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Review

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Calcium channels in photosynthetic eukaryotes: implications for evolution of calcium-based signalling

Author for correspondence:
Colin Brownlee
Tel: +44 1752633331
Email: cbr@mba.ac.uk

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Frédéric Verret[1], Glen Wheeler[1,3], Alison R. Taylor[2], Garry Farnham[3] and Colin Brownlee[1]

[1]Marine Biological Association of the UK, The Laboratory, Citadel Hill, Plymouth PL1 2PB, UK; [2]Department of Biology and Marine Biology, University of North Carolina, 601 S. College Road, Wilmington, NC 28403, USA; [3]Plymouth Marine Laboratory, Prospect Place, The Hoe, Plymouth PL1 3DH, UK

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Summary

Much of our current knowledge on the mechanisms by which Ca^{2+} signals are generated in photosynthetic eukaryotes comes from studies of a relatively small number of model species, particularly green plants and algae, revealing some common features and notable differences between 'plant' and 'animal' systems. Physiological studies from a broad range of algal cell types have revealed the occurrence of animal-like signalling properties, including fast action potentials and fast propagating cytosolic Ca^{2+} waves. Genomic studies are beginning to reveal the widespread occurrence of conserved channel types likely to be involved in Ca^{2+} signalling. However, certain widespread 'ancient' channel types appear to have been lost by certain groups, such as the embryophytes. More recent channel gene loss is also evident from comparisons of more closely related algal species. The underlying processes that have given rise to the current distributions of Ca^{2+} channel types include widespread retention of ancient Ca^{2+} channel genes, horizontal gene transfer (including symbiotic gene transfer and acquisition of bacterial genes), gene loss and gene expansion within taxa. The assessment of the roles of Ca^{2+} channel genes in diverse physiological, developmental and life history processes represents a major challenge for future studies.

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I. Introduction

Photosynthetic eukaryotes occur in widely divergent taxa, including embryophytes (higher plants, bryophytes and pteridophytes), green algae and red algae within the Archaeplastida (or Plantae) group and the diatoms, dinoflagellates, brown algae and haptophytes within the Chromalveolata (Fig. 1). As more is learnt about the genomics, cell biology and physiology of eukaryotic algae, it is becoming clear that, despite their common photosynthetic physiology, they have diverged enormously in many other respects, both between and within the major taxa. Current thinking supports a common evolutionary origin for eukaryote photosynthesis in an early ancestor of the Archaeplastida, via endosymbiosis of a cyanobacterial ancestor (Becker *et al,* 2008). Subsequent secondary (and, in some cases, tertiary) endosymbioses of ancestral red and green algae have enabled photosynthesis to arise in other eukaryote lineages (most notably the Chromalveolata) and have resulted in the complex phylogeny of present-day algae (Keeling, 2009; Moustafa *et al.*, 2009). This review considers a universal feature of eukaryote cells – Ca^{2+} signalling – to explore to what extent features and mechanisms have been retained during evolution and the degree of divergence of Ca^{2+} signalling mechanisms between algal groups. A particular focus is the role of Ca^{2+} channels, which are the primary routes for Ca^{2+} influx into the cytoplasm during the generation of Ca^{2+} signals (e.g. Sanders *et al.*, 2002; Berridge *et al.*, 2003; White & Broadley, 2003; Hetherington & Brownlee, 2004; Bothwell & Ng, 2005; Case *et al.*, 2007; Demidchik & Maathuis, 2007; Cai, 2008)

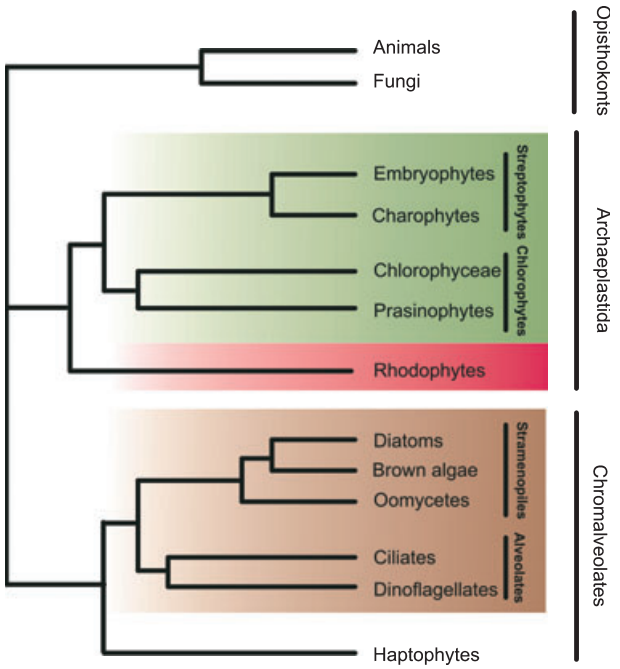


Fig. 1 Schematic tree displaying the currently well-accepted phylogenetic relationships between the major eukaryote groups discussed in this review (based on Keeling, 2009; Moustafa *et al.*, 2009). Eukaryote photosynthesis arose in the Archaeplastida following a primary endosymbiotic event with a cyanobacterial ancestor. Photosynthesis arose in basal chromalveolates via a secondary endosymbiosis involving a red alga (rhodophyte), although recent evidence has emerged for a prior endosymbiotic event involving a green alga (Moustafa *et al.*, 2009). Many lineages within the chromalveolates have subsequently lost their plastid and are no longer photosynthetic (e.g. ciliates, oomycetes, etc.).

It has long been known that the properties of the Ca^{2+} ion render it suitable for the transduction of highly organized spatial and temporal signals (Bothwell & Ng, 2005). The need to maintain average resting cytosolic [Ca^{2+}] at submicromolar concentrations to avoid the toxic consequences of high Ca^{2+} result in large electrochemical potential gradients for Ca^{2+} across cellular membranes. This allows the very rapid modulation of cytosolic [Ca^{2+}] by the regulation of Ca^{2+} influx. A further property of the Ca^{2+} ion is its suitability for rapid reversible binding to macromolecules. Organisms have evolved a vast range of proteins that are able to bind Ca^{2+} reversibly, often with associated changes in conformation and activity. Although these basic properties of Ca^{2+} signalling appear to be universal, it is becoming clear that the components of the Ca^{2+} signalling machinery can differ quite significantly between different groups of organisms. However, most studies of Ca^{2+} signalling in eukaryotes have focused on a limited number of mammalian and plant models. In photosynthetic eukaryotes, the majority of our knowledge of signalling mechanisms has understandably come from studies of embryophytes and green algae, with the assumptions that these groups are likely to have much in common with other photosynthetic eukaryotes in terms of the components underlying the generation of Ca^{2+} signals. As we will see, there are now compelling reasons to abandon these assumptions. Given that many lower plant and algal species have typical 'animal-like' life cycle stages, such as motile gametes, it may not be surprising to find similarities in Ca^{2+} signalling mechanisms that reflect these features. Likewise, embryophytes show a number of unique physiological characteristics, such as H^+-based membrane energization, reduced or absent motile stages and complex metabolic signalling pathways, which may also be reflected in the way in which they use Ca^{2+} signals.

In recent years, full genomic sequences have become available for representatives of a number of eukaryotic taxa, including the major photosynthetic groups. Genome sequencing projects for representatives of the brown algae (Cock *et al.*, 2010) and haptophytes, which include the calcifying coccolithophores, are also nearing completion. It is evident that gene loss and lateral gene transfer have played an important role in the evolution of unicellular eukaryotic algae, as witnessed by recent comparative genomic studies of the diatoms *Thalassiosira pseudonana* and *Phaeodactylum*

tricornutum (Armbrust *et al.*, 2004; Bowler *et al.*, 2008). The implications of this for the evolutionary distribution of signalling and other cellular processes are now becoming clear (Cavalier-Smith, 2003; Armbrust *et al.*, 2004; Bhattacharya *et al.*, 2004; Falkowski *et al.*, 2004; Keeling, 2004; Baldauf, 2008). We are now in a unique position to be able to link the wide-ranging physiological studies that have been carried out with a molecular understanding of Ca^{2+} signalling and the evolution of its components.

II. Physiological features of Ca^{2+} channel activity in eukaryotic photoautotrophs

With the exception of the less well-studied red algae, Ca^{2+} channel activity has been shown to underlie a diverse range of signalling processes in photosynthetic eukaryotes. Here, we highlight some of the key features of channel-mediated Ca^{2+} signal generation in plants and algae.

1. Embryophytes and charophyte algae (Streptophyta)

Ca^{2+} signalling has been studied in a wide range of physiological processes in embryophytes. Examples of key model systems include stomatal guard cells, root hairs and pollen tubes, which have been shown to generate a wide range of Ca^{2+} signals, ranging from slow sustained elevations to rapid transient elevations in response to a wide range of stimuli. These systems have been reviewed in depth by several authors (e.g. McAinsh *et al.*, 2000; Schroeder *et al.*, 2001; Demidchik and Maathuis, 2007; McAinsh & Pittman, 2009; Ward *et al.,* 2009). Although the relative roles of plasma membrane Ca^{2+} influx and release from intracellular stores in bringing about changes in cytosolic Ca^{2+} are well characterized in a small number of plant or algal systems (e.g. Goddard *et al.*, 2000; Oldroyd & Downie, 2006; McAinsh & Pittman, 2009), there are few detailed studies of the intracellular propagation of Ca^{2+} signals, in contrast with the large number of studies on animal systems. A number of pathways that deliver Ca^{2+} to the cytosol have been characterized at the physiological level in embryophytes: plasma membrane depolarization-activated Ca^{2+} channels (DACC); hyperpolarization-activated Ca^{2+} channels (HACC) (Miedema *et al.*, 2008); and vacuolar and endoplasmic reticulum (ER) Ca^{2+} release pathways (McAinsh & Pittman, 2009). However, functional molecular characterization of the ion channels responsible for generating cytosolic Ca^{2+} elevations is limited. Embryophyte counterparts of the well-characterized animal Ca^{2+} channels are restricted to a handful, and many of the typical animal-like Ca^{2+} channels, such as the four-domain, voltage-activated Ca^{2+} channels and inositol-1,4,5-trisphosphate ($InsP_3$) receptors (IP_3Rs), are absent (Nagata *et al.*, 2004; Wheeler & Brownlee, 2008) (see section III. Expansion, loss and horizontal gene transfer of Ca^{2+}-permeable channels in photoautotrophs). A cDNA for a wheat membrane transporter LCT1 shows Ca^{2+} permeability when expressed in yeast, and its involvement in Ca^{2+} uptake or release from intracellular stores has been proposed (Clemens *et al.*, 1998). It is not clear, however, whether LCT1 has a role in the generation of Ca^{2+} signals.

