Alterations in $[Ca^{2+}]_i$ clearly have the potential to act on many cellular processes. Although changes in $[Ca^{2+}]_i$ are clearly linked to pollen tube growth, the mechanisms involved remain unclear. There remains much speculation as to what are the cues and signals which initiate the necessary changes in directional growth of pollen tubes as they grow through the pistil, and these were briefly mentioned earlier in this review. However, these components have not, to date, been tested to see if they alter the $[Ca^{2+}]_i$ of the pollen tube. However, studies aimed at elucidating the controls involved in reorientation of pollen tubes is beginning to throw some light on some of the control mechanisms. Below we discuss some of the evidence for the involvement of Ca^{2+} signaling in modulating pollen tube growth.

Using Ca^{2+}-imaging, a series of studies by Malhó et al. [23, 52, 60] have established a second messenger role for Ca^{2+} in the control of directional growth in *Agapanthus* pollen tubes. Several treatments, all of which resulted in transient elevations of $[Ca^{2+}]_i$, perturb the polarity of the pollen tube, which results in the temporary inhibition of growth (with loss of the apical Ca^{2+} gradient). This was followed by tip swelling and coincided with the establishment of a new apical Ca^{2+} gradient as growth resumed, usually in a different direction [52, 60]. Figure 2B illustrates the rapid reorientation of pollen tube growth, stimulated by localized release of caged Ca^{2+} to one side of the pollen tube [23]. The direction of reorientation could be predicted accurately using fluorescence ratios of the left versus the right of the pollen tube [23]. Modification of external Ca^{2+} locally, to one side of the pollen tube, also resulted in reorientation.

These data, therefore, provide strong evidence that reorientation of pollen tubes can be determined by localized alterations in $[Ca^{2+}]_i$. Use of 'manganese quenching' has allowed the measurement of Ca^{2+} channel activity more directly. Inhibited pollen tubes show greatly reduced Ca^{2+} channel activity, but when they start to swell, prior to reinitiation of growth, considerable localized Ca^{2+} channel activity in the first 20 μm of the pollen tube was measured [60]. These data suggest that Ca^{2+} channel activity in the apical dome plays a critical role in determining pollen tube reorientation. Data suggest that slight alterations in the exact localization of high Ca^{2+} channel activity within the tip region is likely to determine directional growth in pollen tubes. The nature of the type of Ca^{2+} channels is unknown.

Together, these data suggest that spatial alterations in $[Ca^{2+}]_i$ can control the direction of pollen tube growth. Further investigations into the factors involved are required, and it should be borne in mind that there are likely to be many other factors controlling directional growth of the pollen through the pistil tissues towards the ovary. At this stage it would be naive to assume that Ca^{2+} is likely to be the sole means of regulating directional growth.

The role of Ca^{2+} in the SI response of *Papaver rhoeas*. SI is one of the most important genetically controlled mechanisms used by plants to regulate the acceptance or rejection of pollen, in order to prevent fertilization by unwanted pollen. This is, in many species, controlled by a single, multiallelic *S*-locus. Self-fertilization is prevented when pollen carrying an *S*-allele which is genetically identical to that carried by the pistil on which it lands is inhibited, whereas pollen carrying other *S*-alleles is not. SI is an example of a very precise cell-cell communication and signaling system, whereby inhibition of pollen tube growth is a response to a precise, defined signal. Several alleles of the stigmatic *S*-gene of *Papaver rhoeas*, the field poppy, have been cloned and sequenced [66–69]. They encode small, basic glycoproteins (14–15 kDa), and both stigmatic extracts and recombinant S-proteins have been shown to have S-specific biological activity (i.e. they inhibit incompatible pollen). It therefore provides a good model system for studying signal-response coupling. The available data suggest that the stigmatic S-proteins act as signal

molecules that, when perceived by the alighting pollen as 'self', initiate pollen tube inhibition.

Calcium imaging of pollen tubes challenged with S proteins has provided good evidence that Ca^{2+} acts as a second messenger mediating pollen inhibition in the SI response [12, 13, 56]. Transient increases in $[Ca^{2+}]_i$ in pollen tubes were only triggered when biologically active S proteins were used in combination with incompatible pollen. The peak increases in $[Ca^{2+}]_i$ coincided with the cessation of pollen tube growth, and release of caged Ca^{2+} was used to demonstrate a direct link between increases in $[Ca^{2+}]_i$ and the biological response. These data all point to increases in $[Ca^{2+}]_i$ being involved in inhibition of pollen tube growth, and suggest that the S proteins achieve their effect by stimulating changes in $[Ca^{2+}]_i$.

