Update on Genetic Identities of Plant Calcium Channels

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The transient elevation of free calcium ion concentration in the cytosol and organelles of plant cells is a well-established phenomenon. As discussed in this Focus issue on calcium signaling, these elevations occur in response to a range of biotic and abiotic stimuli (McAinsh and Pittman, 2009; Dodd et al., 2010). Recently, transcriptomic analyses coupled with calcium determinations have demonstrated that a specific stimulus (membrane voltage or ozone) can cause a specific free calcium ion transient, leading to a specific transcriptional response (Whalley et al., 2011; Short et al., 2012; Whalley and Knight, 2013). It is also clear that free cytosolic Ca\(^{2+}\) ([Ca\(^{2+}\)\textsubscript{cyt}]) has a role to play in regulating the polar growth of root hairs and pollen tubes (Monsenhausen et al., 2008; Richard et al., 2011). What has yet to be determined fully is the mechanistic basis of these calcium signaling “signatures” and the oscillations of [Ca\(^{2+}\)\textsubscript{cyt}] that occur during polar growth.

There would be sufficient energy in transmembrane Ca\(^{2+}\) gradients to drive passive Ca\(^{2+}\) influx into the cytosol from the apoplast or internal stores (such as the vacuole or endoplasmic reticulum). Pharmacological intervention has implicated such Ca\(^{2+}\) influx in elevating free cytosolic Ca\(^{2+}\) ([Ca\(^{2+}\)\textsubscript{cyt}]) in a variety of Ca\(^{2+}\) signatures (response to environment, stress, hormones, immunity) and during polar growth. With the prediction of passive Ca\(^{2+}\) transport comes the hypothesis that Ca\(^{2+}\)-permeable channel proteins in plasma membranes and endomembranes would be responsible for [Ca\(^{2+}\)\textsubscript{cyt}] elevation. This, in turn, has driven the electrophysiological characterization of these channel proteins and the search for the encoding genes (for review, see Demidchik and Maathuis, 2007; Wheeler and Brownlee, 2008; Dietrich et al., 2010; Dodd et al., 2010; Matzke et al., 2010; Jammes et al., 2011; Peiter, 2011; Hedrich, 2012; Stael et al., 2012; Kursu et al., 2013). Beyond a brief introduction to the various channels for the benefit of readers new to the area, this Update will focus on the latest breakthroughs not addressed by those reviews, their implications, and the renewed efforts now evident in Ca\(^{2+}\) channel research to find those genes. Before going on, a note on terminology. The relevant channels characterized in plant membranes so far do a really good job of transporting K\(^+\) and other cations in addition to Ca\(^{2+}\). The permeability ratio of Ca\(^{2+}\) to K\(^+\) can be as low as 0.5 or as high as 15 (Véry and Davies, 2000; Demidchik and Maathuis, 2007), and this has led many researchers to use terms such as “Ca\(^{2+}\)-permeable (nonselective) cation channel.” Here, the term “calcium channel” will be used liberally, because in the signaling context of this Focus, even a spark or puff of Ca\(^{2+}\) emanating from these proteins could be enough to trigger the next event. The active transporters involved in terminating Ca\(^{2+}\) signals will not be addressed.

**THIS MUCH WE KNOW**

Biophysical studies have clearly demonstrated the existence of voltage-regulated and voltage-independent Ca\(^{2+}\) channels in the plant plasma membrane from a variety of cell types, most notably guard and root cells. Hyperpolarization-activated calcium channels (HACCs) can coexist with depolarization-activated calcium channels (DACCs) and voltage-independent...
calcium channels (VICCs). As can be inferred from the current-voltage relationships in Figure 1, coexistence means that a cell would be competent for Ca^{2+} influx across a very wide voltage range. Therefore, regulation of the transporters generating or recovering a given voltage (primarily H^+-ATPases, K^+, and anion channels) and the possibility of channel sequestration in specific membrane areas are of paramount importance in generating specific changes in [Ca^{2+}]_{cyt} (Miedema et al., 2001; Chen et al., 2012). HACCs operate in guard cell closure and polar growth and are downstream of light, hormones (notably abscisic acid in guard cells; Hamilton et al., 2000), and elicitors. Modeling studies point to the ability of HACCs to stabilize guard cell [Ca^{2+}]_{cyt} and stomatal closure (Chen et al., 2012; Hills et al., 2012). Whether regulators act directly or indirectly is often poorly understood, but they are responsive to (for example) actin, [Ca^{2+}]_{cyt}, cAMP/cyclic GMP (cGMP), heterotrimeric G proteins, phosphorylation, and reactive oxygen species (ROS; for review, see Demidchik and Maathuis, 2007; Dodd et al., 2010; Jammes et al., 2011). Defining the exact channel responding to signaling intermediates and so to specific receptors or sensors is one the great challenges facing the field.