The charophyte algae represent an early diverging group from the streptophyte lineage and are therefore the closest algal relatives of the embryophytes. They share a number of similar features in terms of membrane physiology, such as large negative membrane potentials and membrane energization by an electrogenic H^+ pump (e.g. Beilby, 1989). Voltage-dependent and mechanosensitive plasma membrane Ca^{2+} channel activity has been reported in *Chara coralline*, and has been shown to underlie the slow action potentials (APs) that are characteristic of charophytes and embryophytes (reviewed in Berestovsky & Kataev, 2005; Beilby, 2007), although the molecular counterparts of this physiological activity remain to be characterized. There is evidence for (Biskup *et al.*, 1999; Wacke & Thiel, 2001; Wacke *et al.*, 2003) and against (Tazawa & Kikuyama, 2003) the involvement of Ca^{2+} release from intracellular stores involving $InsP_3$ as a second messenger in the generation of the charophyte AP.

2. Green algae (Chlorophyta)

In considering Ca^{2+} signalling and Ca^{2+} channels in the chlorophyte algae, significant divergences from embryophytes and charophytes are apparent (Wheeler & Brownlee, 2008). In *Chlamydomonas*, Ca^{2+} signalling has been shown to be associated with motile responses (flagellar beat, phototaxis, chemotaxis), sensory responses (flagellar adhesion during mating) and the maintenance and removal of the flagella (flagella length control, flagellar excision) (Kamiya & Witman, 1984; Harz & Hegemann, 1991; Goodenough *et al.*, 1993; Quarmby, 1994; Ermilova *et al.*, 1998; Tuxhorn *et al.*, 1998). Although flagellated sperm are also produced by pteridophytes and bryophytes, no studies have been performed of the Ca^{2+} signalling mechanisms underlying flagellar function in these groups.

The motile responses of chlorophyte algae to light have been studied in considerable detail, and have led to the discovery of a novel class of light-gated ion channels, the channelrhodopsins. In *Chlamydomonas*, light triggers an inward current at the eyespot (the photocurrent) carried by Ca^{2+} and H^+ (Harz & Hegemann, 1991; Harz *et al.*, 1992; Hegemann, 2008). At high light intensities, this inward current depolarizes the cell sufficiently to trigger a secondary inward Ca^{2+} current in the flagella, which mediates a simultaneous shift in flagellar waveform resulting in backwards swimming (the photophobic response) (Holland *et al.*, 1996). The photocurrent amplitude is dependent on the intensity of the light stimulus, whereas the flagella current is

of constant amplitude, representative of an AP triggered by membrane depolarization beyond a threshold value. The photoreceptor current in *Chlamydomonas* is mediated by two light-gated ion channels (channelrhodopsins CHR1 and CHR2) located in the plasma membrane adjacent to the eyespot (Nagel *et al.*, 2002, 2003; Suzuki *et al.*, 2003; Schmidt *et al.*, 2006). Recent characterization of CHR1 RNAi knockdown strains has demonstrated that photocurrents associated with both photophobic and phototactic responses are predominantly carried by CHR1 (Berthold *et al.*, 2008). Although it is clear that the photophobic response is mediated by the subsequent activation of voltage-dependent Ca^{2+} channels in the flagellar membrane, the mechanisms through which the photocurrent triggers the more subtle phototactic changes in flagellar beat are not known. Channelrhodopsins are present in the closely related alga, *Volvox carteri*, but have not been found in other algal genomes (Kianianmomeni *et al.*, 2009).

As with many plant cell types, the visualization of Ca^{2+} elevations in individual cells of the chlorophyte algae has proved problematic, although the recent biolistic loading of dextran-conjugated dyes (Bothwell *et al.*, 2006) has enabled the study of Ca^{2+} signalling during the process of flagellar excision (Wheeler *et al.*, 2008). Deflagellation mediated by the addition of weak organic acids or external Ca^{2+} is associated with rapid cytosolic Ca^{2+} elevations, which either occur across the whole cell or remain localized to the apical region, suggesting that the latter may act as specific signals for flagellar excision.

3. Diatoms

The diatoms are members of the Stramenopile algae, which also include brown algae and the oomycetes (see Fig. 1). The stramenopiles, together with the alveolates (including ciliates and dinoflagellates) and the haptophytes, are members of the chromalveolate supergroup. Stramenopiles retain certain novel and indicative features of algal endosymbiosis, including a double chloroplast envelope. A number of key studies on diatom signalling are beginning to reveal significant novel features and a clear role for Ca^{2+} in the response to environmental perturbations and biotic interactions. For example, aequorin-transformed *Phaeodactylum* cells show rapid cytosolic [Ca^{2+}] elevations in response to mechanical stimuli, and more gradual and sustained increases in response to nutrients such as Fe after a period of Fe-limited growth (Falciatore *et al.*, 2000). There is also increasing evidence that diatoms have a sophisticated suite of sensory mechanisms related to motility (Thompson *et al.*, 2008), CO_2 sensing (Harada *et al.*, 2006), light responses (Takahashi *et al.*, 2007), chemoperception, cell defence and apoptosis (Vardi *et al.*, 1999; Franklin *et al.*, 2006; Vardi *et al.*, 2006; Montsant *et al.*, 2007). More recently, Vardi *et al.* (2008) have shown that diatoms respond to aldehyde production by their neighbours via a Ca^{2+}-dependent apoptosis pathway that involves the activation of genes involved in NO production.

Of particular significance is the discovery of very fast (*c.* 20 ms duration) spontaneous depolarizations of the plasma membrane that closely resemble animal-like APs in the centric diatom *Odontella sinensis* (Taylor, 2009) (Fig. 2). These APs, unlike those of charophytes and embryophytes, exhibit biophysical and pharmacological characteristics that are remarkably similar to Na^+/Ca^{2+}-based APs in animals that result from the operation of four-domain, voltage-dependent Na^+ (Na_v) or Ca^{2+} (Ca_v) channels (Goldin, 1999; Catterall, 2000; Dolphin, 2009). The depolarization phase of the *O. sinensis* AP primarily allows Na^+ influx, but also mediates Ca^{2+} influx (Taylor, 2009). The *O. sinensis* AP is also insensitive to the Na^+ channel blocker tetrodotoxin, which is a property shared with vertebrate cardiac Ca_v and invertebrate Ca_v.

4. Brown algae

A number of well-characterized signalling cascades related to environmental sensing and development have been described in the multicellular marine brown algae of the genus *Fucus*. Fucoid algae produce large immotile eggs that are fertilized by motile sperm. Depolarization-activated Ca^{2+} currents with features of L-type Ca^{2+} currents in animals have been described in the unfertilized egg (Taylor & Brownlee, 1993), and have been proposed to underlie Ca^{2+} influx and cortical Ca^{2+} elevations that occur

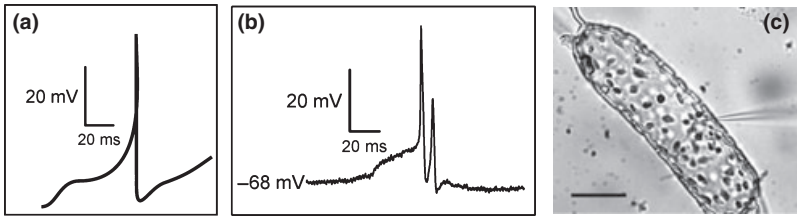


Fig. 2 Action potentials in squid and diatom species. (a) Representative example of an action potential recorded from the giant action of the squid (redrawn from Hodgkin and Huxley, 1952). (b) A spontaneous action potential recorded in single electrode current clamp mode from the diatom *Odontella sinensis* (Taylor, 2009). Note the close similarity in duration and kinetics between (a) and (b). (c) A single cell of *O. sinensis* with intracellular recording electrode in place. Bar, 20 μm

following fertilization (Roberts *et al.*, 1994; Roberts & Brownlee, 1995; Bothwell *et al.*, 2008). Following fertilization, localized Ca^{2+} influx at the germinating rhizoid apex is required for polarization of the zygote and establishment of an embryo pattern (Robinson & Jaffe, 1973; Brownlee & Wood, 1986). Osmotic treatments can elicit either transient or sustained [Ca^{2+}] elevations that initiate at the rhizoid apex and propagate to subapical regions (Taylor *et al.*, 1996; Goddard *et al.*, 2000; Coelho *et al.*, 2002). The magnitude and duration of these [Ca^{2+}] elevations are dependent on the strength of the stimulus (Goddard *et al.*, 2000). These osmotically induced Ca^{2+} signals propagate as fast Ca^{2+} waves that are very similar in speed to those described in many propagating Ca^{2+} signals in animals, and have been shown to spread by localized propagating release of Ca^{2+} from single channels or groups of channels in the ER membrane (Goddard *et al.*, 2000). Significantly, propagating waves of Ca^{2+} release from intracellular stores could be elicited by photorelease of caged $InsP_3$, strongly suggesting a role for this second messenger in Ca^{2+} wave propagation. Patch clamp studies have shown the presence of mechanosensitive Ca^{2+}-conducting channels in the plasma membrane, which may be involved in the triggering of the propagating Ca^{2+} waves (Taylor *et al.*, 1996).

