
*Tansley review*

# The evolution of $Ca^{2+}$ signalling in photosynthetic eukaryotes

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## Contents



**Key words:** $[Ca^{2+}]_{cyt}$ signalling toolkit, calcium (Ca), evolution, photosynthetic eukaryotes, plants, signalling.

## Summary

It is likely that cytosolic $Ca^{2+}$ elevations have played a part in eukaryotic signal transduction for about the last 2 Gyr, being mediated by a group of molecules which are collectively known as the $[Ca^{2+}]_{cyt}$ signalling toolkit. Different eukaryotes often display strikingly similar $[Ca^{2+}]_{cyt}$ signalling elevations, which may reflect conservation of toolkit components (homology) or similar constraints acting on different toolkits (homoplasy). Certain toolkit components, which are presumably ancestral, are shared by plants and animals, but some components are unique to photosynthetic organisms. We propose that the structure of modern plant $[Ca^{2+}]_{cyt}$ signalling toolkits may be explained by their modular adaptation from earlier pathways.

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## I. Introduction

Opinion is divided over how many species of eukaryote share the planet with us but, no matter how obscure the species which we consider, the $Ca^{2+}$ ion has always been found to play a role in their signal transduction (Fig. 1). Photosynthetic organisms are no exception to this rule, and many excellent reviews exist to lead the forgetful through the intricacies of plant $Ca^{2+}$ signalling (Sanders *et al.*, 1999; Rudd & Franklin-Tong, 2001; White & Broadley, 2003; Hetherington & Brownlee, 2004). These authors concur that an elevation in the cytosolic $Ca^{2+}$ concentration ($[Ca^{2+}]_{cyt}$) is required in a number of developmental and physiological pathways, and is generated by the $[Ca^{2+}]_{cyt}$ signalling 'toolkit' (Berridge *et al.*, 2000). Broadly speaking, eukaryotic toolkit components fall into one of four categories – stimuli change the rates of influx and efflux pathways to give $[Ca^{2+}]_{cyt}$ elevations which are interpreted by various $[Ca^{2+}]_{cyt}$ sensors (Box 1). The range of stimuli invoking $[Ca^{2+}]_{cyt}$ changes, and the sheer variety of spatio-temporal patterns these changes can take, have lead to speculation over whether

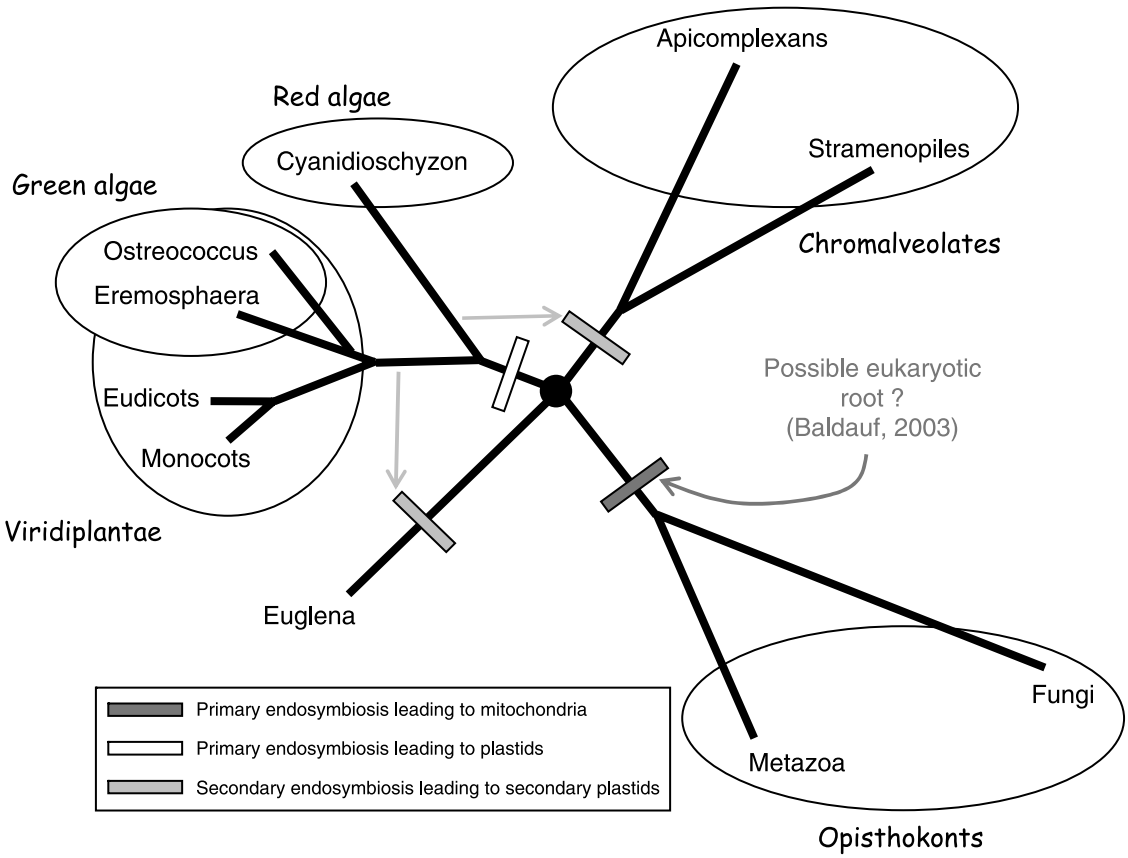



**Fig. 1** The ubiquity of eukaryotic $[Ca^{2+}]_{cyt}$ signalling. This unrooted tree shows the organisms mentioned in this review which have been shown to use $[Ca^{2+}]_{cyt}$ signalling. Distances are roughly indicative of phylogenetic separation, and were calculated by J. H. F. Bothwell using data taken from Baldauf (1999), Yoon *et al*. (2002), Sanderson (2003), Funes *et al*. (2004) and Bhattacharya *et al*. (2004).

specific responses are encoded by specific patterns of $[Ca^{2+}]_{cyt}$ elevations (Scrase-Field & Knight, 2003). It is not our intention to add significantly to this debate. Instead, we would like to focus on an aspect of $[Ca^{2+}]_{cyt}$ signalling which such functional explanations often leave to one side: the relationship between pathway morphology and evolutionary descent.

When faced with any characteristic of a living organism, we tend to ask what purpose it serves. The underlying assumption is that the characteristic has survived millennia of natural selection because it affords the most efficient way of performing a particular task. However, biological systems are not usually considered to have been designed in advance, being pieced together over evolutionary timescales in a manner which has famously led to natural selection being described as a tinkerer, rather than an engineer (Jacob, 1977). This means that a characteristic does not reflect an optimal solution to environmental challenges, but an optimal adaptation to those challenges given the organism's predecessors. Such a characteristic is called an adaptive trait, or said to show adaptation.

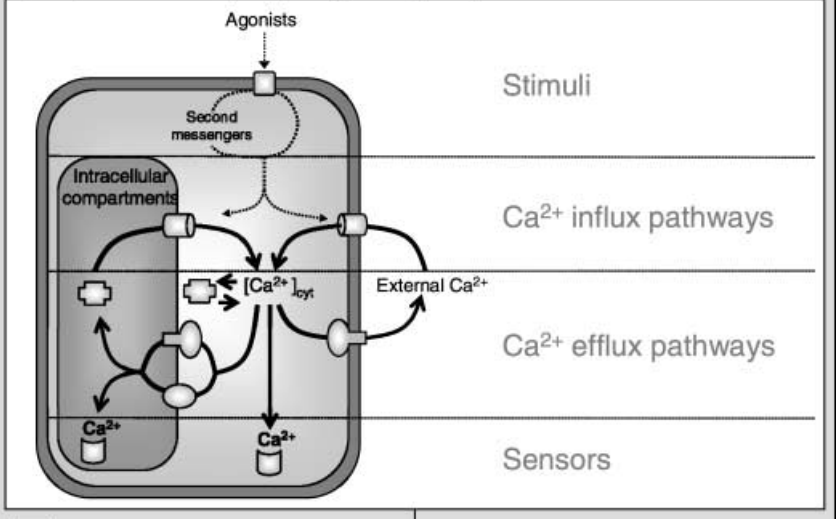
Acknowledgement that $[Ca^{2+}]_{cyt}$ signalling toolkits show adaptation is of particular relevance for two reasons. Firstly, consideration of how $[Ca^{2+}]_{cyt}$ signalling has developed in response to changing selection pressures may help to explain how other complex traits arise. Secondly, understanding that $[Ca^{2+}]_{cyt}$ signalling toolkits have been constructed from a limited set of possible components may shed light on a number of apparent imperfections in $[Ca^{2+}]_{cyt}$ signalling. Without such an outlook, it would be hard to see why some plant kinases have degenerate $Ca^{2+}$-binding subunits which are no longer able to bind the $Ca^{2+}$ ion (Hrabak *et al*., 2003). It would be hard to see why a system based on the precise control of $[Ca^{2+}]_{cyt}$ should mediate $Ca^{2+}$ influx through nonselective channels (White & Broadley, 2003). Indeed, it would sometimes be hard to see why $[Ca^{2+}]_{cyt}$ should be adopted as a signal at all, when a more rapidly diffusing metabolite could presumably do the job as well, if not better. We must, of course, remember that a seemingly ill-adapted process more often reflects faults in our understanding than in evolution. Nevertheless, we shall see that the more we look at plant $[Ca^{2+}]_{cyt}$ signalling, the more we find traces of Jacob's tinkerer.