DACC activity is quickly lost in patch-clamp recordings and may not always be stabilized by microtubule disruption. DACCs have received scant experimental attention, and the inability to resolve depolarization-activated [Ca^{2+}]_{cyt} elevation has brought their significance into question. VICCs are a notable feature of the root cell plasma membrane, where their potential ability to let Na^+ into cells is of significant agronomic importance (Maathuis and Sanders, 2001; Demidchik and Maathuis, 2007). Finally, mechanosensitive (MS) Ca^{2+} channels have been characterized in leaf, root, and pollen plasma membrane (for review, see Hedrich, 2012; Kurusu et al., 2013), where they may be responsible for reporting turgor and topography. Despite the technical challenges, endomembrane Ca^{2+} channels have been characterized at the vacuole, endoplasmic reticulum, chloroplast, and nucleus (Enz et al., 1993; Grygorczyk and Grygorczyk, 1998; for review, see Matzke et al., 2010; Peiter, 2011; Hedrich, 2012; Stael et al., 2012).

Attention has focused on the GLR and CNGC gene families as the most likely sources of genes encoding HACCs, DACCs, and VICCs. Members of both of these families (each comprising 20 members in Arabidopsis [Arabidopsis thaliana]) are predicted to form conventional transmembrane-spanning proteins that would function as channels when assembled into tetramers. Analysis of mutants has implicated members of both families in calcium signaling and [Ca^{2+}]_{cyt}-dependent polar growth (for review, see Dietrich et al., 2010; Dodd et al., 2010; Jammes et al., 2011; Hedrich, 2012). The rice (Oryza sativa) HKT2.4 potassium transporter was proposed to be a plasma membrane Ca^{2+}-permeable nonspecific cation channel on the basis of expression studies in Xenopus laevis oocytes (Tan et al., 2010), but recently, Sassi et al. (2012) reported contradictory data. Recent reports have shed light on two gene families (MSL, for MsS-like, and MCA, for Mldl-complementing activity) encoding transmembrane proteins with potential for MS Ca^{2+} channel activity (for review, see Kurusu et al., 2013). At the vacuole, TWO PORE CHANNEL1 (TPC1) is the slow vacuolar (SV) Ca^{2+}-release channel in Arabidopsis (Peiter et al., 2005), but its role in signaling remains obscure. This is Ca^{2+} channel research: find the gene that encodes the channel, find what the impact is on Ca^{2+} handling, and find a phenotype.

GLRs: MORE EVIDENCE FOR CALCIUM CHANNELS

The tobacco (Nicotiana tabacum) pollen tube contains a D-Ser-activated Ca^{2+} influx channel in the apical plasma membrane that is firmly implicated in growth control (Richard et al., 2011). Leading on from this, the Arabidopsis Atglr1.2 mutant was found to have both aberrant apical [Ca^{2+}]_{cyt} oscillations and morphology. The slower pollen tube growth rate of the Atglr3.7 mutant is suggestive of a role in [Ca^{2+}]_{cyt} regulation (Richard et al., 2011). Analysis of function by electrophysiology has shown that the Atglr3.4 mutant has impaired plasma membrane amino acid-regulated Ca^{2+} channel activity (Stephens et al., 2008), but the acid test is whether a recombinant protein can reconstitute that activity. Otherwise, the phenotype could be just a pleiotropic effect of the mutation. Heterologous expression of GLRs has proved to be problematic, but replacing the pore region of an animal ionotropic Glu receptor with that of AtGLR1.1 or AtGLR1.4 and then expressing the chimera