Early imaging studies of pollen tubes responding to the SI reaction suggested the SI-stimulated Ca_i^{2+} increases were localized in the 'shank' of the pollen tube [12, 13, 70]. Recent studies using ratiometric Ca^{2+} imaging confirmed this and provided further evidence for the propagation of Ca^{2+} waves in pollen elicited by the SI response [56]. Figure 2C illustrates a pollen tube $[Ca^{2+}]_i$ response to addition of incompatible S proteins in both the apical region and the 'shank' region. S-specific increases in $[Ca^{2+}]_i$ were visualized in the subapical and shank regions of the pollen tube virtually immediately after S proteins were added, and $[Ca^{2+}]_i$ in this region increased over several minutes, increasing from ~ 200 nM to > 1.5 μM (as shown in the right hand section of fig. 2C). A coincident diminution of the apical Ca^{2+} gradient was observed at the pollen tube tip (as shown in the left-hand section of fig. 2C), which following some fluctuation was reduced to basal levels within ~ 1 min [56]. These changes appeared to involve not only increases but also a redistribution of $[Ca^{2+}]_i$ and suggested that some of these alterations in $[Ca^{2+}]_i$ might be interpreted as a calcium wave.

We have previously discussed a possible mechanism for the generation of Ca^{2+} waves in pollen tubes with respect to the PI-signaling pathway. The source of the Ca^{2+}, the mechanisms involved in Ca^{2+}-release and the nature of these Ca^{2+} waves with respect to this SI-specific stimulus are unknown, and require further investigation. Clearly IP_3-induced Ca^{2+} release could be involved, though other stores and pathways could also be implicated. Nevertheless, it seems clear that alterations in Ca_i^{2+} in regions of the pollen tube other than the apical region are crucial for some processes regulating pollen tube growth.

Important questions arising from these studies include the question of how these changes in $[Ca^{2+}]_i$ might signal the arrest of pollen tube growth. It is tempting to speculate that it is merely the loss of the apical $[Ca^{2+}]_i$ gradient that mediates this, and based on the importance of this gradient for the growth of pollen tubes in all species investigated, there can be little doubt that this plays a role. However, SI-induced pollen tube inhibition becomes irreversible within ~ 20 min, even when the inhibitory S proteins are removed [71]. Changes in the phosphorylation of pollen proteins are also observed subsequent to the changes in $[Ca^{2+}]_i$ [71, 72], and there is evidence for specific gene transcription in incompatibly challenged pollen [73]. This suggests that the elevated $[Ca^{2+}]_i$ also serves to signal downstream components that probably play key roles in the irreversible inhibition of pollen tube growth. Later discussion will highlight what some of these intracellular targets might be.

An involvement of Ca^{2+} signaling in the fertilization event. Recent work, using confocal imaging of in vitro fertilization in *Zea mays* has demonstrated changes in the $[Ca^{2+}]_i$ of egg cells upon fertilization by isolated pollen sperm cells [74]. A single slow, but transient, elevation in $[Ca^{2+}]_i$ occurred in the egg cell following the fusion of a sperm cell. The spatial and temporal characteristics were strikingly similar to those observed in animal cells during fertilization. It is clear that the interaction between gametes during reproduction in higher plants involves dynamic changes in $[Ca^{2+}]_i$, a situation that has been observed for algae [75, 76], sea urchins [77] and mammals [78]. The spatial and temporal characteristics of these increases vary to some extent, though they all appear to take the form of a Ca^{2+} wave across the cell, originating from the point of fertilization. It will be interesting in the future to see what this information encodes, with respect to the subsequent fertilization processes.

A role for Ca^{2+} signaling in the growth of root hairs

The growth of root hairs closely resembles that of pollen tubes, and appears to be typical of tip-growing cells in general. It has been demonstrated that apical gradients of $[Ca^{2+}]_i$ are also correlated with growth in root hairs [79–84]. Analysis using Ca^{2+}-selective vibrating probes first revealed that Ca^{2+} influx was localized almost exclusively to the tips of growing root hairs of *Sinapis* [79]. More recently, apical $[Ca^{2+}]_i$ gradients have been imaged in root hairs from several species [81–84]. It is of interest that the levels of $[Ca^{2+}]_i$ measured [81, 82] are similar to those in pollen tubes, with tip $[Ca^{2+}]_i$ being broadly comparable (up to > 1 μM) to those in stimulated cells, and the $[Ca^{2+}]_i$ in the regions behind (100–200 nM) being comparable to quiescent cells.