5. Oomycetes

The oomycetes represent a group of stramenopiles that have lost functional photosynthetic plastids and metabolism (Tyler *et al.*, 2006). Many are pathogenic, causing devastating diseases in both aquatic and terrestrial ecosystems. Although much attention has been directed at the understanding of the cellular basis of host responses to these pathogens (Chisholm *et al.*, 2006), relatively little is known about the cellular physiology and signalling in this group. Nevertheless, both biophysical and imaging approaches reveal the central and conserved role of plasma membrane stretch-activated Ca^{2+} channels and Ca^{2+} influx in establishing cytosolic [Ca^{2+}] gradients that co-ordinate the polarized growth of the hyphae in these organisms (Jackson & Heath, 1989; Garrill *et al.*, 1992, 1993; Levina *et al.*, 1994; Hyde & Heath, 1995; Allaway *et al.*, 1997). Moreover, cytosolic [Ca^{2+}] increases rapidly in response to cold shock in *Phytopthora* zoosporangia, and subsequent sustained [Ca^{2+}] elevations regulate cell cycle progression (Jackson & Heath, 1989), suggesting alternate sensory and signalling roles for these ion channels.

6. Alveolates

Ciliates, dinoflagellates and apicomplexans represent the early branching alveolate subgroup of the chromalveolate supergroup. Plastid loss appears to be the more common condition in this clade, with photoautotrophic species restricted to the dinoflagellates with the exception of the photosynthetic apicomplexan *Chromera velia* (Moore *et al.*, 2008). All three taxa play important roles in aquatic ecosystem functioning and some have been well described with respect to their sensory ecology. For example, the membrane physiology and signalling associated with the mechanosensitivity and feeding behaviour of the nonphotosynthetic dinoflagellate *Noctiluca* have been studied extensively (Oami & Naitoh, 1989). These protists use a tentacle to capture prey, and there is evidence for complex spatial patterning of membrane currents (Nawata & Sibaoka, 1987), rapid recruitment of voltage-activated Na^+/Ca^{2+}-permeable channels (Oami *et al.*, 1995), Ca^{2+} influx and Ca^{2+}-dependent cytoskeletal responses (Nawata & Sibaoka, 1987) that initiate tentacle movements, prey capture and phagocytosis. Membrane excitability also regulates Ca^{2+} influx that underlies the rapid mechanically stimulated bioluminescent flash (Eckert & Sibaoka, 1968; Oami, 2004; Chen *et al.*, 2007; Latz *et al.*, 2008). In the related dinoflagellate, *Lingulodinium polyedrum*, Ca^{2+} release from intracellular stores plays a central role in sheer-induced bioluminescence (von Dassow & Latz, 2002).

Further evidence supporting the retention of sophisticated sensory and signalling mechanisms in the alveolate clade can readily be found in nonphotosynthetic models, such as *Paramecium* and *Tetrahymena*. These have been well studied as they rely on physical and chemical cues to locate food items (Hauser *et al.*, 1975; Levandowsky & Hauser, 1978; Leick & Hellunglarsen, 1985; Hellunglarsen *et al.*, 1986; Levandowsky *et al.*, 1988). The ciliate swimming reversal response involves membrane depolarization triggered by anterior mechanoreceptors. This, in turn, activates voltage-gated Na^+ or Ca^{2+} channels in the plasma membrane and, ultimately, Ca^{2+} channels associated with the cilia, generating local changes in cytosolic Ca^{2+} and reversal (reviewed in Plattner & Klauke, 2001). As well as ionotropic-based signalling pathways, an array of metabotropic mechanisms appear to be present in ciliates (Yang *et al.*, 1997; Ramoino *et al.*, 2004, 2005), together with a suite of regulatory Ca^{2+}-binding proteins (Plattner & Klauke, 2001) and mechanisms for regulating Ca^{2+} stores and cytosolic Ca^{2+} levels, such as IP_3Rs (Ladenburger *et al.*, 2006), SERCA (sarco(endo)plasmic reticulum Ca^{2+}-ATPase) pumps (Plattner *et al.*, 1999, 2006), and cyclic nucleotide-, G-protein- and NO-sensitive targets (Rosner *et al.*, 2003; Lucas *et al.*, 2004).

These reports demonstrate that heterotrophic dinoflagellates and ciliates both exhibit a wide range of Ca^{2+}-mediated sensory responses to environmental cues. Photosynthetic dinoflagellates, however, have received less attention with regard to Ca^{2+} signalling, although there is some evidence for mechanosensitive channel activity that underlies Ca^{2+} signalling in the regulation of the cell cycle in response to mechanical stimulation in *Crypthecodinium cohnii* (Lam

et al., 2001, 2005), which may also involve Ca^{2+} release from intracellular stores (Yeung *et al.*, 2006).

7. Excavates

The eugenoid photosynthetic flagellates have proven to be useful models for the study of environmentally controlled behavioural responses, such as gravitaxis (e.g. Hader *et al.*, 2009). Negative gravitaxis in *Euglena gracilis* has recently been shown to involve Ca^{2+} influx via a mechanosensitive transient receptor potential (TRP)-like channel. This was demonstrated by RNAi knockdown of a TRP channel gene, which resulted in the abolition of gravitaxis (Hader *et al.*, 2009). Inhibitor studies suggest the presence of $InsP_3$-mediated signalling in *E. gracilis* (Ohta & Suzuki, 2007), although the absence of significant genomic information precludes comprehensive analysis of Ca^{2+} channels in euglenoids.

8. Haptophytes

Haptophyte phylogeny remains contentious, but there is currently support for the hypothesis that the haptophytes represent a very early diverging branch of the chromalveolate supergroup (Hackett *et al.*, 2007; Patron *et al.*, 2007). Included within the haptophytes are the calcifying coccolithophores, which present an intriguing model system, because of the rapid uptake and transcellular transport of Ca^{2+} that is required to meet the demands of intracellular calcite precipitation by which calcite plates, or coccoliths, are formed in Golgi-derived vesicles and secreted to the cell surface where they form a protective coat (Brownlee & Taylor, 2004; Taylor *et al.*, 2007). Calcification involves arguably the largest sustained unidirectional Ca^{2+} flux across the plasma membrane of any organism (Brownlee & Taylor, 2004). The calcite coccoliths are formed in Golgi-derived vesicles and the calcification process clearly involves the strict separation of Ca^{2+} fluxes underlying signalling and calcification. Patch clamp studies indicate the presence of a range of voltage-activated cation- and anion-permeable channels in coccolithophores (Taylor & Brownlee, 2003). Interestingly, at least two Ca^{2+}-permeable conductances appear to be present in the plasma membrane of the coccolithophore *Coccolithus pelagicus* (Taylor & Brownlee, 2003; Brownlee & Taylor, 2004), one of which exhibits kinetics and permeation that are consistent with Ca^{2+} supply for calcification and the other that is remarkably similar to the AP currents recently characterized in diatoms (Taylor & Brownlee, 2003). How these Ca^{2+}-permeable channels are involved in the uptake of Ca^{2+} for calcification, and/or rapid signalling, is currently being investigated. To date, Ca^{2+} signals have not been investigated in coccolithophores, although Ca^{2+}-dependent blue light-stimulated secretion of polysaccharides has been demonstrated in the noncalcifying prymnesiophyte *Phaeocystis* (Chin *et al.*, 2004; Quesada *et al.*, 2006). It would be of great interest to determine how Ca^{2+} signalling components in coccolithophores are regulated to enable the coordination of Ca^{2+} uptake with Ca^{2+}-dependent regulation of cellular processes.

III. Expansion, loss and horizontal gene transfer of Ca^{2+}-permeable channels in photoautotrophs

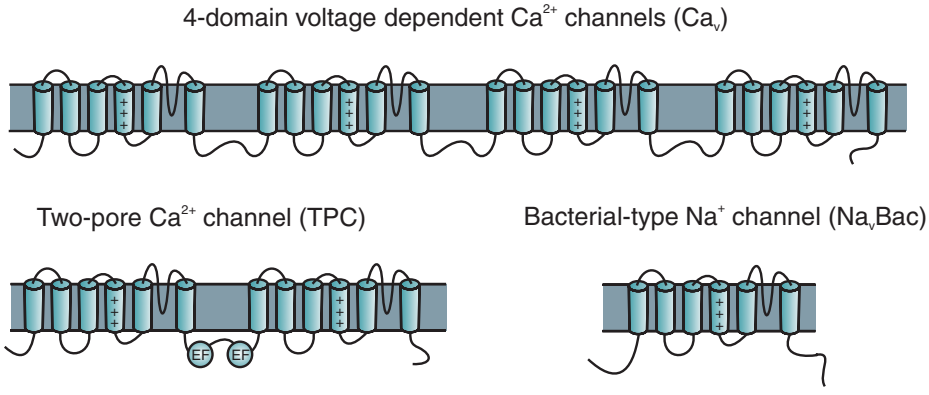
This section considers the main families of eukaryotic Ca^{2+}-permeable channels for which there is good evidence for the involvement in Ca^{2+} signalling. The occurrence in different algal groups is discussed in relation to their possible functional roles. Comparative genomic analyses are presented to reveal some particularly striking patterns of distribution that shed light on the evolutionary origins of Ca^{2+} channels and Ca^{2+} signalling more generally. Gene IDs for all channel genes discussed below are provided in Supporting Information Table S1. Although Table S1 is a comprehensive list of Ca^{2+}-permeable channels based on the current evidence, this is probably not an exhaustive list of all Ca^{2+}-permeable channels present in these organisms. We expect future studies to find further Ca^{2+}-permeable channels in these organisms that have not been identified in the present study, either because of weak similarities with their homologues in the existing databases or, in some instances, incomplete genome sequence data.