![Figure 1. Coresidence of different Ca^{2+} channels in the plasma membrane affords variable Ca^{2+} influx from the apoplast, controlled by membrane voltage. The schematic illustrates the effect of plasma membrane voltage (V_m) on net Ca^{2+} current measured using the "whole cell" patch-clamp configuration (Miedema et al., 2001; Demidchik and Maathuis, 2007). Negative current is generated by Ca^{2+} entry into the cytosol. DACC-mediated current is maximal at less negative plasma membrane voltage than HACC, while VICC-mediated current is effectively ohmic. Together, these channels would have the potential to vary [Ca^{2+}]_{cyt} as plasma membrane voltage changes.](image-url)
in X. laevis oocytes delivered compelling evidence for Ca\(^{2+}\) transport by plant GLRs, despite the dissimilarity of their pore regions to those of the ionotropic Glu receptors (Tapken and Hollmann, 2008). Expression of AtGLR3.7 and AtGLR3.4 (but not AtGLR2.1) in X. laevis oocytes resulted in nonendogenous Ca\(^{2+}\) influx currents, but these were not activated by amino acids (Roy et al., 2008). A recent breakthrough from the Spalding laboratory involved successful expression of AtGLR3.4 in human embryonic kidney (HEK) cells. GFP tagging clearly showed that AtGLR3.4 localized in the plasma membrane, while electrophysiological analysis demonstrated a high level of Ca\(^{2+}\) permeability facilitated by AtGLR3.4 (Vincill et al., 2012). This Ca\(^{2+}\) influx route was activated by the same amino acids (Asn, Ser, Gly) that caused AtGLR3.4-dependent [Ca\(^{2+}\)]\(_{\text{cyt}}\) elevation in HEK cells. Moreover, a previous study on Arabidopsis hypocotyls had shown that Asn- and Ser-dependent Ca\(^{2+}\) influx current required AtGLR3.4 (Stephens et al., 2008). So why didn’t amino acid activation of AtGLR3.4 occur in Roy’s study using oocytes (Roy et al., 2008)? Is this a protein-processing problem or an indication of the host cell’s capacity to provide interacting proteins or regulators?

This year, Vincill et al. (2013) identified AtGLR3.2 as an interacting protein for AtGLR3.4, but one with as yet no clear effect on the latter’s channel activity in HEK cells. Although both of these plasma membrane proteins reside at the sieve plate in roots, their activities ultimately manifest in the pericycle with a protease located at the sieve plate in roots, exogenous Glu causes an accumulation of extracellular ATP (Dark et al., 2011) that, in turn, can activate plasma membrane Ca\(^{2+}\) influx channels via ROS production by a NADPH oxidase (Demidchik et al., 2009). A schematic of this possible relationship is shown in Figure 2.

CNGCs: FROM RECEPTOR TO CHANNEL, A NEW ROLE IN THERMOSENSING AND ADVANCES IN UNDERSTANDING STRUCTURE-FUNCTION RELATIONSHIPS

Although the mechanisms of cyclic nucleotide elevation remain poorly understood, CNGCs as downstream targets have been studied in the greatest detail in Arabidopsis. AtCNGC2 forms a cyclic nucleotide-regulated HACC when heterologously expressed, and loss of function results in loss of a native plasma membrane cyclic nucleotide-regulated HACC (for review, see Dietrich et al., 2010). AtCNGC2 has now been shown to be a component in the perception of peptides generated as damage-associated molecular patterns of the immune response. Binding of an extracellular peptide to its cognate AtPepR1 receptor elevates cGMP levels (possibly by the receptor’s guanylyl cyclase domain), which then causes AtCNGC2-dependent elevation of [Ca\(^{2+}\)]\(_{\text{cyt}}\) (Qi et al., 2010; Ma et al., 2012). This is summarized in Figure 2. Analysis of mutants has implicated CNGCs in immunity, senescence, salt and heavy metal stress responses, and (polar) growth (for review, see Dietrich et al., 2010; Jammes et al., 2011; Hedrich, 2012). In Arabidopsis, AtCNGC7 has now been found localized to the plasma membrane at the flank of the pollen tube (Tunc-Ozdemir et al., 2013a). The AtCNGC7/8 double mutant is male sterile, with mutant pollen bursting at germination (Tunc-Ozdemir et al., 2013a), suggesting that AtCNGC7 and AtCNGC8 could function as Ca\(^{2+}\) channels to coordinate pollen growth. AtCNGC16 is also implicated in pollen tube growth (Tunc-Ozdemir et al., 2013b). A role in nutritional stress is now likely, as boron deficiency increases the expression of AtCNGC19 in roots (Quiles-Pando et al., 2013). This raises an interesting possibility of vacuolar involvement in boron stress, as AtCNGC19 and AtCNGC20 have now been found localized at the tonoplast (Quiles-Pando et al., 2013; Yuen and Christopher, 2013).