There is strong evidence from a variety of sources that Ca^{2+} influx is responsible for the apical gradient, and is correlated with growth. This suggests that these fluxes are essential for tip growth and may be involved in

directing growth in both root hairs and pollen tubes. As expected, inhibition of the apical $[Ca^{2+}]_i$ gradients by a variety of treatments generally results in inhibition of growth. Furthermore, $[Ca^{2+}]_i$ distributions during root hair development in *A. thaliana* have recently been described [82], and studies of the *rhd-2* mutant, defective in sustained root hair growth, have provided strong evidence that implicates $[Ca^{2+}]_i$ in regulating the tip growth process. However, there are data, using *Limnobium* root hairs, which suggest that the magnitude of the Ca^{2+} flux entering the root hair tip does not determine growth rate [80].

Regulation of root hair directional growth. The question of whether this tip-based $[Ca^{2+}]_i$ gradient orientates apical growth has been investigated [83]. An asymmetrical calcium influx across the root hair tip, which was artificially generated, stimulated a change in the direction of tip growth towards the high point of the new $[Ca^{2+}]_i$ gradient. However, this reorientation, unlike those stimulated in pollen tubes, was only temporary, and the root hairs soon established growth in the original direction. Similarly, in root hairs which had their path of growth blocked by mechanical means, reorientation occurred, but as the root tip passed the obstacle, growth returned to the original direction. Ca^{2+} imaging revealed that when the root hair changed direction, the gradient also reoriented, and when growth returned to the original direction, so did the $[Ca^{2+}]_i$ gradient. Thus, the tip-focused $[Ca^{2+}]_i$ gradient was always centred at the site of active growth. These data suggest that while the tip-focused $[Ca^{2+}]_i$ gradient is an important factor in mediating apical growth, it is not the primary determinant of directional growth in root hairs. It suggests that root hairs either have a predetermined direction of growth, or that the root hair growth process is less susceptible to directional signals than extending pollen tubes. Additional cues, therefore, are likely to regulate root hairs in a manner different from those regulating pollen tubes. One suggestion is that they may have predetermined polarity.

Ca^{2+}-signaling during the symbiosis of root hair cells and *Rhizobium*. The ability to fix atmospheric nitrogen is facilitated by the symbiotic relationship between leguminous plants and *Rhizobium* bacteria. This provides us with another example of a physiological process whereby changes in $[Ca^{2+}]_i$ appear to play key cell-signaling roles. In brief, this process involves the interaction between bacterial-derived Nod factors and root hairs at a specific developmental stage [85]. This is when they have just, or are about to, arrest polarized growth. The Nod factors themselves are race-specific lipochitooligosacharides. Although the initial stages of the interaction with respect to the identification of Nod factor receptors awaits elucidation, the interaction is known to induce several distinct physiological responses of the root hairs. First, the root hair undergoes 'deformation'. Subsequent swelling of the root hair is followed by 'curling' which appears to be outgrowth from that swelling [86, 87].

As mentioned above, root hair deformation is one of the earliest responses to Nod factors, and occurs some 60 min or so after application. This is followed by reinitiation of root hair extension. The hypothesis that root hair deformation in *V. sativa* (vetch) induced by Nod factors is the reinitiation of growth directed by reestablishment of an apical Ca^{2+} gradient has been recently tested [84]. This showed that root hairs terminating growth were susceptible to deformation, and demonstrated that these root hairs respond initially by tip swelling, followed by reestablishment of polarity, with a new tip emerging from the swelling. Using Ca^{2+} imaging, these hairs were shown to have characteristics typical of tip-growing cells, including a tip-focused calcium gradient of 1–2 μM at its peak. As illustrated in figure 2E, the most recently formed 'zone I' root hairs, which still exhibit tip growth, had a high apical $[Ca^{2+}]_i$ (as shown in panel i), whereas older root hairs which have ceased to extend did not (see panels ii and iii). This confirms the idea that root hair growth is correlated with a tip-focused gradient, as in pollen tubes. Swollen tips of root hairs treated with Nod factor exhibit a $[Ca^{2+}]_i$ that is 6–10 times higher than in untreated root hairs. This high $[Ca^{2+}]_i$ soon polarized, and tip growth was reactivated, as illustrated in figure 2E (panels iv, v and vi). These data provide good evidence that reinitiation of growth, mediated by $[Ca^{2+}]_i$, is responsible for Nod factor-induced deformation of root hairs [84].