1. Voltage-gated Ca^{2+} channels

Four-domain, voltage-gated Ca^{2+} channels (Ca_v) Ca_v belong to the voltage-gated cation channel superfamily, which also includes K^+ and Na^+ channels (Strong *et al.*, 1993; Anderson & Greenberg, 2001; Hille, 2001). Ca_v mediate fast Ca^{2+} influx in response to plasma membrane depolarization, participating in the generation of Ca^{2+}-based APs and allowing rapid elevation of intracellular $[Ca^{2+}]$. In animals, Ca_v control processes such as excitation–contraction coupling, secretion, neurotransmission and gene expression. Ca_v are multi-subunit complexes, with the pore-forming $\alpha 1$ subunit consisting of four repeated domains connected by intracellular loops, and each domain comprising six transmembrane (TM) segments designated S1–S6. Voltage sensing is facilitated by the S4 segment, which presents positively charged residues (R and K), whereas channel selectivity is mainly determined by acidic residues (D and E) located in the re-entrant P-loop connecting the S5 and S6 segments (Catterall, 2000). Phylogenetic analyses suggest that Ca_v arose through two intragenic duplications of voltage-gated K^+ channels, which consist of four separate single domains arranged in a tetramer. It is proposed that subsequent evolution of Ca_v towards selectivity for Na^+ created four-domain Na^+ channels (Na_v), enabling fast Na^+-based APs prior to or during the appearance of the

Table 1 Distribution of the major classes of Ca^{2+}-permeable channels identified in photosynthetic eukaryote genomes

		VIC superfamily			Ligand gated						
Taxonomic group	Species name	Ca_v	TPC	Na_vBac	GLR	Cys-loop	P2XR	IP_3R	TRP	CNGC	MscS
Animals	*Homo sapiens*	10	2	0	18	46	7	3	27	6	0
Embryophytes	*Arabidopsis thaliana*	0	1	0	20	0	0	0	0	20	10
	Physcomitrella patens	0	9	0	2	0	0	0	0	8	16
Chlorophyceae	*Chlamydomonas reinhardtii*	9	0	0	1	3	0	1	19	3	7
Prasinophytes	*Ostreococcus lucimarinus*	4	0	0	0	0	3	0	0	0	3
	Ostreococcus tauri	4	0	0	0	0	2	0	1	0	1
	Micromonas RCC299	8	0	0	0	1	2	0	5	2	6
Rhodophytes	*Cyanidioschyzon merolae*	0	0	0	0	0	0	0	0	0	2
Diatom	*Thalassiosira pseudonana*	1	1	4	2	0	0	0	5	2	6
	Phaeodactylum tricornutum	0	2	3	0	0	0	0	4	0	7
Brown algae	*Ectocarpus siliculosus*	4	2	0	3	0	0	1	18	0	3
Oomycete	*Phytophtora sojae*	2	1	0	0	0	0	0	9	0	3
	Phytophtora ramorum	2	1	0	0	0	0	0	9	0	2



Ca_v, voltage-dependent Ca^{2+} channel; CNGC, cyclic nucleotide-gated channel; Cys, cysteine; GLR, glutamate receptor; IP_3R, inositol-1,4,5-trisphosphate receptor; MscS, mechanosensitive ion channel; Na_vBac, single-domain, bacterial-type channel; P2XR, purinergic P2X receptor; TPC, two-pore Ca^{2+} channel; TRP, transient receptor potential; VIC, voltage-gated ion channel.

The table includes Ca^{2+}-permeable channels identified by annotators during genome sequencing projects and in previous comparative genomic studies (Armbrust *et al.*, 2004; Matzusaki *et al.*, 2004; Nagata *et al.*, 2004; Derelle *et al.*, 2006; Tyler *et al.*, 2006; Merchant *et al.*, 2007; Palenik *et al.*, 2007; Bowler *et al.*, 2008; Rensing *et al.*, 2008; Worden *et al.*, 2009; Cock *et al.*, in press). The genomes were additionally searched for candidate Ca^{2+} channel homologues, using a combination of BLAST searches with animal, plant and algal proteins and Pfam searches for protein models with ion transport (PF00520), P2XR (PF00864), Cys-loop (PF02931 & PF02932) and GLR (PF00060) domains. The genomes of *Physcomitrella patens*, *Chlamydomonas reinhardtii*, *Ostreococcus lucimarinus*, *Ostreococcus tauri*, *Micromonas* RCC299, *Thalassiosira pseudonana*, *Phaeodactylum tricornutum*, *Phytophthora sojae* and *Phytophthora ramorum* are available at the Joint Genome Institute (http://www.jgi.doe.gov). The genomes of *Arabidopsis*, *Cyanidioschyzon merolae* and *Ectocarpus siliculosus* are available at the *Arabidopsis* Information Resource (http://www.arabidopsis.org), the *Cyanidioschyzon merolae* Genome Project (http://merolae.biol.s.u-tokyo.ac.jp/) and the *Ectocarpus* genome project (http://bioinformatics.psb.ugent.be/genomes/view/Ectocarpus-siliculosus), respectively.

first neuromuscular system in metazoans (Strong *et al.*, 1993; Anderson & Greenberg, 2001; Hille, 2001).

To date, Ca_v have been cloned and functionally characterized almost exclusively in the Opisthokont lineage. Based on primary sequence conservation, animal Ca_v are divided into four subfamilies, designated Ca_v1, Ca_v2, Ca_v3 and Ca_v novel, which differ in their biophysical and pharmacological properties, tissue distribution and physiological roles (Ertel *et al.*, 2000; Littleton & Ganetzky, 2000). Phylogenetic analyses suggest that the diversification of the four Ca_v subfamilies may coincide with, or predate, the emergence of metazoans (Jeziorski *et al.*, 2000; Anderson & Greenberg, 2001). In *Saccharomyces cerevisiae*, CCH1 (Calcium Channel Homologue 1) is localized at the plasma membrane where it mediates Ca^{2+} influx during the yeast response to mating α-pheromone, cold stress and ion toxicity (Paidhungat & Garrett, 1997; Peiter *et al.*, 2005a,b; Iida *et al.*, 2007). A role for CCH1 in the yeast animal-like capacitative Ca^{2+} entry has also been demonstrated (Locke *et al.*, 2000).

Analysis of Archaeplastida genomes indicates that Ca_v homologues are conspicuously absent from embryophytes (such as *Arabidopsis thaliana*, *Oryza sativa* and *Physcomitrella patens*) and the red algae *Cyanidioschyzon merolae* and *Galdieria sulphuraria* (Table 1). However, multiple Ca_v homologues are present in the genomes of the chlorophyte algae, including *Chlamydomonas*, *Ostreococcus* and *Micromonas* (Nagata *et al.*, 2004; Wheeler & Brownlee, 2008; Table 1). A single Ca_v homologue is present in the genome of the centric diatom *T. pseudonana*, whereas, surprisingly, no homologue can be found in the raphid diatom *P. tricornutum* (Table 1). Ca_v homologues are also present in other nonphotosynthetic chromalveolates, such as the oomycetes (*Phytophthora sojae* and *Phytophthora ramorum*) and the ciliates (*Paramecium tetraurelia* and *Tetrahymena thermophila*) (Table 1), where there is both physiological and genomic evidence for four-domain Ca^{2+}_v channels that contribute to the regulation of motility (Eckert & Brehm, 1979; Brehm *et al.*, 1980; Naitoh & Sugino, 1984) and temperature sensing (Kuriu *et al.*, 1998) (Table 1). Our unpublished analyses also indicate that Ca_v homologues are present in the haptophyte *Emiliania huxleyi* genome. The Ca_v homologues in diatoms and chlorophyte algae have low sequence similarities compared with animal Ca_v, and do not correspond to any of the designated animal Ca_v subfamilies. This suggests that the animal Ca_v subfamilies may have arisen after the divergence of the Opisthokonts. It is important to note that the low similarity of algal Ca_v makes it difficult to deduce their ion selectivity and other properties from sequence data alone. In particular, several algal Ca_v exhibit

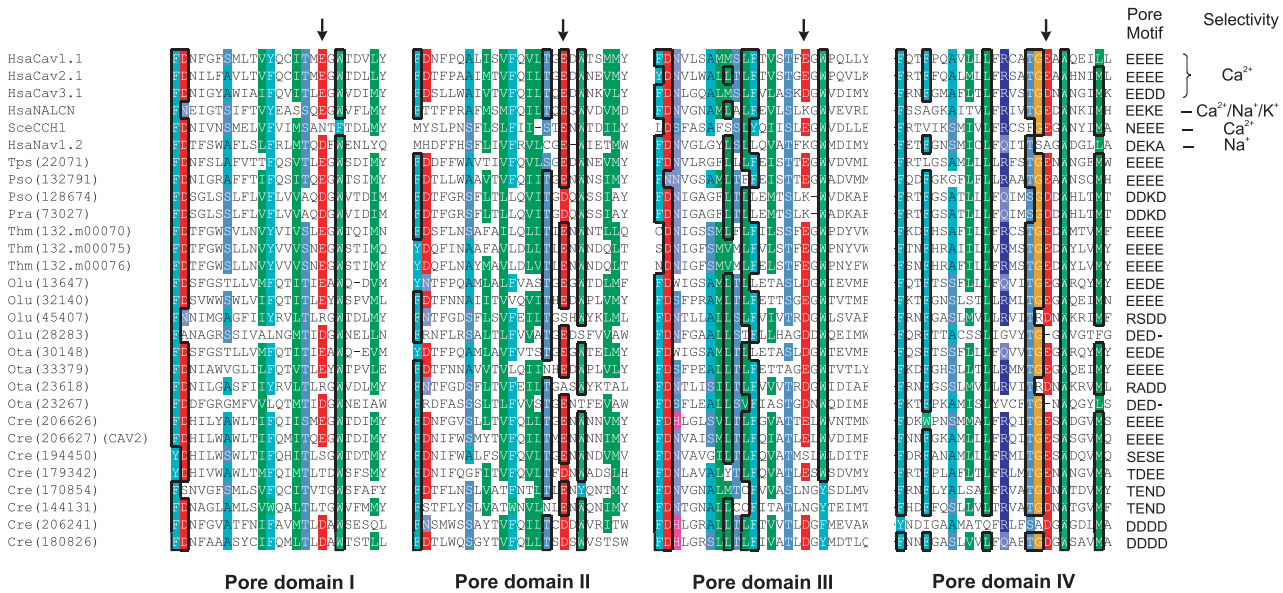


Fig. 3 Multiple sequence alignment of the four pore domains of four-domain channels. Sequences are from *Homo sapiens* (Hsa), *Saccharomyces cerevisiae* (Sce), *Thalassiosira pseudonana* (Tps), *Phytophthora sojae* (Pso), *Phytophthora ramorum* (Pra), *Tetrahymena thermophila* (Thm), *Ostreococcus lucimarinus* (Olu), *Ostreococcus tauri* (Ota) and *Chlamydomonas reinhardtii* (Cre). Accession numbers corresponding to RefSeq on the National Center for Biotechnology Information (NCBI) database are: HsaCa$_v$1.1 (NP_000060.2), HsaCa$_v$2.1 (NP_001123304), HsaCa$_v$3.1 (NP_000711), HsNACLN (NP_443099), SceCCH1 (NP_011733) and HsNav1.2 (NP_001035232). For all other sequences, the gene/protein ID used in each genome resource database is indicated in parentheses. Sequences were aligned using ClustalW (Thompson *et al.*, 1994) included in BioEdit program version 7.0.5.2 (Hall, 1999). When shared by at least 75% of the aligned sequences, similar residues are shaded; identical residues are shaded and boxed. Black arrows indicate residue located in the P-loops critical for channel selectivity. To the right of each sequence is indicated the motif created by the four residues present in the P-loop. Where known, the ion selectivity of the channels is indicated.