The recent findings that CNGCs participate in heat stress signaling in Arabidopsis and Physcomitrella patens provide a mechanistic basis for the well-documented heat-induced [Ca\(^{2+}\)]\(_{\text{cyt}}\) elevation (Finka et al., 2012; Gao et al., 2012). Heat stress raises cAMP in Arabidopsis and activates a root cell plasma membrane HACC that is also activated by membrane-permeant cAMP (Gao et al., 2012). This HACC is absent from the Atcngc6 mutant, leading to aberrant heat shock protein expression and lowered thermostolerance. In contrast, the Atcngc2 mutant and P. patens cngc6 mutant (orthologous genes) were found to be hyperthermosensitive (Finka et al., 2012). Loss of CNGCB activity in moss plasma membrane caused the loss of one of three heat-activated Ca\(^{2+}\) channels and increased the likelihood of the other two opening. Accordingly, the heat-induced [Ca\(^{2+}\)]\(_{\text{cyt}}\) signature was altered, and it is envisaged that these CNGCs act as heteromers in thermosensing (Finka et al., 2012). This year, Tunc-Ozdemir et al. (2013b) demonstrated that heat increases cGMP levels and that the Atcngc16 mutant has heat- and drought-sensitive pollen germination and

pollen tube outgrowth. Use of such mutants will help elucidate the role of \([\text{Ca}^{2+}]_{\text{cyt}}\) in thermosensing. In addition to heat, phytohormones also elevate cGMP, resulting in a suite of phosphorylated proteins (Ishrner et al., 2012). More proteins responsible for cAMP/cGMP elevation now need to be identified.

One of the main puzzles presented by the CNGCs is how they respond to cAMP/cGMP, an issue relevant to salinity stress and K⁺ nutrition. Although several papers report channel activation in native membranes (for review, see Dietrich et al., 2010), Maathuis and Sanders (2001) found inhibition by cAMP and cGMP of both Na⁺ influx into Arabidopsis roots (tracer) and Na⁺ influx into root protoplasts (patch clamp). AtCNGC3 has since been implicated in Na⁺ transport into roots (Gobert et al., 2006). cAMP/cGMP could play a further role in salinity tolerance by regulating the activity of the plasma membrane SOS1 Na⁺ extruder through a putative cyclic nucleotide-binding domain (Oh et al., 2007). More recently, Caballero et al. (2012) found that a membrane-permeant cyclic nucleotide inhibited K⁺ uptake by roots of the Atak1/Athak5 mutant that lacks key K⁺ uptake transporters and suggested that a CNGC could provide a compensatory uptake pathway. Structural studies on CNGCs are now gathering pace, with this year’s finding that Phe-to-Trp substitutions between the cytosolic C-terminal cyclic nucleotide-binding domain and the calmodulin (CaM)-binding site prevent mutant rescue by AtCNGC7 (Tunc-Ozdemir et al., 2013a). Previous studies (for review, see Dietrich et al., 2010) have established Ca²⁺-dependent CaM binding of the CNGC C terminus, and the current model is that \([\text{Ca}^{2+}]_{\text{cyt}}\) increases promote CaM binding with subsequent displacement of the cyclic nucleotide, resulting in channel closure. Fischer et al. (2013) showed that AtCNGC20 can bind all five Arabidopsis CaM isoforms (but not CaM-like proteins) and have demonstrated the existence of a conserved Ile-Gln motif that constitutes an additional CaM-binding site. Intriguingly, in contrast to Yuen and Christopher (2013), AtCNGC20 localized to the plasma membrane in the study by Fischer et al. (2013).