A clear role for $[Ca^{2+}]_i$ in the mediation of these characteristic responses of root hairs to Nod factors has recently been established. Ca^{2+} imaging in root hairs of *Vigna unguiculata* has enabled the identification of a very early Ca^{2+} response to *R. meliloti* Nod factors, within seconds of the stimulus being applied [88]. There appeared to be a sustained increase in $[Ca^{2+}]_i$, up to a plateau. Although a distinct subcellular locale for this increase could not be defined, it was possible to demonstrate a direct correlation between the increases in $[Ca^{2+}]_i$ and the curling and deformation responses of the root hair. Ca^{2+} imaging has been used to study the responses of root hairs of *M. sativa* (alfalfa) following the application of *Nod* factors from *R. meliloti* [89]. After a lag period of ~ 9 min, repetitive transient elevations in $[Ca^{2+}]_i$ in the form of asymmetric Ca^{2+} 'spikes' were observed, in response to addition of Nod factors. These oscillations, with a periodicity of 60 s, continued for several hours. Unexpectedly, the spatial patterns of the changes in $[Ca^{2+}]_i$ were distinctive (as illustrated in fig. 2D), and originated in the nuclear region of the root hair [89]. The increases in $[Ca^{2+}]_i$ in the nuclear region were ~ 700 nM and are comparable

to those in stimulated cells. It is of interest to note that, although most root hairs respond by producing Ca^{2+} spikes, the timing of the responses of adjacent root hairs were apparently not coordinated.

These studies suggest that there are at least three distinct phases or patterns to the Ca^{2+} increases produced in response to Nod factors in root hairs. These comprise an early plateaulike increase in $[Ca^{2+}]_i$, which is followed by transient repetitive increases, in the form of Ca^{2+} spikes. Finally, a reinitiation of tip growth is stimulated, which is accompanied by a reestablishment of high apical $[Ca^{2+}]_i$. We will discuss the significance of differences in the spatial and temporal patterns in $[Ca^{2+}]_i$ later.

The study of changes in $[Ca^{2+}]_i$ observed in whole plants and tissues in response to environmental stimuli

We now move on to studies using the photoprotein aequorin. The use of this technology has enabled the study of Ca^{2+}-based signal-response coupling in whole plants or tissues, rather than generally being limited to studying Ca^{2+} responses in individual cells (see earlier). This approach has so far been used to investigate more environmental stimuli, such as variations in wind and temperature and the response to touch. This contrasts with the systems previously described, which have concentrated on more specific signal molecules as stimuli. These types of stimuli affect the final morphology of a plant by influencing aspects of 'plant form' [90]. It is therefore of interest to investigate how these stimuli may bring about morphological changes and to investigate a role for Ca^{2+} as a second messenger, which may mediate these responses.

Demonstration of an involvement of $[Ca^{2+}]_i$ in mediating these types of environmental responses was obtained by subjecting *Nicotiana plumbaginifolia* seedlings to touch stimuli. A consequent dramatic elevation in $[Ca^{2+}]_i$ was observed in the seedlings [26]. Reductions in external temperature from 20 °C to 0–5 °C also resulted in transient increases in $[Ca^{2+}]_i$ [26]. Interestingly, changes in $[Ca^{2+}]_i$ were not observed for transition from ambient to higher temperatures, including those thought to represent a heat shock. This clearly suggests that $[Ca^{2+}]_i$ plays a signaling role in the chilling or freezing tolerance of the seedling, but not in heat shock responses. It is worth noting that the timing of the elevations in $[Ca^{2+}]_i$ observed in response to the different stimuli varied. For example, very short transients were induced by touch, and longer transients were observed in response to cold shock.