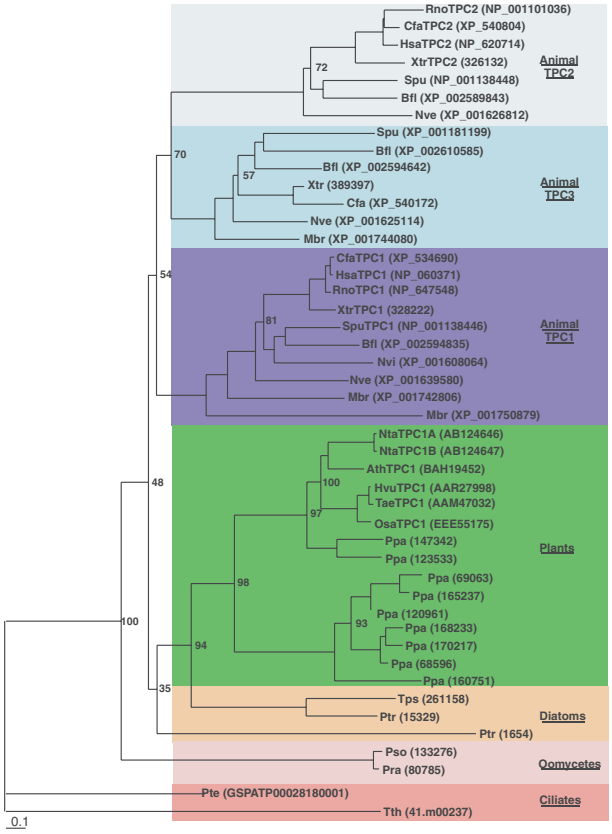


Fig. 4 Phylogenetic tree of two-pore Ca^{2+} channels (TPCs). TPC sequences are from *Nicotiana tabacum* (Nta), *Arabidopsis thaliana* (Ath), *Hordeum vulgare* (Hvu), *Triticum aestivum* (Tae), *Oryza sativa* (Osa), *Physcomitrella patens* (Ppa), *Thalassiosira pseudonana* (Tps), *Phaeodactylum tricornutum* (Ptr), *Rattus norvegicus* (Rno), *Canis familiaris* (Cfa), *Homo sapiens* (Hsa), *Xenopus tropicalis* (Xtr), *Strongylocentrotus purpuratus* (Spu), *Branchiostoma floridae* (Bfl), *Nematostella vectensis* (Nve), *Monosiga brevicollis* (Mbr), *Phytophthora ramorum* (Pra), *Phytophthora sojae* (Pso), *Paramecium tetraurelia* (Pte) and *Tetrahymena thermophila* (Tth). Sequences have been aligned using ClustalW. N- and C-terminal extensions have been removed for the phylogenetic analysis. The tree was generated using the neighbour-joining method. Numbers at the nodes indicate bootstrap values obtained for 100 replicates. Protein accession numbers are indicated in parentheses.

differences from their animal counterparts in the critical P-loop residues involved in ion selectivity, suggesting that they may be permeable to cations other than Ca^{2+} (Fig. 3). However, recent evidence from *Chlamydomonas* supports a role for algal Ca_v in Ca^{2+} signalling. RNAi and insertional mutant lines impaired for the expression of the CAV2 channel are defective for both the photophobic response and the mechanoshock response. Immunolocalization demonstrated that CAV2 is localized in the flagella where it has been proposed to drive the Ca^{2+} influx responsible for flagella waveform conversion (Fujiu *et al.*, 2009).

Two-pore Ca^{2+} channels (TPCs) TPCs consist of two repeated domains linked by a cytosolic loop presenting two EF-hands. According to the scenario of voltage-gated ion channel superfamily evolution (Anderson & Greenberg, 2001), TPCs may have evolved from an intermediate between one-domain K^+ channels (K_v) and four-domain Ca_v. Genome analysis reveals that TPCs are present in animals, embryophytes and chromalveolates, but are absent in green and red algae (Table 1). In plants, TPCs are generally present as a single copy per genome, except in *Nicotiana tabacum* and *Physcomitrella patens* which have two and nine TPCs, respectively. In animals, three TPCs are commonly found in sea urchin and vertebrate genomes, although TPC3 is absent in most primates and some rodent species (Brailoiu *et al.*, 2009). Phylogenetic analyses show that TPCs form three main groups related to animals, photosynthetic organisms (embryophytes and diatoms) and non-photosynthetic chromalveolates (Fig. 4). The observation that diatom TPCs are phylogenetically closer to plant TPCs than those from other chromalveolates (ciliates) (Fig. 4) raises an intriguing question about their origin. Cloning of a TPC was first reported in rat kidney, but attempts to determine its electrophysiological characteristics by heterologous expression in mammalian CHO cell lines and *Xenopus* oocytes were unsuccessful (Ishibashi *et al.*, 2000). More recently, animal TPC1 and TPC2 have been shown to function as NAADP receptors controlling Ca^{2+} release from acidic organelles, which can trigger Ca^{2+}-induced Ca^{2+} release from sarcoplasmic reticululm (SR)/ER (Brailoiu *et al.*, 2009; Calcraft *et al.*, 2009). In embryophytes, TPCs were initially proposed to mediate Ca^{2+} influx at the plasmalemma in rice and wheat (Kurusu *et al.*, 2005; Wang *et al.*, 2005). In *A. thaliana*, TPC1 has been shown to localize to the tonoplast, where it is responsible for the well-characterized Ca^{2+}-activated slow vacuolar (SV) current regulating germination and stomatal movement (Peiter *et al.*, 2005a,b). However, other studies have shown that TPC1 is not essential for the generation of Ca^{2+} signals induced by biotic and abiotic stress, and it has been proposed that TPC responds to, rather than initiates, cellular Ca^{2+} signals (Pottosin *et al.*, 1997; Ranf *et al.*, 2008). TPCs have weak selectivity for Ca^{2+} and are also permeable to monovalent cations (Peiter *et al.*, 2005a,b; Ranf *et al.*, 2008). In addition, the *Arabidopsis* mutant *fou2*, expressing a gain-of-function allele of TPC1, presents a three-fold higher $[Ca^{2+}]/[K^+]$ vacuolar ratio and a gene expression profile reminiscent of K^+ starvation (Beyhl *et al.*, 2009). These observations have led to the hypothesis that embryophyte TPCs play a major role in the control of vacuolar membrane potential and potassium homeostasis (Beyhl *et al.*, 2009). Their roles in chromalveolates have yet to be determined.

Single-domain, bacterial-type channels (Na_vBac) Na_vBac are bacterial-type, single-domain Na^+ channels that have been proposed to function as homotetramers (Ren *et al.*, 2001; Koishi *et al.*, 2004; Nurani *et al.*, 2008). Genome