## II. Homology vs homoplasy

Patterns of $[Ca^{2+}]_{cyt}$ changes are often remarkably similar across species. For example, when treated with 20 mM

## Box 1 | The plant $[Ca^{2+}]_{cyt}$ signalling toolkit

Components of the plant $[Ca^{2+}]_{cyt}$ signalling tookit fall into one of four categories, as shown below. The resulting $[Ca^{2+}]_{cyt}$ elevations adopt one of two patterns; a gradient or a wave. In a gradient the location of peak $[Ca^{2+}]_{cyt}$ remains constant, in a wave it moves. Oscillations may occur in either gradients or waves, and peak $[Ca^{2+}]_{cyt}$ is rarely seen to rise above 1 μM. Non-protein components include metabolites such as NAADP, cADPR, phosphoinositides, and reactive oxygen species, which are broadly conserved across eukaryotes. For more detail on toolkit components, see Day *et al.*, 2002; White & Broadley, 2003; Reddy & Reddy, 2004).




**Stimuli**

Numerous, including:
- Calcium sensor (CAS)
- LysM modulation receptor kinases (NFR1, NFR5)
- S locus
- Blue Light Receptor
- NAPDH oxidase (RBOHC)

**Ion channels**

***Plasma membrane channels***
- Ligand gated (*e.g.* CNGC, GLR)
- Voltage gated (*e.g.* HACC, DACC)

***Endomembrane channels***
- Ligand gated (*e.g.* by $IP_3$)
- Voltage gated (*e.g.* SV, VVCa)

**ATPases**
- $P_{2A}$ type (mainly ER localized), also called ECA
- $P_{2B}$ type (mainly PM localized), also called ACA

**Exchangers**
- $Ca^{2+}$/anion exchanger (CAX)

**$Ca^{2+}$ buffers**
- Endomembrane (often unknown $Ca^{2+}$-binding domain, *e.g.* Calnexin, Calreticulin, Calsequestrin)
- Cytosolic (*e.g.* sensor relays, see below)

**$Ca^{2+}$ binding proteins**

***Sensor relays (bind to effectors)***
- EF-hand (*e.g.* Calmodulins, Calcineurin B-like proteins (CBL))
- Unknown $Ca^{2+}$-binding domains (*e.g.* 14-3-3)

***Sensor responders (contain effectors)***
- EF-hand (*e.g.* $Ca^{2+}$ dependent protein kinases (CDPK), which contain CRK; $Ca^{2+}$/calmodulin-dependent protein kinases (CCaMK))
- C2 (a.k.a. CalB) domain (*e.g.* phospholipases)
- Annexin fold (*e.g.* annexins)

caffeine under certain conditions, the green alga *Eremosphaera viridis* displays $[Ca^{2+}]_{cyt}$ oscillations. These reach $[Ca^{2+}]_{cyt}$ peaks of *c.* 700 nM, display a period of *c.* 1 min and are dependent on extracellular $Ca^{2+}$ (Bauer *et al.*, 1997). They thus bear a remarkable similarity to the response of rat neuronal cells to 5 mM caffeine (Fig. 2), which also show $Ca^{2+}_{ext}$-dependent $[Ca^{2+}]_{cyt}$ oscillations with periods of *c.* 1 min, although the peak $[Ca^{2+}]_{cyt}$ is only *c.* 200 nM (Usachev & Thayer, 1999).

Two extremes bound any explanation for such similarities. The first extreme is homology, in which two patterns in two different organisms are similar because they are generated by the same pathway. This pathway may have been inherited by each organism from a common ancestor, in which case it is called orthologous, or it may have been independently constructed in each organism from the same components, a process which often reflects developmental constraints and which is called parallelism. The second extreme is homoplasy, also known as convergence, in which the efficacy of natural selection in moulding an optimal characteristic leads to the creation of similar patterns from different pathways (Hall, 2003).

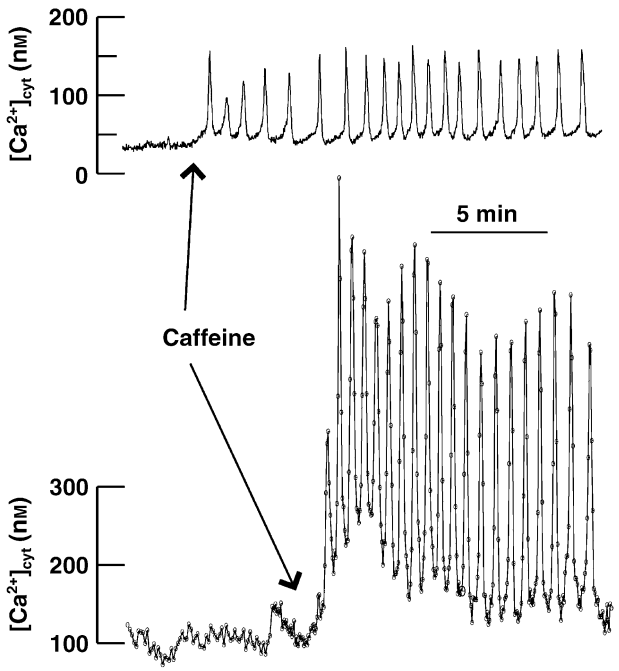



**Fig. 2** Interspecific comparison of caffeine-induced $[Ca^{2+}]_{cyt}$ oscillations. Rat dorsal root ganglion neurones (top) were ester-loaded with Indo-1 AM (© Blackwell Science Ltd. Reproduced, with permission, from Usachev & Thayer, 1999). *Eremosphaera viridis* (bottom) was microinjected with fura-2 10 kDa dextran (Reproduced, with permission, from Bauer *et al*., 1997).

## 1. Probable $[Ca^{2+}]_{cyt}$ signalling homology: nodulation in legumes

Around 30–60 Myr ago (Wikström *et al*., 2001), one eudicot clade of flowering plants developed the ability to host nitrogen-fixing bacteria in a symbiosis characterized by the formation of distinctive nodules on the root hairs of infected plants (Soltis *et al*., 1995). Their descendants include the legumes, a group of crop plants, which are commercially important because the nitrogen-fixing nodules allow them to be grown on poor soils. The formation of nodules is triggered by a bacterial invitation to prospective hosts, in which the bacteria release lipo-chito-oligosaccharide chains, called Nod (for nodulation) factors, and the plants respond by diverting the normal process of root hair development to encompass their bacterial allies.

As part of this diversion of normal development, root hairs display a variety of $[Ca^{2+}]_{cyt}$ elevations which are thought to trigger some, or all, of the necessary changes in metabolism and gene expression. It is currently unclear whether all these $[Ca^{2+}]_{cyt}$ elevations are produced by a single Nod-factor-activated pathway, or whether multiple pathways operate in parallel (Wais *et al*., 2000; Esseling *et al*., 2004). Nonetheless, challenging root hairs of *Medicago sativa* (Ehrhardt *et al*., 1996), *Phaseolus vulgaris* (Cárdenas *et al*., 1999), *Medicago truncatula* (Wais *et al*., 2000; Shaw & Long, 2003), *Pisum sativum* (Walker *et al*., 2000) or *Lotus japonicus* (Harris *et al*., 2003) with nanomolar amounts of Nod factors results in a lag of *c*. 10 min followed by the onset of '$[Ca^{2+}]_{cyt}$ spiking': nuclear localized $[Ca^{2+}]_{cyt}$ elevations which recur, with periods of between 0.5 and 3 min, for a couple of hours (Fig. 3a). In some cases, a distinct second response (Cárdenas *et al*., 1999; Shaw & Long, 2003) results in $[Ca^{2+}]_{cyt}$ elevations near the root hair tip (Fig. 3b).