Subsequent studies, using synthetic aequorins with higher luminescence signals [27], has enabled spatial information to be obtained by imaging $[Ca^{2+}]_i$. For instance cold shock was observed to have a dramatic effect by increasing the $[Ca^{2+}]_i$ in the cotyledons and roots of seedlings, but had little effect on $[Ca^{2+}]_i$ in hypocotyls [27]. The cells in the cotyledons displayed an increase, and then subsequent decrease over time, that suggested a 'wavelike' response. A small localized number of cells initially respond before other cells, distributed in adjacent areas, respond by increasing $[Ca^{2+}]_i$ in the same manner [27]. This type of response, for a tobacco leaf exposed to a cold shock, is shown in figure 2F. This shows sequential photometric images of a leaf, taken 10 s apart, following cold-shock treatment and illustrates the transient increase in the $[Ca^{2+}]_i$ of the cells of the leaf. It is of interest to note that not all of the cells respond in the same way and at the same time.

Studies have shown that even if a whole seedling is subjected to the same stimulus, different tissues appear to have differential sensitivity. Cotyledons appear to be significantly less sensitive to cooling than roots. For example, $[Ca^{2+}]_i$ in root tissue of *N. plumbagifolia* seedlings was observed to respond to controlled temperature reductions ahead of the cotyledons [91]. This suggests that the temperature-sensing mechanisms of these two tissues vary, presumably in line with their unique physiological requirements. Application of a stimulus to a defined region of the plant has established that long-range signaling is also likely to play a role in these types of responses. By subjecting the roots, but not the shoots, of a plant to cold treatment, it was observed that increases in $[Ca^{2+}]_i$ were stimulated in the leaves, which were some distance from the site of the stimulus [91]. This was the first suggestion of a coordinated response of a whole plant, using long-range Ca^{2+} signaling [91]. However, despite an apparently identical level of stimulation, not all the leaves responded, and those that did respond did not respond in the same way. We will discuss the significance of this later.

It is becoming apparent that $[Ca^{2+}]_i$ from different pools may be mobilized by different stimuli. Studies on the response of seedlings to environmental stimuli [92] suggest that touch and wind stimulation mobilize the same Ca^{2+} pools. This is thought to involve Ca^{2+} release from intracellular stores. The cold shock response, on the other hand, appears to largely utilize extracellular Ca^{2+} ($[Ca^{2+}]_e$). Further technological advances, which have allowed aequorin to be targeted to distinct intracellular, subcellular locations has enabled more detailed analysis of subcellular localization of changes in $[Ca^{2+}]_i$. Apo-aequorin expressed both in the cytosol and on the cytoplasmic face of the vacuole was used to report fluctuations in $[Ca^{2+}]_i$ in distinct subcellular 'microdomains' of *A. thaliana* seedlings [31]. Again, the effect of cold shock resulted in a large increase in $[Ca^{2+}]_i$. The largest increases in $[Ca^{2+}]_i$ were measured in the cytosol. In contrast, $[Ca^{2+}]_i$ adjacent to

the vacuolar membrane was much lower than this, but took longer to return to resting levels [31]. This significant observation clearly indicates differences in spatiotemporal changes in $[Ca^{2+}]_i$, which suggests the utilization of different pools of Ca^{2+} which can be simultaneously mobilized by a single stimulus. There is some evidence that suggests that IICR from the vacuole is a feature of the cold or 'chilling' response [31].

The question of specificity for plant cell Ca^{2+} responses

There is obviously more information encrypted in the changes in $[Ca^{2+}]_i$ of a cell than would immediately meet the eye. If we consider the guard cell studies described earlier, it is clear that the responses to auxin and ABA, which have opposite effects on guard cell turgor and stomatal aperture, are both mediated by increases in $[Ca^{2+}]_i$ [30, 32]. How this may be accomplished has led to discussion of the elements that could aid in the generation of specificity of response via changes in $[Ca^{2+}]_i$.

Mechanisms whereby specificity can be encoded include the concept of the 'calcium signature' [93, 94]. Certain features of Ca^{2+} signals are thought to 'encrypt' the information that is necessary for the generation of a specific response to a stimulus. Information may be passed on, within and between cells, using temporal and spatial patterning. This could use digital 'encoding', in the form of variations in amplitude of the increases in $[Ca^{2+}]_i$ that are often referred to as Ca^{2+} transients. The duration of the transient is also likely to contain information. In situations where repetitive transients (oscillations) are observed, the frequency of these is likely to encode information.