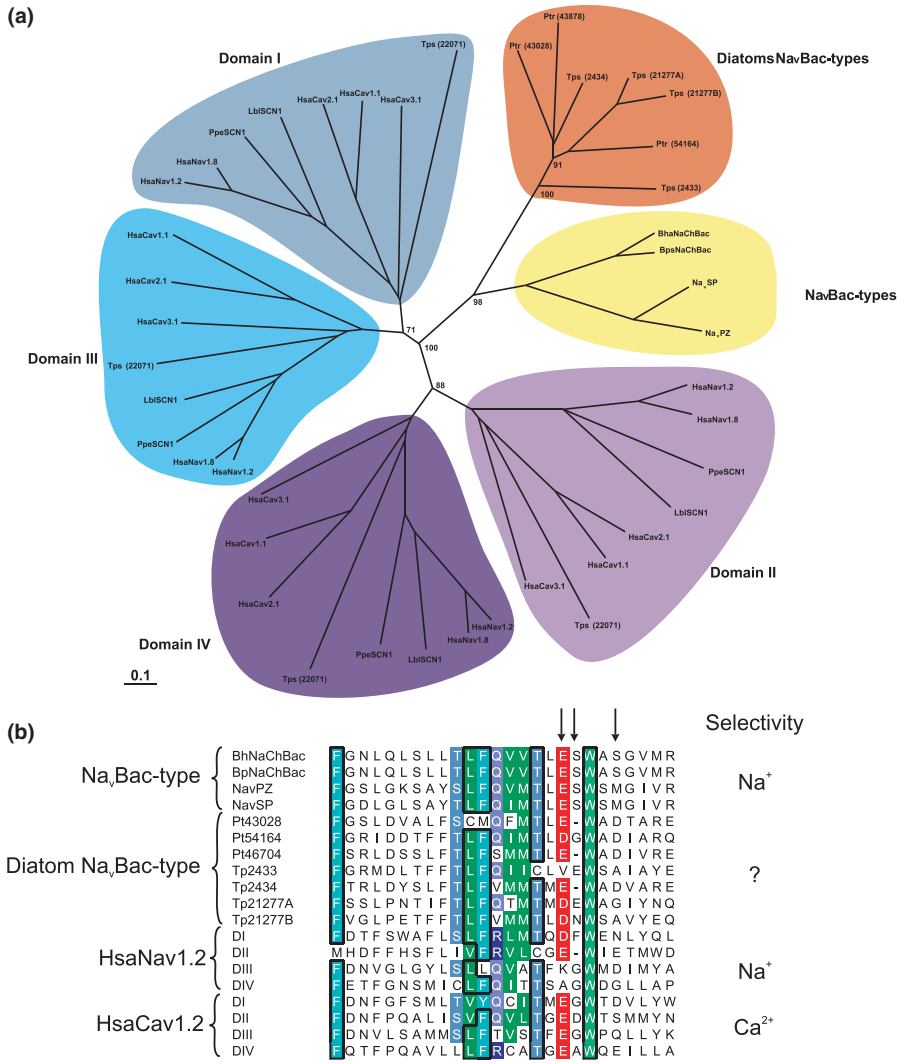


Fig. 5 Radiogram of single-domain, bacterial-type channels (Na_vBac), diatom Na_vBac types and domains I–IV of various voltage-dependent Ca^{2+} (Ca_v) and Na^+ (Na_v) channels. (a) Sequences are from *Homo sapiens* (Hsa), *Thalassiosira pseudonana* (Tps), *Phaeodactylum tricornutum* (Ptr), *Polyorchis penicillatus* (Ppe), *Loligo bleekeri* (Lbe), *Bacillus halodurans* (Bha), *Bacillus pseudofirmus* (Bps), *Paracoccus zeaxanthinifaciens* (NavPZ) and *Ruegeria pomeroyi* (NavSP). Sequences were aligned using ClustalW. N-and C-terminal extensions have been removed for the phylogenetic analysis. The tree was generated using the neighbour-joining method. Numbers at the nodes indicate bootstrap values obtained for 100 replicates. Protein accession numbers are $HsaCa_v1.1$ (NP_000060.2), $HsaCa_v2.1$ (NP_001123304), $HsaCa_v3.1$ (NP_000711), HsNav1.2 (NP_001035232), HsaNav1.8 (NP_006505.2), PpeSNCN1 (AAC38974), LblSCN1 (BAA03398), BhaNaChBac (BAB05220), BpsNaChBac (AAR21291), NavPZ (CAD24428) and NavSP (AAR26729). For all other sequences, the gene/protein ID used in each genome resource database is indicated in parentheses. (b) Multiple sequence alignment of the pore regions of Na_vBac, diatom Na_vBac types and domains I–IV (DI–DIV) of four-domain channels from *Homo sapiens* (Hsa), *Thalassiosira pseudonana* (Tps), *Phaeodactylum tricornutum* (Ptr), *Bacillus halodurans* (Bha), *Bacillus pseudofirmus* (Bps), *Paracoccus zeaxanthinifaciens* (NavPZ) and *Ruegeria pomeroyi* (NavSP). Arrows indicate residues critical for Na^+ selectivity of NaChBac. Sequences have been aligned using ClustalW. When shared by at least 75% of the aligned sequences, similar residues are shaded; identical residues are shaded and boxed.

analyses have indicated that Na_vBac are present in proteobacteria and actinobacteria, but absent in cyanobacteria. By mediating Na^+ influx in alkaliphilic Bacilli, Na_vBac contribute to the Na^+ cycle, supporting motility, chemotaxis and pH homeostasis (Ito *et al.*, 2004; Padan *et al.*, 2005). When expressed heterologously in mammalian cells, Na_vBac differ from four-domain Na_v by their 10- to 100-fold slower kinetics of voltage activation, inactivation and recovery from inactivation (Ren *et al.*, 2001; Ito *et al.*, 2004). Moreover, whereas Na^+ selectivity in Na_v is primarily determined by the 'DEKA' pore motif, Na_vBac organized in tetramers form an EEEE motif typical of the Ca_v1 and Ca_v2 subfamilies (Yue *et al.*, 2002). This observation has led to the proposal that the *Bacillus halodurans* Na_vBac (NaChBac) gene encodes a Ca^{2+} channel (Durell & Guy, 2001). Analysis of algal genomes indicates that diatoms, so far uniquely, possess Na_vBac-type genes: four in *T. pseudonana* and three in *P. tricornutum* located on

chromosomes 2 and 3, respectively. Diatom Na_vBac may have arisen via horizontal gene transfer from a prokaryote. Indeed, analysis of the *P. tricornutum* genome has indicated that 7.5% of the total predicted genes have a bacterial origin (Bowler *et al.*, 2008). Our phylogenetic analysis indicates that each domain of mammalian Na_v is more related to its Ca_v counterpart than to Na_vBac, supporting the hypothesis that mammalian Na_v arose more recently via Ca_v, rather than through a series of duplication events from bacterial Na_vBac (Fig. 5). Diatom Na_vBac types form three groups, two comprising *T. pseudonana* and *P. tricornutum* Na_vBac types. This suggests that an ancestral Na_vBac was transferred to the common ancestor of both diatoms, followed by a series of gene duplications. Sequence alignment of the pore regions shows that none of the diatom Na_vBac channels has full identity with the FxxxTxExW signature of Na_vBac (Fig. 5b), suggesting that this has not been fully conserved in the diatom genes. Interestingly, some differences located in or near to the signature correspond to substitution by aspartate residues which, when introduced by site-directed mutagenesis in NaChBac, have been shown to change channel selectivity towards Ca^{2+} (Yue *et al.*, 2002). Whether or not the diatom Na_vBac proteins form functional channels, and what their roles might be, requires further investigation.

2. Ligand-gated ion channels

Animals possess an extensive family of ligand-gated ion channels, including cysteine (Cys)-loop receptors (serotonin, nicotinic acetylcholine, γ-aminobutryic acid (GABA) and glycine receptors), ionotropic glutamate receptors (GLRs) and purinergic P2X receptors (P2XRs). A decade ago, the surprising discovery of a large family of ionotropic GLRs in embryophytes indicated that this class of ion channel most probably arose in a common eukaryote ancestor of animals and plants (Lam *et al.*, 1998). The evidence now available from the analysis of algal genomes suggests that the other major classes of ligand-gated cation channels (Cys-loop receptors and P2XRs) are also present in photosynthetic eukaryotes, prompting a similar re-evaluation of their cellular role and evolution.

Ionotropic GLRs Ionotropic GLRs are nonselective cation channels that mediate excitatory neurotransmission in the central nervous system of animals. An extensive group of GLR homologues exists in *Arabidopsis* with proposed signalling roles in cold resistance, root elongation and resource allocation (Dubos *et al.*, 2005; Meyerhoff *et al.*, 2005; Li *et al.*, 2006). The agonists glutamate and glycine both induce elevations in cytosolic Ca^{2+} in plant cells, suggesting the GLRs may act as ligand-gated, Ca^{2+}-permeable channels (Dennison & Spalding, 2000; Meyerhoff *et al.*, 2005). However, the evidence linking embryophyte GLRs directly to Ca^{2+} signalling is limited. Electrophysiological characterization of plant GLRs in heterologous systems has been problematic, although recent progress has indicated that AtGLR1.1 and AtGLR1.4 possess functional nonselective cation pores, and that AtGLR3.4 and AtGLR3.7 demonstrate constitutive nonselective cation channel activity (Roy *et al.*, 2008; Tapken & Hollmann, 2008). In addition, cytosolic [Ca^{2+}] elevations and the associated plasma membrane depolarization induced in *Arabidopsis* by glutamate and other amino acids are very reduced in *Arabidopsis glr3.3* mutants, suggesting that this protein could act directly as a ligand-gated, Ca^{2+}-permeable channel (Qi *et al.*, 2006).

GLR homologues are present in cyanobacterial genomes, although heterologous characterization indicated that GluR0 from *Synechocystis* acts as a glutamate-gated, K^+-selective channel, rather than a nonselective cation channel (Chen *et al.*, 1999). The presence of GLR homologues, at low numbers, in the *Physcomitrella*, *Chlamydomonas*, *Thalassiosira* and *Ectocarpus* genomes (Table 1) suggests that they have a widespread distribution amongst photosynthetic eukaryotes, and that the expansion of the GLR family in angiosperms occurred relatively recently.

Cys-loop receptors In vertebrates, the Cys-loop receptor class of ion channels plays a major role in fast synaptic transmission and can be broadly subdivided into two major groups: the excitatory cation-selective channels (including serotonin and nicotinic acetylcholine receptors) and the inhibitory anion-selective channels (including GABA and glycine receptors). The $5\text{-}HT_3$ serotonin receptor and certain isoforms of the nicotinic acetylcholine receptors exhibit Ca^{2+} permeability in addition to Na^+ and K^+ permeability (Lee *et al.*, 2009). Homologues of the Cys-loop receptors have been identified in bacterial genomes, indicating that they may have a widespread phylogenetic distribution (Tasneem *et al.*, 2005). A Cys-loop receptor from the cyanobacterium, *Gloeobacter violaceus*, acts as an H^+-gated cation channel in heterologous expression systems. Conserved residues suggest that aspects of the channel gating mechanisms may be conserved in prokaryotes and eukaryotes, but clearly the ligand-binding properties are very different (Bocquet *et al.*, 2007).

The Cys-loop receptors appear to be absent from the majority of photosynthetic eukaryote genomes examined, including the embryophytes. However, proteins with homology to Cys-loop receptors are present in the genomes of *Micromonas* and *Chlamydomonas*. These proteins contain several conserved residues important in the gating process, although the canonical cysteines are missing from the Cys-loop in the *Chlamydomonas* proteins, as is also the case in the *Gloeobacter* ion channel (Bocquet *et al.*, 2007). Cys-loop-like receptors may therefore be relatively widespread amongst the algae, although, without further

experimental characterization, we cannot predict their cellular function or their mode of activation.