This conservation of $[Ca^{2+}]_{cyt}$ patterns seems to be matched by conservation of the toolkit components identified so far, which suggests that these responses are homologous. Work on *L. japonicus* nodulation mutants has shown that a pair of Nod factor receptor kinases, NFR1 (Radutoiu *et al*., 2003) and NFR5 (Madsen *et al*., 2003), are required to trigger nodulation. LYK3 and LYK4, the LysM domain-containing receptor-like

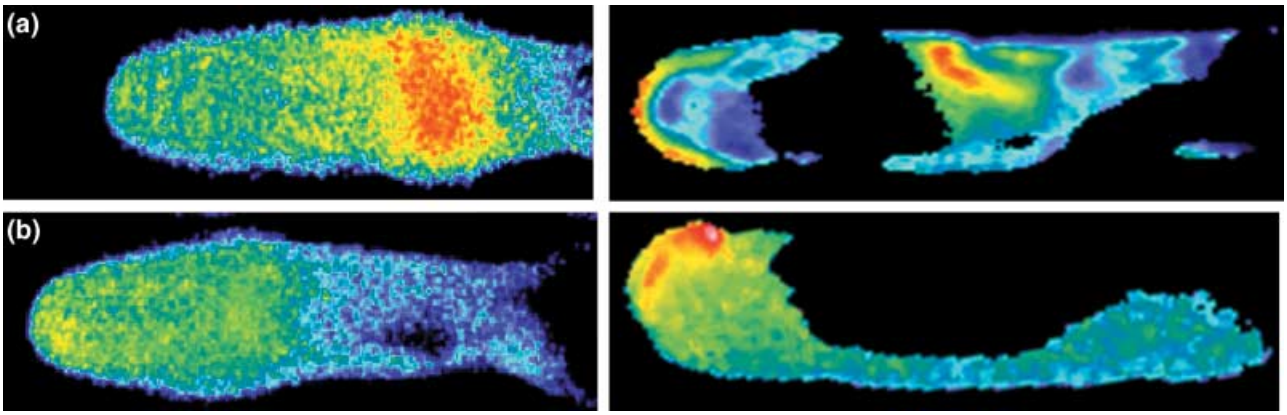



**Fig. 3** Interspecific comparison of Nod factor-induced $[Ca^{2+}]_{cyt}$ spiking. *Phaseolus vulgaris* root hairs (on the right) were microinjected with fura-2 70 kDa dextran (© Blackwell Science Ltd. Reproduced, with permission, from Cárdenas *et al*., 1999); *Medicago truncatula* root hairs (on the left) were microinjected with Calcium Green-1 10 kDa dextran and Texas Red 10 kDa dextran (© American Society of Plant Biologists. Reproduced, with permission, from Shaw & Long, 2003). Each root hair is approx. 10 μM wide. (a) About 10 min after application of 10 nM Nod factor, $[Ca^{2+}]_{cyt}$ spiking is seen in the perinuclear region of root hairs, and oscillations can persist for several hours. (b) Within 10 min of application of 10 nM Nod factor, $[Ca^{2+}]_{cyt}$ elevations are sometimes seen in the tips of root hairs (Ehrhardt *et al*., 1996; Walker *et al*., 2000).

kinases of *M. truncatula*, are NFR1/5 orthologues (Limpens *et al.*, 2003), and, again, without these nodulation will not occur. The identity between NFR1/5 and LYK3/4 is not exact; NFR5 has three LysM domains (Madsen *et al.*, 2003), but NFR1 (Madsen *et al.*, 2003), LYK3 and LYK4 (Limpens *et al.*, 2003) have only two. Given that the LysM domain is thought to be responsible for oligosaccharide binding (Radutoiu *et al.*, 2003), such variation presumably underlies receptor specificity, and may explain why chitin oligomers whose structures are closely related to those of Nod factors elicit $[Ca^{2+}]_{cyt}$ spiking in *P. sativum* (Walker *et al.*, 2000) and *M. truncatula* (Oldroyd *et al.* 2001) but not in *P. vulgaris* (Cárdenas *et al.*, 1999).

A more contentious analysis of $[Ca^{2+}]_{cyt}$-spiking mutants (Wais *et al.*, 2000 vs Esseling *et al.*, 2004) has revealed three further loci named DMI1-3, which do not make bacterial infections and which have orthologues in all legumes studied to date. DMI1 is a novel protein which may form a cation channel and probably interacts with other proteins (Ané *et al.*, 2004). DMI2, which is also referred to as SYMRK (symbiosis receptor-like kinase) or NORK (nodulation receptor kinase), is a membrane-bound serine/threonine protein kinase (Endre *et al.*, 2002; Kistner & Parniske, 2002; Stracke *et al.*, 2002); DMI3 is an orthologue of the *Pisum sym9* gene (Mitra *et al.*, 2004), and a member of the $[Ca^{2+}]_{cyt}$ sensor-responder family known as the $Ca^{2+}$ and calmodulin-dependent protein kinases, or CCaMKs (Lévy *et al.*, 2004).

Exactly which $[Ca^{2+}]_{cyt}$ signalling pathway DMI1-3 belong to is still unclear. Until recently, DMI1-3 were thought to be components in the nuclear $[Ca^{2+}]_{cyt}$-spiking pathway (Wais *et al.*, 2000), but this view may need revision, following the discovery that DMI mutants are able, when handled extremely delicately, to display root hair deformation (Esseling *et al.*, 2004). DMI1-3 may be involved in the tip-high $[Ca^{2+}]_{cyt}$ pathway, rather than the nuclear one, although this remains to be confirmed either way (Shaw & Long, 2003; Esseling *et al.*, 2004).

## 2. Probable $[Ca^{2+}]_{cyt}$ signalling homoplasy: $Ca^{2+}$ release from endomembrane stores

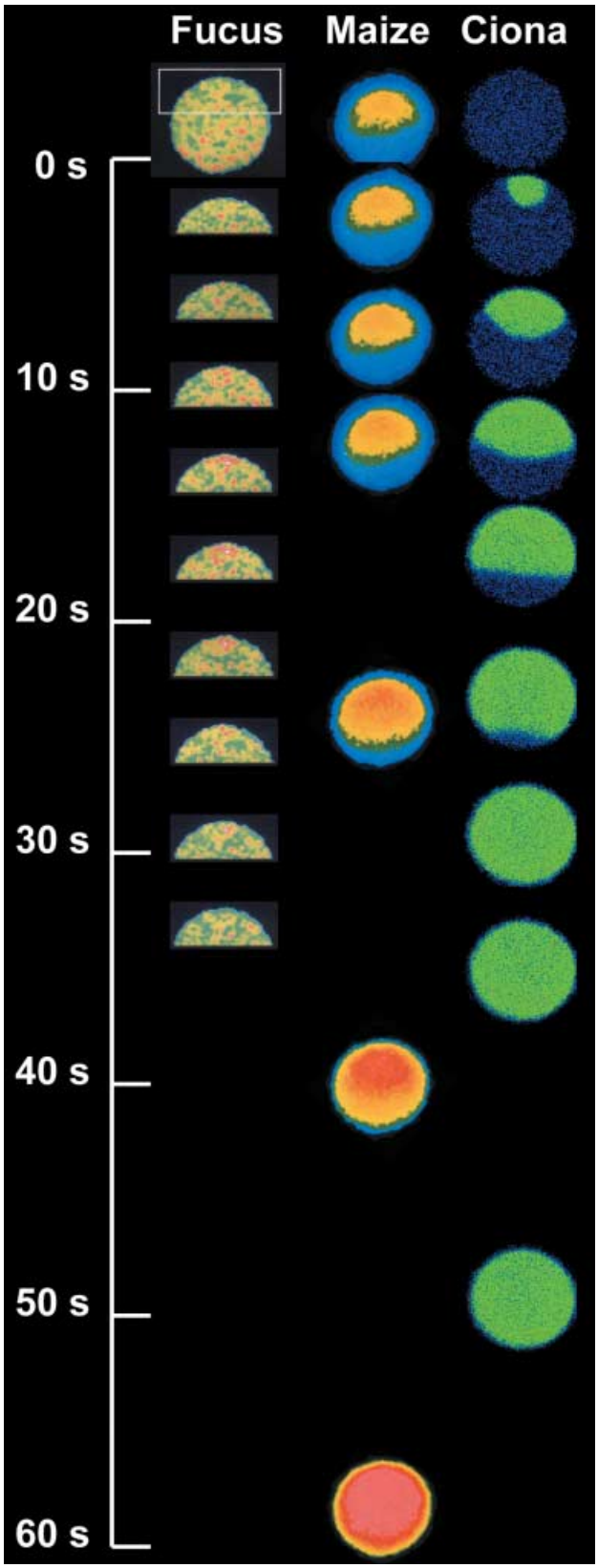
While homology is often an intuitively appealing explanation for any similarities which exist between stimulus-evoked $[Ca^{2+}]_{cyt}$ patterns, convergent evolution of complex traits is not unknown in either the animal or plant world, explaining, among other things, similarities between transcriptional regulation in bacteria and yeast (Conant & Wagner, 2003) and intelligence in animals (Emery & Clayton, 2004).

It is likely that a further example of convergence occurs in the mechanisms by which $[Ca^{2+}]_{cyt}$ elevations are propagated. There is little common morphological ground to be found between metazoa and plants, which diverged between 1 and 2 Gyr ago (Feng *et al.*, 1997; Sanderson, 2003), but the early development of both begins with a sperm fertilizing an egg. In metazoa, sperm entry is accompanied by a $[Ca^{2+}]_{cyt}$ fertilization wave, whose speed is conserved at *c.* 10 μm $s^{-1}$ in all species studied to date (Jaffe, 2002). A ten-fold slower $[Ca^{2+}]_{cyt}$ fertilization wave has been seen in *Zea mays* (Digonnet *et al.*, 1997; Antoine *et al.*, 2000), and no wave at all can be seen in the stramenopile *Fucus serratus* (Roberts *et al.*, 1994) (Fig. 4).