Spatial patterning is also regarded as important. There may be heterogeneity within the cell and or tissues of the plant. Localization of the increases in $[Ca^{2+}]_i$, and their origin and propagation through the cell and tissues may be important. Alterations in $[Ca^{2+}]_i$ may be regionally confined, or they may take the form of Ca^{2+} waves, and move across cells or tissues. Coordination of these intracellular responses is likely to be important.

The strength of the signal, and whether $[Ca^{2+}]_i$ reaches a threshold, may determine the resulting response. In animal cells this information appears to be in the form of quarks, blips, sparks, puffs [15] which all occur in highly localized regions of the cell, and are all regarded as fundamental and elementary changes in $[Ca^{2+}]_i$. Ca^{2+} spikes, oscillations and waves [15] are believed to result from the recruitment of these elementary release events, which have been visualized in single cells [17, 18].

Temporal patterning and 'calcium signatures'

We have described several model plant systems which have been demonstrated to use $[Ca^{2+}]_i$ as a second messenger. These include stomatal guard cells responding to ABA, pollen tubes responding to reorientation stimuli and SI proteins; root hairs responding to Nod factors; and leaves responding to chilling (see fig. 2). From the patterns of changes in $[Ca^{2+}]_i$, it is clear that there are a variety of calcium signatures generated.

Digital encoding of the calcium signals and the formation of a calcium signature in the form of amplitude, duration and frequency of the increases in $[Ca^{2+}]_i$ are thought to be one way in which specificity is encoded. Using the example of *N. plumbaginifolia* seedlings, different stimuli, in the form of chilling, wind and touch, elicit transient increases in $[Ca^{2+}]_i$. However, the temporal nature of these transients varies [26]. As each of these stimuli elicits specific characteristic responses in the plants, the duration of Ca^{2+} transients are interpreted to be specific signals. Stomatal guard cells and their response to ABA provide another example of the generation of $[Ca^{2+}]_i$ transients. Whereas some stimuli trigger a single transient, other stimuli may trigger multiple increases in the form of oscillations. Furthermore, the $[Ca^{2+}]_i$ responses in both guard cells and in *N. plumbaginifolia* protoplasts have been found to be directly proportional to the strength of the stimulus, and this affects the pattern of the oscillations [40, 95]. This is an important observation that is consistent with the responses of mammalian cells to changes in the concentrations of agonists [17], suggesting that the same underlying mechanisms for controlling and relaying information through changes in $[Ca^{2+}]_i$ may apply.

The same stimulus can also trigger different responses with respect to $[Ca^{2+}]_i$. One example which we have described is that of the root hair to Nod factors. In root hairs of *Vigna unguiculata*, a very rapid increase in $[Ca^{2+}]_i$, within seconds of *R. meliloti* Nod factor being applied, was observed; this was sustained in the form of a plateau [88]. A later response has been observed in *M. sativa* [89]. Following the application of *R. meliloti* Nod factors, there was a lag period of ~ 9 min, after which repetitive asymmetric Ca^{2+} 'spikes' were observed, as illustrated in figure 2D. These oscillations, with a periodicity of 60 s, continued for several hours [89]. Even later than these responses, increases in $[Ca^{2+}]_i$ in swollen root hairs of *Vicia sativa*, which precede reinitiation of tip growth are observed in response to *R. leguminosarum* bv *viciae* Nod factors [84]. Although these are all responses to Nod factors in root hairs, the calcium signatures are clearly different, and they may be responsible for different aspects of the response. Although it is not yet clear, the amplitude, duration and frequency of these increases in $[Ca^{2+}]_i$ may all contribute to the specific responses within the plant cell.

Spatial patterning and 'calcium signatures'

Although the amplitude, duration and frequency of these increases in $[Ca^{2+}]_i$ are important in encoding specificity of response, the spatial patterning of these increases is considered to be equally important. Figure 2 illustrates some of the diversity in the spatial patterning of alterations in $[Ca^{2+}]_i$. There is clearly heterogeneity with respect to the $[Ca^{2+}]_i$ response to stimuli, and the localization of increases in $[Ca^{2+}]_i$ are generally not uniformly distributed within the cell/tissues. For example, within the cytoplasm of the guard cell, there are localized 'hot spots' of high $[Ca^{2+}]_i$ triggered in response to ABA, as illustrated in figure 2A. In some situations, this localization has been correlated with distinct regions of the cell. For example, in root hairs stimulated by Nod factor, increases in $[Ca^{2+}]_i$ are clearly highly localized (as shown in fig. 2D) and associated with the region of the cell containing the nucleus [89]. Similar localization of transient increases in $[Ca^{2+}]_i$ in the 'nuclear region' have been observed in pollen tubes stimulated by S proteins [12, 13]. This may point to the specific use of particular Ca^{2+} stores in certain responses. The specific distribution and positioning of increases in $[Ca^{2+}]_i$ may also be important in sending messages to particular intracellular targets with distinct localities within the cell.