P2XRs Extracellular ATP plays an important signalling role in animal cells, mediated via the P2X and P2Y purinoceptor families, which both play important roles in Ca^{2+} signalling, notably in fast synaptic transmission. The P2XRs are ATP-gated cation channels with a relatively high permeability to Ca^{2+} (Egan & Khakh, 2004), whereas the P2YRs are G-protein-linked receptors which act to trigger the release of Ca^{2+} from internal stores. There is increasing evidence that ATP may also act as an extracellular signal in embryophytes, with proposed roles in the fine control of growth or in the response to wounding (Roux & Steinebrunner, 2007). The application of extracellular ATP (or its analogues) also triggers cytosolic [Ca^{2+}] elevations in embryophytes, which are inhibited by chelation of extracellular Ca^{2+} or by the addition of the P2XR inhibitor, pyridoxal-phosphate-6-azophenyl-2′,4′-disulphonate (PPADS), suggesting that a P2X-type receptor may be responsible (Jeter *et al.*, 2004; Demidchik *et al.*, 2009). However, P2XRs have not been identified in any embryophyte genomes and the molecular identities of the embryophyte purinoceptors remain unknown.

P2XRs are not universally present in animals as they were not identified in *Caenorhabditis elegans* and *Drosophila* genomes (Khakh & North, 2006). However, P2XRs have recently been identified in the photosynthetic prasinophyte *Ostreococcus tauri* (Table 1) and in the social amoeba *Dictyostelium discoideum*, indicating a wide distribution amongst diverse phylogenetic groups (Fountain *et al.*, 2007, 2008). Heterologous expression studies have confirmed that P2XRs from these organisms function as ATP-gated cation channels (Fountain *et al.*, 2007, 2008). Localization studies have indicated the P2XR is present in the contractile vacuole in *Dictyostelium discoideum* and not the plasma membrane, suggesting a novel role in osmoregulation (Fountain *et al.*, 2007). The P2XR from *Ostreococcus*, *OtP2X*, exhibits a low Ca^{2+} permeability (P_{Ca}/P_{Na} = 0.4) relative to other P2XRs, which may be a result of the substitution of a highly conserved aspartate residue in the second TM domain (Fountain *et al.*, 2008). Several additional P2XR homologues with a lower sequence similarity to animal P2XRs have been detected in *Ostreococcus* and are also present in the genome of the related prasinophyte, *Micromonas*. P2XRs were not found in any of the other algal genomes within the scope of this review and their cellular role within the prasinophytes remains unclear.

3. IP_3Rs

IP_3Rs play a central role in Ca^{2+} signalling in animal cells, mediating Ca^{2+} release from intracellular stores, which contributes to the spatial propagation of cytosolic Ca^{2+} elevations. Animal IP_3Rs are large proteins of *c.* 2700–2800 amino acids, which form homo- or heterotetrameric nonselective cation channels and are primarily, although not exclusively (Durell & Guy, 2001), localized to the ER. IP_3Rs are primarily activated by the binding of $InsP_3$ and Ca^{2+}, but the regulation of ion channel activity is complex and may also be modulated via phosphorylation and interactions with other regulatory proteins. The role of IP_3R in embryophytes is the subject of much debate. IP_3Rs appear to be absent from embryophyte genomes, although a signalling role for $InsP_3$ has been identified in a range of cellular processes, such as stomatal guard cell closure, pollen tube elongation and the response to environmental stimuli, such as temperature shock or hyperosmotic stress (Gilroy *et al.*, 1990; FranklinTong *et al.*, 1996; DeWald *et al.*, 2001; Ruelland *et al.*, 2002; Tang *et al.*, 2007). This role has been supported by electrophysiological studies indicating $InsP_3$-activated Ca^{2+} currents in isolated plant vacuoles, although attempts to identify the proteins responsible have not been successful. The evolution and phylogenetic distribution of IP_3Rs is therefore of great interest to plant biologists and may provide an insight into the cellular mechanisms of $InsP_3$-mediated signalling in embryophytes.

Our knowledge of IP_3Rs is derived almost exclusively from studies on animal cells. However, recent evidence has indicated that IP_3Rs are found in diverse phylogenetic groups. An IP_3R homologue in the ciliate, *Paramecium*, exhibits $InsP_3$-binding activity *in vitro* and localizes to the contractile vacuoles, suggesting a role in osmoregulation (Ladenburger *et al.*, 2006). An IP_3R homologue has also been identified as an abundant protein in the *Chlamydomonas* flagella proteome (Pazour *et al.*, 2005). As intracellular stores are absent from flagella, this protein may mediate Ca^{2+} entry across the flagella membrane. This phylogenetic distribution indicates that IP_3Rs are clearly present in diverse eukaryotes, but analysis of the available plant and algal genomes indicates that they are absent from a significant number of these organisms (Table 1). IP_3Rs may therefore have developed from a common eukaryote ancestor and have been lost on multiple occasions throughout evolution. Without a fuller understanding of the role of IP_3Rs in nonanimal eukaryotes, it is very difficult to understand why this may have occurred. $InsP_3$-mediated signalling processes associated with deflagellation have been identified in *Chlamydomonas* (Quarmby *et al.*, 1992). In addition, the $InsP_3$ signalling processes associated with osmoregulation in the brown alga, *Fucus* (Goddard *et al.*, 2000; see above), may be mediated by an IP_3R, and an IP_3R homologue is present in the genome of the related brown alga, *Ectocarpus* (Table 1). Detailed characterization of the molecular and physiological roles of these IP_3R homologues is required if we are to understand why they are apparently absent from embryophytes despite the presence of many $InsP_3$-mediated signalling processes.

4. TRP channels

The TRP channels are nonselective cation channels which, in animal cells, are primarily found in the plasma membrane and mediate the TM flux of Ca^{2+} and Na^{+} ions (Ramsey *et al.*, 2006). TRP channels act in the generation of cytosolic [Ca^{2+}] elevations, either through direct Ca^{2+} entry or through depolarization of the plasma membrane and activation of voltage-gated Ca^{2+} channels (Minke, 2006). TRP channels are themselves only weakly voltage gated, and are instead activated by many diverse stimuli, including intracellular ligands (phosphatidylinositol-4,5-bisphosphate (PIP_2) and diacylglycerol (DAG)), receptor activation (G-protein-coupled receptors and receptor tyrosine kinases) and direct activation by environmental stimuli (heat and mechanical shock) (Ramsey *et al.*, 2006). Mammalian TRP channels comprise an extensive and diverse group of ion channel subunits with seven major subfamilies (Minke, 2006). Although TRP channels possess the six-TM architecture of voltage-gated K^{+} channels and are proposed to form a tetrameric structure, they exhibit little sequence similarity to the voltage-gated ion channels.

TRP channels have not been identified in embryophyte genomes. However, analysis of the available algal genomes indicates that they are abundant amongst the majority of algae. TRP channels were not found in the marine picoeukaryote, *Ostreococcus lucimarinus*, but are not absent from this lineage altogether, as a TRP homologue is present in *Ostreococcus tauri* (Table 1). We know very little about the functions of TRP channels in algae. Mechanosensitive TRP channels are implicated in osmoregulatory responses in animals (Lin & Corey, 2005), and we may speculate that they may also underlie the Ca^{2+} signalling observed in diatoms and brown algae in response to osmotic stimuli (Taylor *et al.*, 1996; Falciatore *et al.*, 2000; Goddard *et al.*, 2000). A TRP channel homologous to human polycystin-2 (TRPP2, PKD2) has been characterized from *Chlamydomonas*, (Huang *et al.*, 2007). Polycystin-2 is responsible for mechanosensation in the primary cilia of kidney epithelial cells, and defects in TRPP2 are a primary cause of autosomal polycystic kidney disease in humans. In *Chlamydomonas*, CrPKD2 also localizes to the flagellar membrane, suggestive of a conserved signalling role in this organelle. RNAi knockdown of *CrPKD2* severely reduces mating efficiency, suggesting that CrPKD2 may mediate a flagellar Ca^{2+} influx which initiates a signalling cascade during gamete fusion (Huang *et al.*, 2007).

5. Cyclic nucleotide-gated channels (CNGCs)

Embryophyte genomes contain an extensive family of CNGCs that may play a major role in Ca^{2+} signalling in the absence of other groups of Ca^{2+}-selective channels. Animal CNGCs are nonselective cation channels that are activated by cyclic nucleotide binding and are only weakly voltage gated. On the basis of electrophysiological studies of heterologously expressed channels, plant CNGCs may exhibit differing ionic selectivities. Although CNGCs have been linked to roles in development and disease resistance, there is currently limited evidence linking embryophyte CNGCs directly to Ca^{2+} signalling events. However, AtCGNC2 has recently been shown to mediate Ca^{2+} influx in response to bacterial elicitors, resulting in NO formation and the subsequent hypersensitivity response (Ali *et al.*, 2007). Embryophyte CNGCs are related to Shaker-like K^{+} channels consisting of a six-TM domain with a C-terminal cyclic nucleotide-binding domain, but lack the conserved GYGD motif found in K^{+}-selective channels (Talke *et al.*, 2003). Related ion channels are found in the genomes of chlorophyte algae and the diatom, *T. pseudonana*, although sequence similarity is low. Analysis of the CNGCs found in the 'green' lineage indicates that the expansion of the CNGCs in angiosperms (Table 1) may have occurred largely after the divergence of the mosses. *Physcomitrella* contains eight CNGC homologues, four of which cluster with the Group IV CNGCs (including AtCNGC2, AtCNGC4 and AtCNGC19), suggesting that they may have a conserved function between mosses and angiosperms. The remaining moss CNGCs form a phylogenetic subgroup which is distinct from the major subgroups of CNGCs found in angiosperms.

6. Mechanosensitive ion channels (MscS)

In prokaryotes, there is a diverse family of MscS which are required for adaptation to osmotic shock (Martinac, 2004). Changes in membrane tension during hypo-osmotic shock lead to pore formation and osmotic adjustment though a 'safety valve' mechanism. The MscS family is diverse, comprising proteins with 3–11 TM regions (Pivetti *et al.*, 2003). The three C-terminal TMs are the most conserved region of MscS proteins and form part of the core pore region in the functional channel. Structural studies have indicated that the *Escherichia coli* MscS channel folds as a membrane-spanning heptamer that undergoes a conformational change and forms a pore in response to membrane tension (Steinbacher *et al.*, 2007).