**MS CHANNELS: Ca²⁺ PERMEABILITY, A NEW GENE, AND A POTENTIAL ROLE IN [Ca²⁺]cyt OSCILLATIONS**

Ca²⁺ channels are central to mechanosensing in plants, which is key to growth and development as well as the response to changes in environmental conditions. Of the 10 AtMSL proteins that were identified through similarity to bacterial MS channels, AtMSL2 and AtMSL3 are plastidic, and although channel activity has not been determined, they are involved in osmoregulation and plastid division (Veley et al., 2012; for review, see Stael et al., 2012; Kurusu et al., 2013). Electrophysiological characterization of single Arabidopsis mutants of MSL9 and MSL10 suggested that these are root plasma membrane MS channels with some permeability for Ca²⁺ (Haswell et al., 2008). Further analysis established that heterologously expressed AtMSL10 can provide a MS channel activity with a moderate preference for anions, but Ca²⁺ selectivity was not addressed (Maksaev and Haswell, 2012). AtMSL9 and AtMSL10 are now depicted in the literature as anion channels (Kurusu et al., 2013), but it is premature to dismiss them as Ca²⁺-permeable channels. In the original study by Haswell et al. (2008), current was still generated when external Ca²⁺ was lowered from 50 to 1 mM in the presence of a K⁺ channel blocker. This would still provide a good driving force for Ca²⁺ influx, and Ca²⁺ was not removed completely to see if that abolished current. While AtMSL9 and AtMSL10 primarily but not exclusively localize to the plasma membrane of root cells, their roles in \([\text{Ca}^{2+}]_{\text{cyt}}\) signaling remain to be established, and single mutants, the double mutant msl9-1; msl10-1, and even the quintuple mutant msl4-1;msl5-2;
msl6-1;msl9-1;msl10-1 have shown no clear phenotype (Haswell et al., 2008).

MCAs were first identified in Arabidopsis because of their ability to complement the Mid1 yeast (Saccharomyces cerevisiae) mutant (Nakagawa et al., 2007). MCAs comprise two transmembrane domains, contain an EF hand, but show no significant similarity to known ion channels (for review, see Kurusu et al., 2013). All MCAs characterized thus far have localized to the plasma membrane and mediate Ca\(^{2+}\) uptake, with the N-terminal and EF hand regions as necessary structures for transport (for review, see Kurusu et al., 2013). Electrophysiological analyses of AtMCA1 expressed in X. laevis oocytes demonstrated that AtMCA1 can provide a MS cation channel activity of small conductance (34 pS; Furuichi et al., 2012). While a MCA gene family typically holds two homologs in dicots, only one homolog could be found in the Poaceae (Kurusu et al., 2013). In Arabidopsis, MCA1 and MCA2 seem to play functionally different roles, with mca1-null showing an impaired touch response in the primary root while mca2-null showed lower Ca\(^{2+}\) uptake in the roots (Nakagawa et al., 2007; Kurusu et al., 2013). A role for MCA1 in Ca\(^{2+}\) influx in response to mechanical stimulation at the plasma membrane was also supported by studies reporting the effect of hypoosmotic shock on rice OsMCA1 suppression lines and tobacco overexpressor cells. Induced transcript levels during the G1 phase suggested an additional role for NtMCA2 and AtMCA1 (for review, see Kurusu et al., 2013) during the cell cycle. Studies on plant MS channels now need to extend to piezo proteins; these are animal and insect MS channel subunits, and a single possible gene has been identified in Arabidopsis (At2g48060; Hedrich, 2012). These together with the MSL and MCA proteins are the clear candidates for testing a new model of [Ca\(^{2+}\)]_cyt oscillations, in which Ca\(^{2+}\) released from periplasmic arabinoxylan glycoproteins enters the cytosol through plasma membrane MS Ca\(^{2+}\) influx channels (Lamport and Várnai, 2013).