The ability to elicit the formation and propagation of Ca^{2+} waves has been observed in plant cells [22, 56, 74], as illustrated for a pollen tube in figure 2C, is another means whereby specificity may be encoded by spatial patterning of $[Ca^{2+}]_i$ within the cell. In this way, relatively small increases in $[Ca^{2+}]_i$ may be elicited in a defined region of the cell, and then this message may be propagated to other parts of the cell. This propagation is likely to involve generation of further increases in $[Ca^{2+}]_i$ which spread across or through the cell in a particular pattern. This has been likened to a 'flood' or a 'tide' of $[Ca^{2+}]_i$. This wavelike movement of $[Ca^{2+}]_i$ may propagate the signal within the cell. In this way, the response of a cell may be integrated using information from a single message (the original point source of Ca^{2+}). Ca^{2+} waves appear to be a distinctive feature of fertilization, in animal cells [96], algal cells [76] and plant cells [74]. This may be a characteristic 'Ca^{2+} signature' evoked by the fertilization event, with increases in Ca^{2+} propagating from the point of initial fertilization across the cell. In contrast, the Ca^{2+} waves observed in pollen tubes clearly have a different mode of action (see fig. 2C), and the result is inhibition of growth.

The concept of 'physiological address'

In order to explain the considerable variability of responses of cells to specific stimuli, the concept of 'physiological address' in plant cells has been suggested [93, 94]. During development, individual cell types, tissues and, indeed, whole plants, are exposed to a variety of stimuli. Depending on the combination of these stimuli, it is thought that they acquire a unique complement of signaling components. In this way, it is thought that different cells, even if they are adjacent to each other, may respond differently to the same stimulus. We have already previously described some of the variability in responses to the same stimuli, for example for guard cells [20, 33, 34], for root hairs [89] and for seedlings exposed to environmental stimuli [91]. It has been established that, with respect to the stomatal guard cell response to ABA, the temperature and water stress that the plants were previously exposed to dictated whether increases in $[Ca^{2+}]_i$ were observed to precede stomatal closure [34]. This illustrates the importance of the concept of 'physiological address'. This unique programming, therefore, currently provides a good explanation for the unique responses of cells, tissues and plants to particular stimuli.

While specificity is likely to involve both calcium signature and physiological address, there is obviously further complexity within the system, which may allow 'fine tuning'. The concept of 'cross-talk' between signaling pathways is likely to play a role in this. By this we mean that pathways are unlikely to be exclusive and linear. Completely different pathways are likely to operate at the same time, and it is the communication between these 'independent' pathways that is likely to be important in generation of specificity. A further consideration for the generation of a specific response via changes in $[Ca^{2+}]_i$ therefore probably also includes the distribution of 'downstream' signaling components that exist as targets for the increase in $[Ca^{2+}]_i$.

The intracellular targets of elevated $[Ca^{2+}]_i$

The nature of some of the intracellular targets for elevated $[Ca^{2+}]_i$ are addressed in other articles within this issue, and we will therefore only highlight a selected few of these. Among the elements which may be triggered by a Ca^{2+}-signaling pathway are protein kinases and phosphatases, which may influence gene expression. Other components affected by $[Ca^{2+}]_i$ may be more structural, such as the cytoskeleton and exocytosis.

A signaling role for $[Ca^{2+}]_i$ in the activation of gene transcription has been identified for the response of plants to red/far-red light. This stimulus is detected by a soluble receptor referred to as phytochrome A (*PhyA*) and results in the development of chloroplasts and anthocyanin synthesis, which involves gene transcription [97]. As in the absence of red light increases in $[Ca^{2+}]_i$-stimulated gene expression and chloroplast development [97, 98], this provides evidence that Ca^{2+} signals can be decoded to influence gene transcription.