All of the photosynthetic eukaryotes analysed in the present study have one or more copies of an MscS-like (MSL) sequence, suggesting that they play important roles in eukaryotes as well as prokaryotes. Recent studies in both *Arabidopsis* and *Chlamydomonas* have proposed an intracellular role for MSL channels to release ions from the chloroplast and allow correct development of the plastid (Haswell & Meyerowitz, 2006, Nakayama *et al.*, 2007). Characterization of the chloroplast-localized *Chlamydomonas* MSC1 channel indicated a strong preference for anions over cations (Nakayama *et al.*, 2007). MSL channels may also

play a role in plasma membrane ion conductances during osmoregulation. A recent study has demonstrated that MSL channels are responsible for the mechanosensitive ion conductances observed in protoplasts from *Arabidopsis* root cells (Haswell *et al.*, 2008). However, mutant plants lacking mechanosensitive ion conductances attributed to MSL channels did not exhibit sensitivity to osmotic stress (Haswell *et al.*, 2008). The high conductance of plant MSL channels suggests that they may initiate osmoregulatory signalling processes via the depolarization of the plasma membrane or alteration of turgor pressure, but are unlikely to contribute directly to the generation of cytosolic [Ca^{2+}] elevations (Haswell *et al.*, 2008). In many other photosynthetic eukaryotes, TRP channels could also contribute to mechanosensory processes. Loss of the TRP channels may be a contributory factor in the relative expansion and diversification of the MSL channel family in the embryophytes (Table 1).

7. Annexins

Annexins form a family of Ca^{2+}- and phospholipid-binding proteins found in most eukaryotes, except yeasts (Gerke & Moss, 2002). Annexins serve a wide variety of cellular roles in structural organization and intracellular signalling, and certain annexins (e.g. human annexin A5) may also act as atypical Ca^{2+}-permeable channels (Moss & Morgan, 2004). Recent results have indicated that annexins from embryophytes may also function as Ca^{2+}-permeable channels and may therefore play a direct role in the modulation of cytosolic [Ca^{2+}] in plant cells (Laohavisit *et al.*, 2009). Annexins were present in the majority of algal genomes analysed here (with the exception of *Chlamydomonas* and *Cyanidioschyzon merolae*), although it remains to be seen whether these may also function directly in Ca^{2+} transport across cellular membranes. The majority of annexins contain a core domain comprising four annexin repeats, each containing a conserved Ca^{2+}-binding domain. The annexin homologues found in the *Ectocarpus* genome comprise eight annexin repeats, and may therefore have resulted from a gene duplication event in a manner analogous to annexin A6 in humans.

IV. Conclusions

In animals, the roles of many Ca^{2+} channel types is clear. For example, the large number of Ca_v is related to the higher level of complexity and regulation of fast mechanical and electrical processes, such as muscle contraction and nervous propagation at the cellular and tissue level. However, the presence of Ca_v in photosynthetic organisms is not related to multicellularity (Wheeler & Brownlee, 2008; Taylor, 2009). In photosynthetic eukaryotes that have actively swimming life cycle stages, a role for Ca_v in the generation of fast membrane depolarizations through APs is apparent. In *Chlamydomonas reinhardtii*, the Ca_v homologue, CAV2, has been shown to mediate flagella Ca^{2+} influx observed during the photoshock and photophobic response (Fujiu *et al.*, 2009). However, which physiological roles have led to the retention of Ca_v in nonmotile unicellular organisms, such as diatoms, coccolithophores, *Ostreococcus* and *Phytophthora* spp? Fast AP-based signalling, as demonstrated in the diatom *O. sinensis*, may be related to fast responses to grazing or other stress (Taylor, 2009), allowing the coordinated release of signalling/defence molecules, such as aldehydes, in diatoms and the activation of stress response pathways (Vardi *et al.*, 2006; Brownlee, 2008; Vardi *et al.*, 2008). Fast plasma membrane-based signalling may also underlie the requirement for fast metabolic regulation in response to rapidly fluctuating environmental conditions, such as light and nutrients. The presence of Ca_v in the diatom *T. pseudonana*, but not in *P. tricornutum*, may be related to differences in their ecophysiology or differences in their metabolic requirements as, for example, silica formation is facultative in *P. tricornutum* but obligatory in *T. pseudonana.* However, without further characterization of Ca_v and other channels in diatoms, we may only speculate on their functional roles.

Expansion, loss and horizontal gene transfer all appear to have played a central role in the distribution of Ca^{2+}-permeable channels amongst photosynthetic eukaryotes. The broad phylogenetic distribution of many classes of ion channels suggests that these ion channel families were present in the very earliest eukaryotes. However, the nonuniform distribution of many ion channel families between closely related phylogenetic clades suggests multiple gene loss events, although lateral gene transfer (both endosymbiotic gene transfer and horizontal gene transfer) also appears to have contributed to the complex distribution patterns observed. The phylogeny of Na_vBac channels, in particular, supports a horizontal gene transfer from prokaryotes into diatoms. The evolutionary history of those ion channel classes with only a few representatives (such as IP_3R, P2XR and Cys-loop receptors) remains less clear. However, unlike the Na_vBac channels, the broad distribution of these representatives between several eukaryote kingdoms (e.g. Opisthokonta, Archaeplastida, Amoebozoa and Chromalveolata) suggests that they were present in very early eukaryotes. Greater sampling of many more algal genomes is required to fully examine these phylogenetic relationships, and will no doubt reveal multiple levels of complexity (Rogers *et al.*, 2007).

Without a knowledge of the physiological roles of particular ion channels from diverse taxa, it is not currently possible to determine the factors driving the loss of ion channel families. We can speculate that, as differing physiologies evolved in photosynthetic eukaryotes, differing selective pressures resulted in the loss of entire ion channel families

and, in some cases, the expansion of others. This is well demonstrated in embryophytes, where many classes of ion channel appear to have been lost, but CNGCs and GLRs are significantly expanded. The physiological reasons underlying gene losses are likely to be diverse. The most obvious example relates to the loss or reduction in motility in certain lineages, which may have resulted in the loss of specific Ca_v associated with motile responses (such as those identified in ciliates and *Chlamydomonas*), although Ca_v also clearly play a role in nonmotile organisms. There are several examples of gene loss occurring within closely related groups. For example, the TRP channels in *Ostreococcus lucimarinus* and Ca_v in *P. tricornutum* may have been lost relatively recently. Examining the roles of TRP and Ca_v channels in *Ostreococcus tauri* and *T. pseudonana*, respectively, may help us to understand the selective pressures leading to the loss of an entire class of ion channels from related organisms.

The presence of both TPCs and Ca_v in photosynthetic organisms suggests that intragenic duplication events from a precursor of the voltage-gated K^+ channel occurred before the divergence of the Opisthokonts and the Archaeplastida (2 Gya). This is supported by phylogenetic analysis of the *T. pseudonana* Ca_v, showing that domains I/III and II/IV are most similar to each other, and that each domain from $TpCa_v$ is more similar to animal Ca_v than to the other domains of $TpCa_v$ (see Fig. 3). The chromalveolate Ca_v and TPCs may have originated from either the eukaryotic heterotroph host or by endosymbiotic gene transfer from red or green algae. Ca_v and TPC are absent from the genomes of the red algae *Cyanidioschyzon merolae* and *Galdieria sulphuraria*, but, as these red algae are representative of only one of the seven red algal lineages (the Cyanidiales) and are a specialized group of thermophilic algae with extremely small genomes (Yoon *et al.*, 2006), additional screening of red algal genomes is required to identify whether Ca_v and TPCs arose from red or green algal endosymbionts.

Many algae produce toxins which act directly on ion channel activity. For example, brevetoxins and ciguatoxins from marine dinoflagellates result in the hyperactivation of Na_v channels, whereas other dinoflagellate toxins, such as the saxitoxins, block Na^+ channel activation. Domoic acid, produced by the diatom *Pseudo-nitzschia*, acts as an agonist of ionotropic kainate receptors. It has been demonstrated recently that Na^+ channel diversification has been driven by predator–toxic prey interactions in vertebrates (Geffeney *et al.*, 2005). Algal toxins may also have impacted on the early evolution of the Na_v channels in animals (Anderson *et al.*, 2005) and arguably influenced the evolution of ion channels in algae themselves. As algae possess homologues of voltage-gated and ionotropic channels, we may speculate that ion channel toxins from neighbouring algae could have resulted in selective pressure for the evolution of alternative or insensitive ion channels. Experimental evidence is now required to examine the sensitivity of algal ion channels to these compounds.

In conclusion, this review draws together current evidence across the photosynthetic eukaryote taxa that points to the widespread retention of ancient Ca^{2+} channel genes, as well as the occurrence of horizontal gene transfer, gene loss and gene expansion within taxa. The analysis also uncovers evidence for fundamental differences between Ca^{2+} signalling mechanisms in embryophytes and algae. A recent comparison between plants and algae in the green lineage has indicated that embryophytes have lost a range of Ca^{2+} channel types that are widespread in animal, algal and other groups (Wheeler & Brownlee, 2008). We conclude that many classes of Ca^{2+} channel are of ancient origin in the eukaryotes, and that signalling mechanisms based on their action have been retained in most groups, but have been substantially modified or lost in the embryophytes. The evolutionary drivers of such loss in the embryophytes cannot be explained by any single factor. Rather, the adoption of an H^+-based energization of the membrane potential, loss of motile-like cycle forms and a terrestrial lifestyle may all have been contributory factors. A combination of the physiological characterization of algal ion channels, together with a detailed survey of many more algal genomes, is required in order to understand the evolutionary stages at which these losses have occurred and the selective pressures driving these events.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Table S1 The identities of the Ca^{2+}-permeable channels found in photosynthetic eukaryote genomes.

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