**ROS-RESPONSIVE CHANNELS: SKOR AND ANNEXINS**

ROS continue to enjoy significant research interest due to their participation in the life and death of plants, ranging from germination and growth to immunity and death. Plasma membrane Ca\(^{2+}\) channels can be responsive to ROS, depending on the cell type, which ROS is present, and at which membrane face (Demidchik et al., 2003, 2007). Only recently have these channels started to be linked with genes. The Shaker-like STELAR K+ OUTWARD RECTIFIER (SKOR) is a member of the K+ channel superfamily but has an experimentally determined Ca\(^{2+}\) permeability that could result in Ca\(^{2+}\) influx at the plasma membrane (Gaymard et al., 1998). The Atskor mutant accumulates less Ca\(^{2+}\) in its leaves than the wild type, consistent with SKOR-dependent retrieval of Ca\(^{2+}\) from the transpiration stream in the roots (Gaymard et al., 1998). K+ transport by the heterologously expressed AtSKOR responds positively to exogenous hydrogen peroxide, and although Ca\(^{2+}\) permeation was not tested, such results beg the question of whether AtSKOR is acting as a de facto ROS-activated calcium channel in vivo (García-Mata et al., 2010). A well-executed study on wheat (Triticum aestivum) root protoplasts that combined patch clamping with flux determination delivered “no consistent indication” of Ca\(^{2+}\) permeation through the K+ efflux pathway (Gillilham et al., 2006). It may now be timely to reexamine this question, perhaps by combining patch clamp with Ca\(^{2+}\) imaging (Gradogna et al., 2009) and by exploiting the ability to mutate AtSKOR for functional analysis (Poréé et al., 2005).

Plant annexins have been mooted to form Ca\(^{2+}\) channels for some time, but there is now firm evidence from in vitro and in vivo studies that they have some form of transport capacity (Hofmann et al., 2000; Laohavisit et al., 2009, 2010, 2012; Laohavisit and Davies, 2011a). Plant annexins are small (32–36 kD) amphipathic proteins that are capable of Ca\(^{2+}\)-dependent and -independent binding to anionic phospholipids. They are present throughout the plant kingdom, and an excellent recent review by Clark et al. (2012) charts their phylogeny from green algal ancestors onward. Annexins are seemingly ubiquitously distributed throughout the angiosperm body, from pollen and egg cells to the mature vegetative organs (for review, see Laohavisit and Davies 2011b; Clark et al., 2012). They also appear free to be distributed throughout the plant from their site of production via phloem sap (Laohavisit and Davies 2011b; Clark et al., 2012). In stark contrast to conventional Ca\(^{2+}\) channels, which will be transported from the Golgi to reside probably exclusively in specific membranes, plant annexins appear able to occupy more than one cellular location simultaneously and may also occupy lipid rafts (for review, see Laohavisit and Davies, 2011b; Clark et al., 2012). This provides scope for rapid, stimulus-driven recruitment to a membrane region not necessarily reliant on vesicle delivery.

Purified maize annexins conduct Ca\(^{2+}\) across planar lipid bilayers (PLB), changing from being voltage independent to hyperpolarization activated (Fig. 1), when malondialdehyde is incorporated into the PLB to mimic lipid peroxidation (Laohavisit et al., 2009, 2010). Thus, in vivo, interaction with malondialdehyde (which forms in membranes during stress responses known to involve ROS and [Ca\(^{2+}\)]_cyt elevation; for review, see Laohavisit et al., 2010) could restrict annexin-mediated Ca\(^{2+}\) transport to more negative voltages, and this may have an impact on a Ca\(^{2+}\) signature. Greater progress on in vivo function has been made with Arabidopsis ANNEXIN1 (AtANN1), one of eight in this plant. In addition to a presence in the cytosol, proteomic, immunolocalization, and GFP studies have identified AtANN1 at the plasma membrane, endoplasmic reticulum, vacuole, mitochondria, chloroplast, and cell wall (for review, see Laohavisit and Davies, 2011b). Its localization and expression in roots correspond well with the presence of a plasma membrane Ca\(^{2+}\) conductance that is involved in root cell elongation and is activated...
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by hyperpolarization and extracellular hydroxyl radicals (OH\(^{-}\); Demidchik et al., 2003; Foreman et al., 2003; Laohavisit et al., 2012). Accordingly, the AtANN1 knockout mutant was found to lack this Ca\(^{2+}\) channel in epidermal and root hair apical plasma membrane, with mutant root hairs found to be shorter than wild-type root hairs (Laohavisit et al., 2012). Mutant root hairs still retained the constitutive (i.e. not ROS-activated) HACC first described by Véry and Davies (2000), showing that this channel is encoded by a different gene and helping to explain why hairs could still grow.