The elucidation of the machinery by which the metazoan fertilization waves, and other metazoan $[Ca^{2+}]_{cyt}$ elevations, are propagated is one of the most elegant achievements of signal transduction research and readers are referred elsewhere for a more detailed description (Berridge *et al.*, 2003). Briefly, metazoan cells have a number of endomembrane $Ca^{2+}$ stores, and agonists can cause $Ca^{2+}$ to be released from any of these by the activation of three main pathways. In the first pathway, agonists bind G protein-coupled receptors. The G proteins then stimulate one of a variety of phospholipase C (PLC) isoforms, which cleave phosphatidylinositol 4,5 bisphosphate ($PIP_2$), generating inositol-1,4,5-trisphosphate ($IP_3$), which binds to the $IP_3$ receptor ($IP_3R$) on the endoplasmic reticulum (ER). In the second pathway, agonists cause intracellular generation of cyclic ADP-ribose (cADPR) and nicotinic acid adenine dinucleotide phosphate (NAADP), both of which are synthesized by the same enzyme, ADP ribosyl cyclase. In the third pathway, $[Ca^{2+}]_{cyt}$ elevation causes activation of ryanodine receptors (RyR) on the ER. Activation of $IP_3R$, the NAADP receptor, or RyR will cause increases in their $Ca^{2+}$ permeablility, and hence release of $Ca^{2+}$ from the ER and other endomembrane stores (Berridge *et al.*, 2003).

The interplay between $Ca^{2+}$ influx and efflux determines the spatio-temporal $[Ca^{2+}]_{cyt}$ pattern, so early attempts to mathematically simulate metazoan $[Ca^{2+}]_{cyt}$ elevations tended to focus on describing the behaviour of the $IP_3R$ using the so-called De Young/Keizer, or DYK, model (De Young & Keizer, 1992). This assumes that the $IP_3R$ consists of three subunits, each having stimulatory $IP_3$- and $Ca^{2+}$-binding sites, as well as inhibitory $Ca^{2+}$-binding sites. When the DYK approximation is combined with terms for $Ca^{2+}$ efflux through a nonlinear pump and $Ca^{2+}$ diffusion through a homogenous cytosol, model predictions match experiment extremely well. Varying the intracellular $IP_3$ concentration ($[IP_3]_{cyt}$) gives rise to different $[Ca^{2+}]_{cyt}$ patterns: a low $[IP_3]_{cyt}$ gives a steady low $[Ca^{2+}]_{cyt}$; increasing $[IP_3]_{cyt}$ gives solitary $[Ca^{2+}]_{cyt}$ waves or oscillations; and high $[IP_3]_{cyt}$ leads to cessation of waves and a steady high $[Ca^{2+}]_{cyt}$ (De Young & Keizer, 1992).

Since there is evidence that NAADP (Navazio *et al.*, 2000), cADPR (Allen *et al.*, 1995; Leckie *et al.*, 1998) and $IP_3$ (MacRobbie, 2000) can also stimulate $[Ca^{2+}]_{cyt}$ elevations in plants, it is often assumed that plants and metazoan use the same internal $Ca^{2+}$-release pathways. Indeed, many of the proteins involved in the metazoan response, such as PLC (Koyanagi *et al.*, 1998; Mueller-Roeber & Pical, 2002) and the $IP_3$-removing inositol phosphatases (Berdy *et al.*, 2001; Xiong *et al.*, 2001; Ercetin & Gillaspy, 2004) have been found in




plants, and their expression is both affected by plant hormones (Hunt & Gray, 2001; Ercetin & Gillaspy, 2004) and required for certain $Ca^{2+}$-dependent pathways such as nodulation (Engstrom *et al.*, 2002) and the stomatal closure response (Sánchez & Chua, 2001; Xiong *et al.*, 2001; Hunt *et al.*, 2003).

Despite these similarities, there are caveats which make us suggest that $Ca^{2+}$ release from endomembrane stores is an example of homoplasy, and not homology. While cADPR is generated in plants, no orthologue to the metazoan ADP-ribosyl cyclase has been found in plant genomes (Sánchez *et al.*, 2004). Similarly, no study has ever shown the existence of orthologues of $IP_3R$ or RyR in plants or algae (Nagata *et al.*, 2004). To complicate matters further, the PLCβ isoform which is activated by a variety of G proteins in metazoa (Hartweck *et al.*, 1997) is not found in sequenced plant genomes, which encode the PLCδ isoform and only one Gα protein (Mueller-Roeber & Pical, 2002). In fact, although it is not seriously doubted that phosphoinositides are important regulators of plant cell physiology, the exact species responsible for plant $[Ca^{2+}]_{cyt}$ elevations have yet to be pinned down. Although a rise in $IP_3$ has been observed to follow hormonal and stress signalling (Hunt & Gray, 2001), and addition of $IP_3$ to isolated vacuoles stimulated $Ca^{2+}$-permeable channels (Allen *et al.*, 1995; Muir & Sanders, 1997), more recent work suggests that $IP_6$ is also competent to stimulate $Ca^{2+}$ release from endomembrane stores (Lemtiri-Chlieh *et al.*, 2003), which has lead to the proposal that $IP_3$ is converted to $IP_6$ and is active in that form. For the moment we can only say that if $IP_3$ is involved in plant endomembrane $Ca^{2+}$ release, it probably isn't generated or perceived as it is in metazoa, which may explain much current confusion among plant physiologists.

It is, however, important to realize that spatio-temporal patterns of $[Ca^{2+}]_{cyt}$ elevations are not inextricably linked to certain molecules. Bearing this in mind, it is instructive to look at a mathematical model which was developed to look at $[Ca^{2+}]_{cyt}$ oscillations in cardiac myocytes, in which the $Ca^{2+}$ release sites are not $IP_3$ receptors, but the poorly characterized RyR. This is the Fire-Diffuse-Fire (FDF) model (Keizer *et al.*, 1998), in which a cluster of $IP_3$ receptors is replaced by a release unit which is activated when $[Ca^{2+}]_{cyt}$ rises above a certain threshold. All the biology of the receptor model is then approximated by the choice of threshold, and once the threshold is passed, $Ca^{2+}$ release occurs. No assumptions need to be made about the identity of the $Ca^{2+}$ release agent or the $Ca^{2+}$

**Fig. 4** Interspecific comparison of $[Ca^{2+}]_{cyt}$ elevations during fertilization. *Fucus serratus* eggs (© Company of Biologists Ltd. Adapted, with permission, from Roberts *et al*., 1994) and metazoan *Ciona intestinalis* eggs were microinjected with Calcium Green-1 dextran. *Zea mays* eggs were ester loaded with fluo-3 AM (© Company of Biologists Ltd. Adapted, with permission, from Digonnet *et al*., 1997). Figs have been adapted to fit on the same timescale, with $t = 0$ being the time of sperm entry into the egg.

release site, yet the FDF model is also able to simulate a wide variety of $[Ca^{2+}]_{cyt}$ patterns, including waves (Ponce-Dawson *et al.*, 1999) and oscillations (Keizer *et al.*, 1998).

So, although interspecific stimulus-evoked $[Ca^{2+}]_{cyt}$ patterns are often extremely similar, perhaps best exemplified by the caffeine-induced $[Ca^{2+}]_{cyt}$ oscillations seen in both green algae and mammals, these similarities may not necessarily reflect conserved mechanisms of generation but could easily result from the similarity in wiring between two systems, each composed of very different components. Having persuaded ourselves of this, we now turn to see how the components of $[Ca^{2+}]_{cyt}$ signalling toolkits are organised and may vary.

## III. The structure and variation of $[Ca^{2+}]_{cyt}$ signalling pathways

$[Ca^{2+}]_{cyt}$ signalling pathways are complex phenomena. To describe them properly, the traditional reductionist, bottom-up approach, in which metabolites and proteins are individually characterized, is increasingly being complemented by a top-down, systems biology approach, in which mathematical models of information transfer are used to make sense of large datasets, especially the relevant genomes and proteomes.

Between them, these approaches are revealing that $[Ca^{2+}]_{cyt}$ signalling toolkits consist of a number of levels, each of which forms a discrete combinatorial system (Fig. 5). On the bottom rung lie amino acids, which are held in common by most cellular life. These may be combined to form a number of polypeptide domains, such as the $Ca^{2+}$-binding EF hand

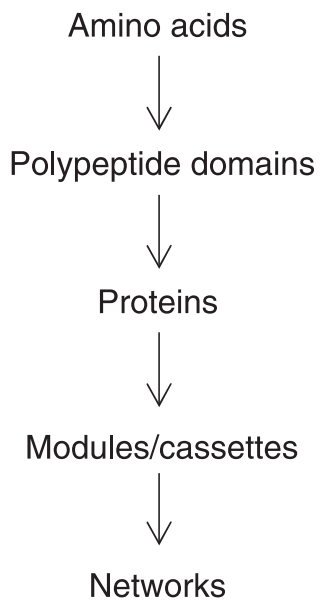



**Fig. 5** Levels of the $[Ca^{2+}]_{cyt}$ signalling tookit. Each level consists of a number of discrete components which may be combined in many ways to create new components for the next level of organization. This allows the generation of a diverse modular system, which is then subject to natural selection.

(Kretsinger & Nockolds, 1973), which are, again, broadly conserved across living organisms. Domains may then be combined to form proteins, and it is here that we begin to see major interspecific differences between toolkit components.

### 1. Protein variation

Orthologous proteins do not necessarily behave the same way in all eukaryotes. For example, SOS2, which is a plant calcineurin B like (CBL) sensor relay, binds a kinase, SOS3, in contrast to yeast CBLs (Gong *et al.*, 2004) which bind phosphatases. Those plant $Ca^{2+}$-binding proteins which do interact with kinases activate serine/threonine kinases, rather than the tyrosine kinases prevalent in metazoa (Reddy & Reddy, 2004). Plant $Ca^{2+}$-permeable glutamate receptor channels seem to be activated by glycine, whereas orthologous metazoan channels are activated mainly by glutamate (Dubos *et al.*, 2003).