One way in which $[Ca^{2+}]_i$ may pass on information within the cell is through Ca^{2+}-binding proteins such as calmodulin (CaM) and calcium-dependent CaM-independent protein kinases, which are generally now known as calmodulin-domain-like protein kinases (CDPKs) [99, 100]. CaM and CDPKs have been identified in a variety of subcellular locations in plants [101]. This suggests that they are likely to play a key role in decoding the information encrypted in spatiotemporal changes in $[Ca^{2+}]_i$. As the name suggests, CDPKs contain a CaM domain as part of the structure of the native enzyme and are therefore directly activated by Ca^{2+} [100, 101].

Both animal and plant cells are known to possess CaM, which mediates a wide variety of the cellular responses to elevated $[Ca^{2+}]_i$. Activated CaM (i.e. Ca^{2+}-bound) has been shown to induce gene expression in plant cells [97], which suggests a key role for Ca^{2+}-CaM in mediating developmental responses. This also provides evidence that CaM is an important downstream target for elevated $[Ca^{2+}]_i$ in plants. We have already discussed the role of elevated $[Ca^{2+}]_i$ in plants in response to touch stimulation [26, 92]. There is good evidence that this stimulus can result in the increased expression of a family of CaM-related genes, referred to as the *TCH* genes [102, 103]. These examples imply that increases in $[Ca^{2+}]_i$ may transmit messages to the nucleus, thereby activating gene transcription, through the activities of Ca^{2+}-binding proteins.

Other targets thought to be mediated by increases in $[Ca^{2+}]_i$ are more structural in function. One example which we draw attention to is the cytoskeleton. As microfilament and microtubule assembly and disassembly are thought to be influenced by $[Ca^{2+}]_i$ [104], it is also considered likely that the cytoskeleton is directly or indirectly influenced by the changes in $[Ca^{2+}]_i$. There is increasing evidence that the cytoskeleton, as well as having a structural role, may also have a signaling role. In many eukaryotic cells, actin-binding proteins function as stimulus-response modulators, translating signals into alterations in the cytoarchitecture [105]. There is evidence emerging that this is likely to be true for plant systems, and that this may be modulated by alterations in $[Ca^{2+}]_i$. For example, a kinesin-like CaM-binding protein (KCBP) has been identified in *Arabidopsis*, and Ca^{2+}-CaM has been shown to regulate the interaction between KCBP and tubulin. This suggests that this interaction may be regulated by $[Ca^{2+}]_i$ [106]. This clearly indicates 'cross-talk' between signaling pathways and 'structural' elements, whereby the cell architecture may be influenced by these signals. Several of the responses mediated by $[Ca^{2+}]_i$, which we described earlier, such as the root hair deformation response to *Nod* factor [84], the responses of seedlings to wind and touch [26, 92], pollen tube reorientation [23, 52, 60] and inhibition stimulated by S proteins [12, 22, 56] are likely to involve alterations in the cytoskeleton as an end product of the stimulus. Many other targets for Ca^{2+} probably remain to be identified. Their future identification will, no doubt, provide much data towards the elucidation of how the information contained within Ca^{2+} signals are processed in plant cells.

Future perspectives

Ten years ago we had little idea about the levels and distribution of $[Ca^{2+}]_i$ in plant cells. We also had no idea that $[Ca^{2+}]_i$ was involved as a second messenger in these cells. Great leaps forward have been achieved over this time period, and we now have indisputable evidence that $[Ca^{2+}]_i$ has this role in plant systems. We have described some of the well-characterized systems, and we expect more examples of different stimulus-response-coupled systems to emerge in the future. Although there is no doubt that increases in $[Ca^{2+}]_i$ are stimulated in response to physiological stimuli, the mechanisms whereby this is achieved are largely unknown. Future studies will, no doubt, elucidate in detail the pathways involved in the mobilization of $[Ca^{2+}]_i$ under physiological conditions. Combined with further advances in technology, we should expect to look forward to considerable advances in our understanding of Ca^{2+} signaling in plant cells in the future. We have discussed the perceived importance of the Ca^{2+} 'signature'. The spatiotemporal 'encoding' of the signals is a relatively new concept in the mammalian field, and we expect that in the near future the plant field will develop in a similar direction. More detailed information about the downstream elements that are triggered by Ca^{2+} signaling pathways will enable further understanding of stimulus-response coupling mediated by $[Ca^{2+}]_i$.

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