Annexins appear as potentially multifunctional proteins in vitro, so their lipid- and actin-binding properties could mean that they are involved in regulating the trafficking of a more conventional Ca\(^{2+}\) channel to a membrane, and care needs to be taken in interpreting the loss of channel activity in an annexin mutant. However, recombinant AtANN1 formed an OH\(^{-}\)- and hyperpolarization-activated Ca\(^{2+}\) conductance in PLB that very strongly resembled the OH\(^{-}\)-activated Ca\(^{2+}\) channel conductance of the wild-type plasma membrane (Laohavisit et al., 2012). Despite conservation of the salt bridges thought to be involved in selectivity, the estimated permeability ratio of calcium to potassium P\(_{\text{Ca}}\)/P\(_{\text{K}}\) of 0.6 is much lower than that of animal annexins (Laohavisit et al., 2012), and the mechanism through which AtANN1 forms a transport route now needs to be elucidated. Animal counterparts form transport routes in vitro by destabilizing or inserting into bilayers, promoted by voltage, pH, GTP, and ROS, with annexin concentration determining voltage dependence (for review, see Laohavisit and Davies, 2011b). The structural differences between plant and animal annexins that could be relevant to functional diversification have been reviewed recently by Clark et al. (2012). Posttranslational modification may alter membrane association and the S-glutathionylation of AtANN1 severely limits membrane interaction (Konopka-Postupolska et al., 2009), so this could help restrict OH\(^{-}\)-activated Ca\(^{2+}\) influx by this annexin. Interacting protein partners have been considered recently by Laohavisit et al. (2011b) and Clark et al. (2012). The position of an annexin is likely to be central to its contribution to Ca\(^{2+}\) signaling. How plant annexins can become extracellular remains to be determined (Laohavisit et al., 2011b; Clark et al., 2012), but their ability to increase [Ca\(^{2+}\)]\(_{\text{cyt}}\) by acting at the apoplast face of the plasma membrane has now been demonstrated (Laohavisit et al., 2009). The maize annexins found to form a Ca\(^{2+}\) conductance in PLB were also capable of transiently increasing the [Ca\(^{2+}\)]\(_{\text{cyt}}\) of Arabidopsis protoplasts, but whether this was by directly forming a Ca\(^{2+}\) influx pathway or through the activation of other channels was not determined (Laohavisit et al., 2009).

ENDOMEMBRANES: TPC1 REMAINS CONTROVERSIAL, AND NEW INSIGHT FROM MODELING

After years of controversy on whether the SV channel was Ca\(^{2+}\) permeable, the debate now is about its function. Peiter et al., (2005) showed unequivocally that SV current magnitude varied with the expression of TPC1 in Arabidopsis, while Gradogna et al. (2009) combined patch clamping with Ca\(^{2+}\) imaging to show TPC1-dependent Ca\(^{2+}\) transport. Hamada et al. (2012) have found that rice OsTPC1 forms a voltage-regulated Ca\(^{2+}\) conductance in the HEK cell plasma membrane. A Ca\(^{2+}\)-binding site that confers sensitivity to vacuolar Ca\(^{2+}\) has now been identified, and the finding of an N-terminal di-Leu motif (EDPLI) that directs AtTPC1 to the tonoplast rather than the plasma membrane helps resolve the debate about the localization of that protein (Peiter, 2011; Hedrich, 2012; Larisch et al., 2012). In contrast, immunoblotting of rice plasma membrane still suggests that OsTPC1 resides there, and further analysis reveals a role in [Ca\(^{2+}\)]\(_{\text{cyt}}\) response to a fungal xylanase linking to phytoalexin synthesis (Hamada et al., 2012). Given the large magnitude of SV currents (which should permit vacuolar Ca\(^{2+}\) efflux), the lack of effect of AtTPC1 loss of function on [Ca\(^{2+}\)]\(_{\text{cyt}}\) in a wide range of signaling scenarios has been surprising (Ranf et al., 2008; Islam et al., 2010). However, whole seedling or organ studies may mask cell-specific responses; for example, the Atann1 root [Ca\(^{2+}\)]\(_{\text{cyt}}\) response to ROS was identical to that in the wild type, but genotypic differences were apparent at the epidermis (Laohavisit et al., 2012). Perhaps the finding that polyunsaturated fatty acids drastically inhibit SV activity (Gutla et al., 2012) may help explain why Attpc1 presents as the wild type in some [Ca\(^{2+}\)]\(_{\text{cyt}}\) studies. The polyunsaturated fatty acid a-linoleic acid is a potent SV inhibitor and is synthesized during wounding and pathogen attack (Gutla et al., 2012). Is experimental handling in signaling studies enough to trigger polyunsaturated fatty acid synthesis and inhibit the SV, leaving Attpc1 to phenocopy the wild type?