How does such variation arise? A single gene may be altered by recombination, nucleotide substitution, insertion or deletion, but all these can often disrupt the function of the gene product to the detriment of the whole organism, as seen in most point mutations in $[Ca^{2+}]_{cyt}$ signalling toolkit components (e.g. Schumacher *et al.*, 1999). Every once in a while, however, a mutation in a $[Ca^{2+}]_{cyt}$ toolkit component can lead to a subtle enough alteration of function to hint at new possibilities whilst allowing survival. As examples, we proffer both the single amino acid alteration in the yeast VCX1 transporter which results in a shift in specificity from $Ca^{2+}$ towards $Mn^{2+}$ (Del Pozo *et al.*, 1999) and the gradual coevolution of symbiont and host in rhizobial nodulation (Aguilar *et al.*, 2004).

Such examples notwithstanding, it is more likely that an organism will survive if a mutation retains the old function while creating a new one. While this can happen through developmental variation and differential expression/splicing, as in some plant $Ca^{2+}$-binding proteins (Persson *et al.*, 2003; Kolukisaoglu *et al.*, 2004), it can also happen through gene duplication, in which one of the two resulting genes will be made redundant, and so freed from a functional role. A duplicated gene, also known as a paralogue, may then evolve in one of three ways: one copy acquires a new function, while the other maintains the old function (neofunctionalization); both copies share the work of the original (subfunctionalization); or one copy is lost (degeneration). Individual and local tandem duplications of many components of the $[Ca^{2+}]_{cyt}$ handling toolkit are seen in the available plant genomes (Navazio *et al.*, 1998; Felleisen *et al.*, 2000; Baxter *et al.*, 2003; Vandepoele *et al.*, 2003; Kolukisaoglu *et al.*, 2004), often occuring through the actions of transposons (Zhang & Wessler, 2004).

### 2. Module variation

Duplication is not limited to single genes. Fragments of chromosomes, or even whole genomes, may also be duplicated

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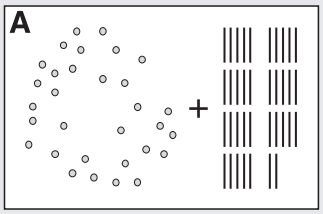
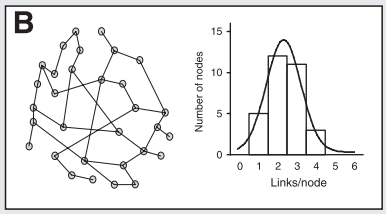
## Box 2 | Small Worlds

Attention has turned in recent years to describing complex biological systems, or networks (Strogatz, 2001). Networks have topology (the order in which things happen) and dynamics (the rates at which things happen), and different mathematical approaches are better at describing different facets of a network.

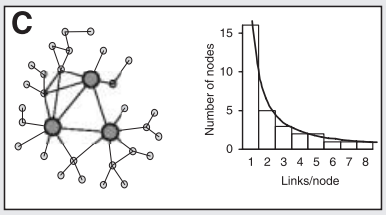



Consider a system of 31 nodes and 37 links, which may be made between any two nodes (A).

It is simplest to think of a node as a single molecule, but hierarchical network organization is possible, with clusters of nodes forming the nodes of a higher-level structure (Barabási & Bonabeau, 2003).




If new nodes are added at random to an existing collection, the result is a random network (B). The network shown has a mean of 2.4 links per node, and the number or links per node follows a normal distribution, shown here to the right of the network diagram.




Contrariwise, if new nodes are added preferentially to the highlighted 'hubs' – nodes which are already well connected – the result will be a non-random, so-called 'small-world' network (C). The network shown has the same mean number of links per node as the network in (B), but the distribution is very different.

A number of complex biological systems have been shown to exhibit small-world network topology, such as metabolic pathways (Jeong *et al.*, 2000; Almaas *et al.*, 2004) and gene interactions (Tong *et al.*, 2004).

(Anderson & Stebbins, 1954; Lawton-Rauh, 2003), with *Arabidopsis* having undergone a couple of rounds of polyploidy (Blanc *et al.*, 2000; Vision *et al.*, 2000; The Arabidopsis Genome Initiative, 2000; Bowers *et al.*, 2003). Natural selection will tend to favour such duplication if a group of proteins on the duplicated region work efficiently together, and can be adapted for use in a variety of pathways. Such groups are known as 'modules' (Hartwell *et al.*, 1999) or 'cassettes' (Mori & Schroeder, 2004) and may be considered to form the next level of toolkit construction.

There is little doubt that plant $[Ca^{2+}]_{cyt}$ signalling toolkits employ modules. At its simplest, selection over time for efficient functional interactions between proteins creates gene fusions, such as those which have given rise to many varieties of $Ca^{2+}$-binding protein kinases (Hrabak *et al.*, 2003). However, there are tantalising hints of larger $[Ca^{2+}]_{cyt}$ signalling toolkit modules in the *Arabidopsis thaliana* genome, most notably in the repetitive activation of hyperpolarization-activated $Ca^{2+}$-permeable plasma membrane cation channels by reactive oxygen species (Mori & Schroeder, 2004) and the *CaM/ACA4/CPK14* gene cluster on chromosome 2 which is duplicated as *CaM/ACA11/CPK32* on chromosome 3 (J. H. F. Bothwell, unpublished data).

## 3. Network variation

Recent mathematical topological analysis (Strogatz, 2001) suggests that individual proteins and protein modules (Jeong *et al.*, 2000; Almaas *et al.*, 2004; Tong *et al.*, 2004) fit together to form networks which exhibit so-called 'small-world' properties (Box 2). We may consider a random network (Box 2) as the way in which random variation throws up the substrate for evolution but, if we filter this through natural selection, preferential survival will lead to preferential attachment. A system growing in this way will thus tend to become a small-world network (Barabási & Albert, 1999; Lenski *et al.*, 2003).

Explaining how a complex system might vary poses an intuitive problem. It has been argued that, in a complex system, components are so well adapted to each other that they resist further change (von Mering *et al.*, 2003; Fernández *et al.*, 2004). Altering any component causes the system to fail, so no further change will occur. Such reasoning has been used explicitly to explain the conservation of $[Ca^{2+}]_{cyt}$ wave speeds in metazoa and plants as reflecting the conservation of the propagating machinery (Jaffe, 2002) and is implicit in many studies which look for plant homologues of metazoan $[Ca^{2+}]_{cyt}$ signalling phenomena.

Although the existence of such a mechanism, which would effectively limit the power of Darwinian adaptation, is hotly

contested (Dennett, 1995), there is nonetheless a way out of any theoretical impasse. The recent 'toolkit remodelling hypothesis' (Berridge *et al.*, 2003) points out that many $[Ca^{2+}]_{cyt}$ elevations are able to regulate the activities and transcription of $[Ca^{2+}]_{cyt}$ signalling toolkit components (Yang & Poovaiah, 2002) and suggests that this is one way in which the effects of altering individual components may be buffered. If correct, this would allow $[Ca^{2+}]_{cyt}$ patterns to remain constant while toolkit components were changed and, eventually, even replaced. Homology could thus be turned into homoplasy.

## IV. A putative course of descent for plant $[Ca^{2+}]_{cyt}$ signalling

We have briefly described how $[Ca^{2+}]_{cyt}$ signalling toolkits are constructed across several levels (Fig. 5) to produce a complex network. Understanding how such complexity arises has always required an investment in counterintuition, which painstaking work on the 'perfect and complex eye' which so troubled Darwin (Darwin, 1859; Goldsmith, 1990), and similar 'organs of extreme perfection', has repaid. Darwin's speculation that random variation, filtered by natural selection, can generate complex traits in a series of small, gradual steps has been shown to be retrospectively possible through analysis of fossil records and comparative anatomy (Goldsmith, 1990), and prospectively possible in computer simulations of development in digital organisms (Lenski *et al.*, 2003). Can we now imagine creating a $[Ca^{2+}]_{cyt}$ signalling response in a series of finely graded intermediate forms, each one useful to its possessor?

### 1. Prevention of $[Ca^{2+}]_{cyt}$ toxicity in early cells

Evidence for biogenic $CO_2$ fixation in 3.8-Gyr-old rocks from west Greenland hints at the presence of self-replicating organisms (Rosing, 1999). The exact conditions in which these organisms formed are unknown (Nisbet & Sleep, 2001), but given the composition of environments (Krasnov *et al.*, 1995) which are presumed to resemble the prebiotic Earth (Nisbet & Sleep, 2001), it seems reasonable to suppose that then, as now, $Ca^{2+}$ would have been one of the major cations present. Energy derived from redox reactions involving other cations, especially the $Fe^{2+}/Fe^{3+}$ pair, is thought to have driven the replication of early organisms (Martin & Russell, 2003), and the control of cation concentrations in early cells would thus have been of prime importance. Consequently the cation pumping ATPases which effect this control are ubiquitous to cellular life (Gogarten *et al.*, 1989; Palmgren & Axelsen, 1998), and are some of the few proteins found in early cells that are not involved in nucleic acid replication (Martin & Russell, 2003).