The expression of AtTPC1 is greater in guard cells than the mesophyll, and patch clamping has shown that SV characteristics differ between these two cell types, probably due to activities of regulatory proteins or cell-specific posttranslational modifications (Riemmüller et al., 2010). Analysis of the impact of loss of AtTPC1 function on guard cell [Ca\(^{2+}\)]\(_{\text{cyt}}\) dynamics could implicate AtTPC1 only in the priming of the Ca\(^{2+}\) sensitivity of the plasma membrane S-type anion channel (Islam et al., 2010). Recent predictive modeling studies of guard cell [Ca\(^{2+}\)]\(_{\text{cyt}}\) dynamics could not secure a clear role for TPC1 and, rather, emphasized the potential of other voltage-regulated vacuolar Ca\(^{2+}\) channels that now need to be identified at the genetic level (Chen et al., 2012; Hills et al., 2012). TPC1 is unsusited to a role in calcium release induced by an increase in [Ca\(^{2+}\)]\(_{\text{cyt}}\) because it is not self-regulating with regard to [Ca\(^{2+}\)]\(_{\text{cyt}}\) (Chen et al., 2012; Hills et al., 2012). The significance of TPC1 may lie, rather, in an ability to regulate vacuolar Ca\(^{2+}\) accumulation. AtTPC1 expression correlates positively with leaf Ca\(^{2+}\) content (Conn et al., 2012), but analysis of epidermal and mesophyll vacuolar Ca\(^{2+}\) revealed a negative relationship between that parameter and AtTPC1 expression (Gilliam et al., 2011). An
attractive possibility is that TPC1’s function is to release vacuolar Ca\(^{2+}\) to prevent accumulation in specific cell types (Gilliham et al., 2011; Conn et al., 2012). As mentioned earlier, there are now indications of CNGC residence at the tonoplast, GLR residence at the chloroplast, and MSL residence in chloroplasts (Teardo et al., 2011; Velely et al., 2012; Yuen and Christopher, 2013). The chloroplast protein translocon TIC complex conducts K\(^+\) in PLB, and its capacity for Ca\(^{2+}\) conduction now needs to be explored (Kikuchi et al., 2013). Mitochondria still present as a black box with regard to Ca\(^{2+}\) channels. It will be interesting to see whether the Arabidopsis genes identified as encoding possible homologs of the animal mitochondrial calcium uniporter Ca\(^{2+}\) channel can conduct Ca\(^{2+}\) (De Stefani et al., 2011; Stael et al., 2012).

**FUTURE DIRECTIONS**

Recent advances have made it clear that there are now even more genes to identify, consider, and even reconsider as encoding Ca\(^{2+}\)-permeable channels. The successful reconstitution of AtGLR3.4 in HEK cells should prove to be a turning point in Ca\(^{2+}\) channel research, permitting structure-function relationships to be explored further. Cellular expression systems may prove to be a useful tool in finding regulatory proteins. Protein-protein interaction studies may help reduce the number of theoretically possible heteromeric GLR and CNGC subunit combinations to be tested. A key challenge for annexin research is establishing their mode of Ca\(^{2+}\) transport. Modeling channel activity in Ca\(^{2+}\)-handling scenarios looks set to be a powerful tool in helping to predict and identify participating channels, particularly for specific signaling pathways. Identifying and characterizing Ca\(^{2+}\) signaling mutants holds much promise for future discoveries (Fan et al., 2012; Raffa et al., 2012), and it is hoped that these will include the identities of more HACCs, the DACCs, and the Ca\(^{2+}\) channels of the organelles.

Received May 3, 2013; accepted July 14, 2013; published July 16, 2013.

**LITERATURE CITED**


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