Fine control of $[Ca^{2+}]_{cyt}$ is particularly vital because $Ca^{2+}$ has an unusually large radius for a divalent cation. The electrostatic attraction which the $Ca^{2+}$ nucleus holds for anions is thus relatively weak, so anions which coordinate with $Ca^{2+}$ are not forced into rigidly packed complexes around the $Ca^{2+}$ nucleus, as they are when coordinating with, say, the smaller $Mg^{2+}$ nucleus (Levine & Williams, 1982). In practical terms, this means that even low $[Ca^{2+}]_{cyt}$ is able to cross-link, and thereby precipitate, flexible molecules carrying a negative charge, which is clearly something to be avoided in a cell consisting of self-replicating organic anions. To avoid such $Ca^{2+}$ toxicity, there seems to have been early adaptation of one family of cation pumps and one family of cation antiporters for $Ca^{2+}$ removal. The pump family became the $P_2$ ATPases, and it is an indication of their importance that, of the five P-type ATPase families found in living organisms, only these and the $P_1$ family are ubiquitous (Palmgren & Axelsen, 1998). The antiporter family became the $Ca^{2+}/H^+$ exchangers, such as the *Arabidopsis* CAX (Cai & Lytton, 2004).

In addition to moving $Ca^{2+}$ across the cell membrane as a final sink, early cells developed intracellular $Ca^{2+}$-binding proteins, which acted to buffer $[Ca^{2+}]_{cyt}$ prior to its removal. It is unlikely that our ancestors appreciated the irony, but those same properties of $Ca^{2+}$ which predispose it to precipitating organic salts also form its Achilles' heel. A large divalent cation may be specifically bound in a flexible, weakly anionic pocket. Flexibility excludes smaller, highly charged cations, which cannot form complexes of the correct shape, and the weak anionic charge allows rapid removal of $Ca^{2+}$ from the pocket, while excluding monovalent cations of similar size, for which the electrostatic interaction is not strong enough (Levine & Williams, 1982).

This structure has been seen in the $P_2$ ATPases (Toyoshima *et al.*, 2000), and in the motif called the helix-loop-helix, or EF hand. As their synonym suggests, EF hands consist of two $\alpha$ helices connected by a flexible loop, which helps to give them remarkable $Ca^{2+}$ specificity (Kretsinger & Nockolds, 1973). Short polypeptides containing four EF hands are found in all domains of life (Babu *et al.*, 1985), suggesting that they came to predominate as $[Ca^{2+}]_{cyt}$ buffers before the separation of the bacterial, archaeal, and eukaryotic lineages, possibly striking an opimal balance between $Ca^{2+}$-carrying capacity and diffusion rate. Whatever the reason for their success, this loose group of polypeptides, which are today called the calmodulins, remain the major bacterial and archaeal $[Ca^{2+}]_{cyt}$ buffers, although they have been supplemented in eukaryotes.

### 2. $[Ca^{2+}]_{cyt}$ elevations as indicators of stress in early cells

The selection advantage that avoiding calcium phosphate precipitation would confer on any system of self-replicating, phosphate-bound nucleotides is clear enough that we presume it to have driven the development of $Ca^{2+}$-ATPases and the calmodulins. It has been suggested that one consequence of the resultant $[Ca^{2+}]_{cyt}$ handling mechanism would be the maintenance of a large inward $[Ca^{2+}]$ gradient, which could be adapted

New Phytologist

to form the basis of a highly sensitive signalling pathway (Sanders *et al.*, 1999). This predisposition of the $[Ca^{2+}]_{cyt}$ handling machinery to become adapted for $[Ca^{2+}]_{cyt}$ signalling is not, in itself, sufficient for $[Ca^{2+}]_{cyt}$ signalling to develop. Although studies on *E. coli* expressing the $Ca^{2+}$-sensitive photoprotein, aequorin, have shown that they are able to regulate $[Ca^{2+}]_{cyt}$ (Jones *et al.*, 1999), there is little evidence that $[Ca^{2+}]_{cyt}$ signalling in bacteria or archaea is as important as it is in eukaryotes (Norris *et al.*, 1996; Herbaud *et al.*, 1998).

Nonetheless, anything tending to compromise the integrity of a cell membrane would result in a steep increase in $[Ca^{2+}]_{cyt}$. It therefore seems likely that the first step towards the adaptation of the existing $[Ca^{2+}]_{cyt}$ handling pathway into a $[Ca^{2+}]_{cyt}$ signalling pathway involved $[Ca^{2+}]_{cyt}$ acting as an indicator of membrane damage. This could simply involve a mutation which allowed the interaction of a $Ca^{2+}$-bound calmodulin with a stress response protein, giving an opportunistic stress response pathway. Some studies have indeed implicated $[Ca^{2+}]_{cyt}$ in bacterial heat shock responses (Freestone *et al.*, 1998; Nazarenko *et al.*, 2003), although the elucidation of $[Ca^{2+}]_{cyt}$ functions in bacteria has not progressed to the stage where we could, with confidence, claim that this finding supports our hypothesis.

## 3. Recruitment of $[Ca^{2+}]_{cyt}$ elevations as integrators in the first eukaryotes

The events underlying the appearance of eukaryotes sometime after 2.7 Gyr ago (Martin & Russell, 2003) are a matter of contention (Katz, 1999; Pennisi, 2004). Although current dogma maintains that at least one endosymbiosis (Katz, 1999), including the formation of mitochondria by the endosymbiotic engulfment of bacteria, was crucial to the establishment of eukaryotic cells, it is debatable whether the eukaryotic lineage predated this endosymbiosis (Cavalier-Smith, 2002; Hartman & Fedorov, 2002; Baluška *et al.*, 2004) or not (Martin & Russell, 2003; Rivera & Lake, 2004).

Whatever the exact history, there is a marked diversification of $[Ca^{2+}]_{cyt}$ signalling toolkit components which appears to accompany the appearance of eukaryotes. The $P_2$-ATPases split into the $P_{2A}$ and $P_{2B}$ subfamilies (Palmgren & Axelsen, 1998); the calmodulins, with four EF hands, are joined by the calcineurins, which have three, and the penta-EF hand family, which have five (Maki *et al.*, 2002); and the β-sheet C2 domains became adapted for $Ca^{2+}$-handling, possibly from an earlier binding function, joining the α-helical EF hand (Rizo & Südhof, 1998).

The sudden expansion of the $[Ca^{2+}]_{cyt}$ signalling toolkit suggests that eukaryotes made more of the opportunities offered by the pre-existing $[Ca^{2+}]$ gradient than prokaryotes. In the absence of any obvious candidates for a eukaryotic cell without endosymbiotic organelles, it is often assumed that endosymbiosis confers a selection advantage (Williams & Fraústo Da Silva, 2003). However, if this is the case, why has primary endosymbiosis been so rare an event?

We suggest endosymbiosis to be an optimal resolution to conflict, rather than an optimizing of reproductive efficiency. The idea that a characteristic of eukaryotes may have evolved to resolve biotic conflict, rather than to optimize the utilization of abiotic resources, has already been mooted to explain sex (Hurst, 1995), and we believe that the struggle for resources which would follow an endosymbiotic event is best resolved in a similar fashion, by the creation of a system for organizing the subsystems which comprise the eukaryotic cell (Box 3). Given the high concentration of $Ca^{2+}$ in the early ocean (Brennan *et al.*, 2004), the ancestral $[Ca^{2+}]_{cyt}$ handling system is ideally – some would say inevitably (Williams & Fraústo Da Silva, 2003) – placed for adaptation into such an integrative system. Not only does the large inward $[Ca^{2+}]$ gradient confer sensitivity (Sanders *et al.*, 1999) but, due to the large variations in metabolic pathways found in bacteria and archaea (Martin & Russell, 2003), the $[Ca^{2+}]_{cyt}$ stress response mechanism would be one of the few shared by host and symbiont, allowing for easier integration.

We make a clear distinction here between $[Ca^{2+}]_{cyt}$ as a carrier of information and $[Ca^{2+}]_{cyt}$ as an organizer of information. As we have seen, in some bacterial pathways, $[Ca^{2+}]_{cyt}$ seems to act as a signal, being causally linked to the conditions it reports, but in many eukaryotic responses $[Ca^{2+}]_{cyt}$ acts largely as an organizer, being a seemingly arbitrary agent by which different cellular pathways may communicate. We do not expect $[Ca^{2+}]_{cyt}$ to be the only organizing entity, and suppose that messengers such as NO would behave in a similar way. The extent to which $[Ca^{2+}]_{cyt}$ elevations act in tandem with these other integrating systems through crosstalk may help explain contradictory findings in different studies, a point which has been well argued already (Scrase-Field & Knight, 2003).

## 4. Intracellular $Ca^{2+}$ stores

One feature of all modern eukaryotic cells is the presence of intracellular compartments, and we suppose that these would also have been a feature of the first eukaryotes. Soon after the establishment of eukaryotes, protein targeting modifications allowed the incorporation of $[Ca^{2+}]_{cyt}$ handling components into the ER, creating the endomembrane $[Ca^{2+}]_{cyt}$ sink (Navazio *et al.*, 1998; Felleisen *et al.*, 2000). The release of $Ca^{2+}$ from endomembrane stores thus became another source of $Ca^{2+}$ for $[Ca^{2+}]_{cyt}$ elevations, and this has been observed in small, unicellular eukaryotes such as yeast (Denis & Cyert, 2002) as well as larger, multicellular ones.

Why should internal $Ca^{2+}$ release become so entrenched? We proffer two possible explanations. Firstly, the more sources of $Ca^{2+}$ release are present in a cell, the more variety of $[Ca^{2+}]_{cyt}$ elevations are possible, and the more flexible the $[Ca^{2+}]_{cyt}$ signalling system can be. Secondly, the small-world networks

## Box 3 | Conflict in endosymbiosis

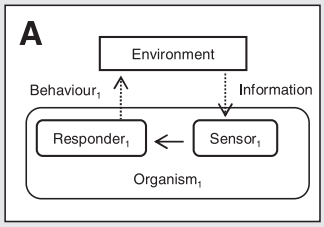
The behaviour of an individual organism, acting under natural selection, tends to be selfish.

Endosymbiosis brings a change of priorities. The optimal relationship between a host and symbiont will vary depending upon the organisms involved.

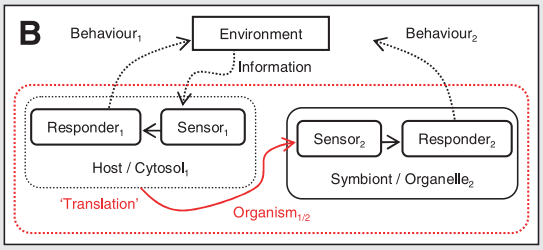
If we consider an aerobic symbiont producing energy from fuel provided by an anaerobic host, the best short-term outcome for either host or symbiont is still selfish – obtain all of the energy without providing any fuel. If both host and symbiont pursue this outcome, a conflict for the available resources will result, turning endosymbiosis into the short-lived relationship of parasite and host, or the even shorter-lived one of predator and prey.

Modern evolutionary theory has applied the techniques of Game Theory to such conflict (Maynard Smith and Price, 1973), and predicted that cooperation can also arise, in which host and symbiont share fuel and energy.

Any successful organism is able to use information from its environment to predict optimal behaviour. At its simplest, e.g. prokaryotic two-component signalling, this requires a sensor and a responder (A).




Following endosymbiosis, there is no longer direct contact between the symbiont and the environment to which its information processing is adapted. Intuitively we might suppose that the symbiontwould rely on its host for information. For example, a mutation may adapt the host sensor-responder pathway (B).

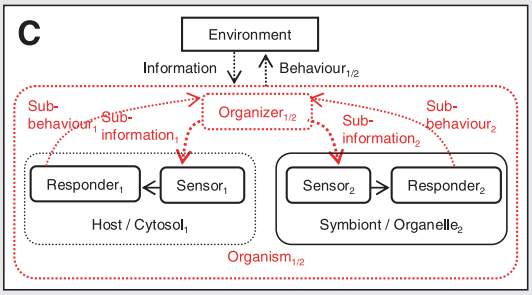



However, such a mutation provides an incentive for the host to cheat – falsifying information to work the symbiont harder for less reward. The positive feedback nature of such cheating would soon spiral out of control and subvert the endosymbiosis.

A second way for the symbiont to gain predictive information would involve a series of mutations in which both host and symbiont cede control of their information processing to an independent system (C). We propose that the collection of second messengersfound in eukaryotic cells serves this function.

Each partner in endosymbiosis is thus made subordinate to the rule of a system which is able to optimize benefit to the community of host and symbiont.

$[Ca^{2+}]_{cyt}$ thus acts as an integrator of information and organizer of responses.




adopted by complex biological systems are, in general, resilient to the effect of random mutations (Jeong *et al.*, 2000; Li *et al.*, 2004), but are especially vulnerable to loss of the more highly interacting components (Albert *et al.*, 2000), and it might be supposed that $Ca^{2+}$ would be such a central agent. Thus the development of internal and external stores may introduce a certain amount of redundancy, guarding against such loss of function, as shown by findings in which external $Ca^{2+}$ entry is up-regulated following depletion of intracellular stores (Csutora *et al.*, 1999).

### 5. An increase in eukaryotic cell size would require rapidly diffusing messengers

We have argued that the successful endosymbiosis required for the establishment of eukaryotes was favoured by the creation of a system for mediating the conflict between host and symbiont. Thus we suppose the existence of $[Ca^{2+}]_{cyt}$ signalling to be unrelated to cell size. Nevertheless, the nature of the eukaryotic cell rendered an increase in cell size possible, and such increases seem to have happened early. Most

New Phytologist

prokaryotic cells have a maximum dimension of < 5 μm, but the earliest putative eukaryotic fossils are an order of magnitude larger. 1.8-Gyr-old acritarchs – microfossils with organic walls but a phylogeny so uncertain that their identification as eukaryotes is not definite – have been found with diameters of 40–200 μm (Zhang, 1986), and larger fossils resembling algae have been claimed to be 2.1 Gyr old (Han & Runnegar, 1992). 1.5-Gyr-old fossils which are more definitely eukaryotic can be > 200 μm in diameter (Javaux *et al.*, 2001).

What changes would such an increase in size bring to an information organizing system, such as signal transduction? The key to a successful signal transduction pathway is the precise and timely interaction of components. Early cells, like modern bacteria and archaea, would have been small enough for the diffusion of proteins to maintain a suitable rate of information transfer. The predominant form of signal transduction may thus be effected by protein–protein interactions, exemplified by prokaryotic two-component signalling (Bray, 1998).

The speed at which a protein can move through a cell is directly proportional to its diffusion coefficient, $D$, which, for most proteins, varies between *c.* 1 and $10 \times 10^{-7}$ cm$^2$ s$^{-1}$ (Swaminathan *et al.*, 1997; Bray, 1998; Dayel *et al.*, 1999). This is comparable to the effective $D$ for $[Ca^{2+}]_{cyt}$ which, because of the constant binding of $Ca^{2+}$ to intracellular buffers, lies between 1.3 and $6.5 \times 10^{-7}$ cm$^2$ s$^{-1}$ (Allbritton *et al.*, 1992). This is too low to allow rapid signal transduction in cells larger than *c.* 20 μm in diameter (Allbritton *et al.*, 1992; Batada *et al.*, 2004). Since any modification which increases the rate at which the slower-diffusing components are able to interact will tend to enhance the speed of signal transduction, slowly diffusing components, such as proteins and $[Ca^{2+}]_{cyt}$, tend to be localized, and smaller, faster-diffusing components communicate between them (Batada *et al.*, 2004), which explains why the speed of $[Ca^{2+}]_{cyt}$ wave propagation is constrained by the speed at which $IP_3$ can diffuse (Gromada *et al.*, 1993).

We find therefore that many slow-diffusing components of a single signalling pathway will tend to cluster together in complexes with other components. These complexes form at certain locations on cell membranes or the cytoskeleton, and are held together by scaffold proteins (Weng *et al.*, 1999; Forgacs *et al.*, 2004). Such complexes, of course, bear more than a superficial resemblance to manifestations of the modules (Hartwell *et al.*, 1999) upon which natural selection may act. Examples of such $[Ca^{2+}]_{cyt}$ signalling complexes exist in metazoa (Berridge *et al.*, 2003), but we await stronger evidence that plant $[Ca^{2+}]_{cyt}$ signalling toolkit complexes exist. This looks most likely to come from proteomic analysis, but we note that components similar to those involved in metazoan $[Ca^{2+}]_{cyt}$ signalling have already been found in plant signalling complexes. For example, KAPP (Kistner & Parniske, 2002) and POLTERGEIST (Yu & Clark, 2003), which are protein phosphatase 2Cs similar to those required by guard cell $[Ca^{2+}]_{cyt}$ signalling and encoded by the *abi* loci (Allen *et al.*, 1999), form multiprotein complexes with the CLAVATA signalling system, and it would not be surprising if they formed similar complexes in their role in guard cell $[Ca^{2+}]_{cyt}$ signalling.

We turn now to faster-diffusing metabolites. Eukaryotes use a number of these, including NAADP ($D \sim 1 \times 10^{-6}$ cm$^2$ s$^{-1}$; Churchill & Galione, 2000), $IP_3$ ($D \sim 2.83 \times 10^{-6}$ cm$^2$ s$^{-1}$; Allbritton *et al.*, 1992), cAMP ($D \sim 2.7 \times 10^{-6}$ cm$^2$ s$^{-1}$; Chen *et al.*, 1999) and NO ($D \sim 3.3 \times 10^{-5}$ cm$^2$ s$^{-1}$; Malinski *et al.*, 1993), but we have already suggested (in Section II.2) that their perception may have evolved separately in different clades. Indeed, if the faster-diffusing metabolites are reserved for covering larger distances, then we might imagine NO, with its extremely high diffusion coefficient, to have developed as a signal in response to the independent adoption of large size by plants and metazoa, as there are no reports of it functioning as a signal in protists or bacteria.

We propose, then, that $[Ca^{2+}]_{cyt}$ is an integrative signal, but not necessarily the means of its own transmission. We also suggest that early single-celled eukaryotes had a basic $[Ca^{2+}]_{cyt}$ signalling pathway before the divergence of plants and animals. As cell size increased, a number of small metabolites were co-opted to speed up $[Ca^{2+}]_{cyt}$ signalling, although we cannot tell at what stage these were adopted. Although fungi, as well as plants, show $IP_3$-dependent $[Ca^{2+}]_{cyt}$ elevations (Silverman-Gavrila & Lew, 2001), the canonical $IP_3R$ has only been found in the metazoa. Given the absence of $IP_3$ receptor homologues in fungi and plants, either $IP_3$ acted as a $Ca^{2+}$ release agent in early eukaryotes, and the initial receptor for $IP_3$ was later supplanted in metazoa by the canonical $IP_3R$, or $IP_3$ was independently adopted as a $Ca^{2+}$ release agent by plants, fungi and metazoa. Further speculation must await the identification of the receptors for $IP_3$ in plants and fungi.

The discovery of ultrasmall eukaryotes which are comparable in size to bacteria (Baldauf, 2003) allows, in theory, a proving ground for our hypothesis that endomembrane signalling by $[Ca^{2+}]_{cyt}$, but not its mediation by $IP_3$, is essential in eukaryotes. *Ostreococcus tauri*, with a cell diameter of *c.* 1 μm, is a promising avenue (Khadaroo *et al.*, 2004) and it is interesting that endomembrane $Ca^{2+}$ release from *Saccharomyces cerevisiae*, which is small, does not appear to involve $IP_3$ (Denis & Cyert, 2002).

## 6. Plastids

The next major expansion of the $[Ca^{2+}]_{cyt}$ signalling toolkit seems to have followed the endosymbiotic events which first gave rise to, and then shuffled, photosynthetic plastids (Fig. 1). This is presumed to have begun with the engulfment of a cyanobacterial symbiont by a eukaryotic host, which has been estimated to have occurred *c.* 1.6 Gyr ago (Yoon *et al.*, 2004), and was followed *c.* 1.3 Gyr ago by secondary endosymbiosis of one eukaryotic cell by another, in which the

symbiont was already acting as a host for a cyanobacterium (Yoon *et al.*, 2004).

Following these endosymbioses, gene fusions created novel $Ca^{2+}$-handling proteins (Nagata *et al.*, 2004). In the protist lineage leading to fungi and metazoa, the interaction of calmodulin-binding domains and tyrosine protein kinases has led to the creation of the $Ca^{2+}$/calmodulin protein kinases, the CaMKs, which are largely absent from green plant genomes. The distribution of $[Ca^{2+}]_{cyt}$ sensor-responders is less clearly associated with lineages in the plantae. The interaction of $Ca^{2+}$-binding EF hands and serine/threonine protein kinases has given rise to the creation of monophyletic calcium-dependent protein kinases, the CDPKs, in both apicomplexans and the viridiplantae (Zhang & Choi, 2001; Billker *et al.*, 2004). However, the heterokont (stramenopile) diatom *Thalassiosira pseudonana* and the recently released genome of the red alga, *Cyanidioschyzon merolae* (Matsuzaki *et al.*, 2004) appear to have, in addition to a calmodulin gene, CaMK homologues, rather than CDPK homologues (J. H. F. Bothwell, unpublished data).

While we await elucidation of whether CaMKs of *Thalassiosira*, *C. merolae* and metaozoa are monophyletic, two complicating phenomena, gene loss and lateral gene transfer, may help to explain this mismatch between sensor-responder phylogeny and lineage phylogeny. Loss of gene families is not unknown (Shiu & Li, 2004), so we might imagine that CaMKs, CDPKs or both were present in the last common ancestor of photosynthetic organisms, but were differentially lost or retained by various clades. Alternatively, if the multiple independent creation of either CaMKs or CDPKs is too improbable, we must invoke lateral gene transfer. It is known that the secondary endosymbiosis which gave rise to the chromalveolate ancestor resulted in the transfer of a large number of genes from the symbiont to the host (Bhattacharya *et al.*, 2004; Funes *et al.*, 2004). Similarly, in flowering plants, real time gene transfer has been seen in tobacco between plastids and the nucleus (Huang *et al.*, 2004), while up to one-fifth of the *Arabidopsis* genome has been estimated to have derived from the chloroplast progenitor (Martin *et al.*, 2002), including the two-component signalling histidine kinases (McCarty & Chory, 2000). Thus it is not too great a leap of the imagination to suppose that CDPKs were among the genes transferred, which may explain, for example, the monophyly of CDPKs in viridiplantae and apicomplexans.

### 7. Multicellularity and toolkit diversification in flowering plants

We come at last to the viridiplantae and flowering plants. We would like to be able to compare the numbers of $[Ca^{2+}]_{cyt}$ signalling toolkit components in red algae and viridiplantae to see whether the appearance of green plants was accompanied by a $[Ca^{2+}]_{cyt}$ toolkit expansion, but we have only one red algal genome, which is of the highly reduced *C. merolae*, and is thus of limited use.

We do, however, see toolkit diversification. Interaction among $Ca^{2+}$-binding EF hands, calmodulin-binding domains and serine/threonine protein kinases has resulted in the CCaMKs, which possess separate binding sites for calmodulin and $Ca^{2+}$, and which are only present in *c.* 80% of flowering plants (Hrabak *et al.*, 2003). In addition, an intriguing class of kinases offer a possible snapshot of adaptation. As their name suggests, the CDPK-related kinases (CRKs) are similar to CDPKs, but their EF hands do not bind $Ca^{2+}$ (Hrabak *et al.*, 2003). Since it has been demonstrated that EF hands can bind other molecules (Ermilov *et al.*, 2001), this may be a case of previously $Ca^{2+}$-binding kinases adapting over evolutionary timescales to mediate novel signal transduction pathways.

Tantalising traces of such adaptation may still be glimpsed in other $[Ca^{2+}]_{cyt}$ signalling pathways. The *DMI1-3* genes involved in Nod-factor symbiosis, and discussed in Section II.1, are also thought to mediate the more ancient arbuscular mycorrhizal symbiosis pathway, suggesting their recruitment from one symbiotic pathway to another (Lévy *et al.*, 2004; Parniske, 2004). More definitely, orthologous reactive oxygen generating respiratory burst oxidase homologues (RBOH) signal to $Ca^{2+}$ channels in a number of tissues (Mori & Schroeder, 2004). The more ancestral RBOHD and RBOHF are active in plant defence (Torres *et al.*, 2002) and development (Kwak *et al.*, 2003), and the younger RBOHC is involved in root hair growth (Foreman *et al.*, 2003), suggesting that ancestral stress response pathways have been adapted to drive development, as we argue in Section IV.3.

In this light, a particularly interesting observation has recently been made concerning natural selection following gene duplications. While not directly involved in $[Ca^{2+}]_{cyt}$ signalling, the receptor-like kinases (RLKs) of the viridiplantae have independently expanded in *Arabidopsis* (through whole genome duplications and tandem repeats) and rice (mainly through tandem repeats). It may be of note that the RLKs involved in defense/disease resistance have been expanded more than those involved in development (Shiu *et al.*, 2004). Given the plasticity required of plant signalling responses, similar selection pressures may have acted to increase the number of CDPKs in flowering plants, and we might expect that a majority of these are also involved in stress response pathways, rather than developmental ones.

## V. Conclusion

The evolutionary dissection of any trait should, ideally, be founded on a wealth of comparative interspecific observations. Unfortunately, such data are limited for $[Ca^{2+}]_{cyt}$ signalling, especially in photosynthetic organisms. Although the current boom in genome sequences will allow useful comparisons to be made (Gutman & Niyogi, 2004), they will not stand alone, and we add our voice to those already calling for more comparative physiological studies (Kellogg, 2004).

Given the environmental and physical constraints under which cells develop, it is to be expected that large eukaryotic cells develop a $[Ca^{2+}]_{cyt}$ based signalling network which keeps $[Ca^{2+}]_{cyt}$ at a steady level of *c.* 100 nM and certainly below 1 μM, which makes use of diffusible components and membrane-bound protein complexes, and whose topology can be described as a small-world network. Given this template, the observed patterns of $[Ca^{2+}]_{cyt}$ elevation will follow.

We hypothesize that $[Ca^{2+}]_{cyt}$ signalling developed as a system to minimize biotic conflict between the host and symbiont during endosymbiosis. This organizing system might subsequently have been adapted to respond to external stimuli, whether biotic or abiotic, and then adapted once more for development. It is not to be expected that such adaptation could be achieved without the aid of other signalling systems. We thus expect that $[Ca^{2+}]_{cyt}$ signalling should involve cross-talk with other signalling components, as $[Ca^{2+}]_{cyt}$ elevations probably did not originate to answer the uses to which they are now put.

We have argued that many of the basic mechanisms for propagating $[Ca^{2+}]_{cyt}$ elevations have developed independently in metazoa and in plants. Thus there is no reason to expect conservation of components, or to invoke evolutionary relationships to explain similarities. A move away from comparative genomics is overdue, and we eagerly await the development of novel techniques for the identification of pathway components. The rewards of such an evolutionary appreciation of pathway structure are great, and it is to be hoped that $[Ca^{2+}]_{cyt}$ signalling, and signal transduction in general, stay in the forefront of research into the evolution of the complexity of living organisms.

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