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REVIEW

Imaging of calcium channels during polarity induction in plant cells

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Abstract

Understanding the molecular basis of polarity induction in plant cells is a research aspect that extends from signal perception and transduction to morphogenesis. A gradient of cytoplasmic ion fluxes generated through ion channels plays a crucial role in subsequent events leading to polar growth. Convincing evidence is now available implicating temporal and spatial distribution of Ca^{2+} in cytoplasm, generated by localized activity of calcium channels, as the early biochemical events associated with polarity induction. Ion channel antagonists are common tools for studying ion channel structure and function. Coupled with a fluorescent dyes, calcium channel antagonists (phenylalkylamine and dihydropyridine), have been used to localize L-type calcium channels. Additionally, the advent of Confocal Laser Scanning Microscopy has made possible the visualization of Ca^{2+} channels in plant cells. Persisting problems of dye loading and their cellular compartmentation have been addressed by developing a variety of experimental protocols. Present article highlights the current state of our understanding of these concepts, methodologies and their applications in different aspects of plant development.

Additional key words: calcium antagonists, confocal laser scanning mirocscopy, voltage-gated ion channels.

Introduction

Polarity is defined as persistent asymmetrical and ordered distribution of structures along an axis. This definition applies equally to polarity at the level of organelles, cells, organs or whole organisms. In seed plants, polarity plays a critical role in the formation of pollen grains, roots, guard cells and trichomes in leaves, as well as during early embryo development. In non-seed plants, cells of green and brown algae, as well as vegetative cells of mosses and ferns exhibit polarity that is critical for normal development (Belanger and Quatrano 2000, Cove 2000). Division of a polar cell generates non-equivalent daughter cells allowing differential development. During tip growth, a cylindrical cell develops that is morphogenetically similar in pollen tubes, root hairs, rhizoids, fungal hyphae and moss protonemata. All polar systems in plants show cell wall expansion and localized exocytosis at the tip. Asymmetric cytoplasmic ion fluxes generated by ion channels are important regulators of exocytotic gradients leading to polar growth. Mechanisms by which ion fluxes may localize the site of growth or development, are broadly considered under two hypotheses (De Rujiter and Malho 2000, Malho *et al.* 2000): *1*) localized Ca^{2+} influx generates an electrical field across the cytoplasm, which in turn exerts a directional driving force on all charged particles within its range; *2*) polarization would result from electrophoretic redistribution of membrane proteins, leading to regional differentiation within the cells. According to the alternative hypothesis, localized ion fluxes serve as informational signals, exerting their effects by communicating with cytoskeleton. Evidences for both proposals are now available.

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Abbreviations: CLSM - confocal laser scanning microscopy; DHP - dihydrophyridine; NPA - naphthylphthalamic acid; PAA - phenylalkylamine; PAF - polar axis formation.
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Types of calcium channels

Specific roles for mechanosensitive Ca^{2+} channels have been proposed in the elongation of root hairs, pollen tubes, algal rhizoids and fungal hyphae (White 2000). The suggested sequence of events for polarized cell growth is: cell wall yielding and its evagination followed by stretching of the plasma membrane, localized activation of stretch-activated Ca^{2+} channels, Ca^{2+} influx and generation of a $[Ca^{2+}]_{cyt}$ gradient from tip to base, secretory-vesicle movement and exocytosis in the region of elevated $[Ca^{2+}]_{cyt}$. However, little is known about the Ca^{2+} channels involved in this process.

Structure of voltage-gated calcium channels

Calcium channels regulated by membrane potential show common features among different organisms (Trewavas 2000). They are composed of four different proteins (α_1, α_2, β and δ) (Fig. 1*A*). The major membrane-spanning and pore-forming subunit (α_1) consists of four homologous domains (I - IV), each composed of six transmembrane segments (not shown). The α_2 protein is linked by disulphide bridges to δ, which in turn interacts with α_1. Inside the cytoplasm, the β-subunit interacts with I - II domains of α_1. Extracellular regions of the channel may be glycosylated and internal regions phosphorylated by protein kinases. Anchorage of the calcium channel to the cytoskeleton and further regulation take place through the C termini of β and α_1. Why such a large structure is necessary to transmit Ca^{2+} is not understood.

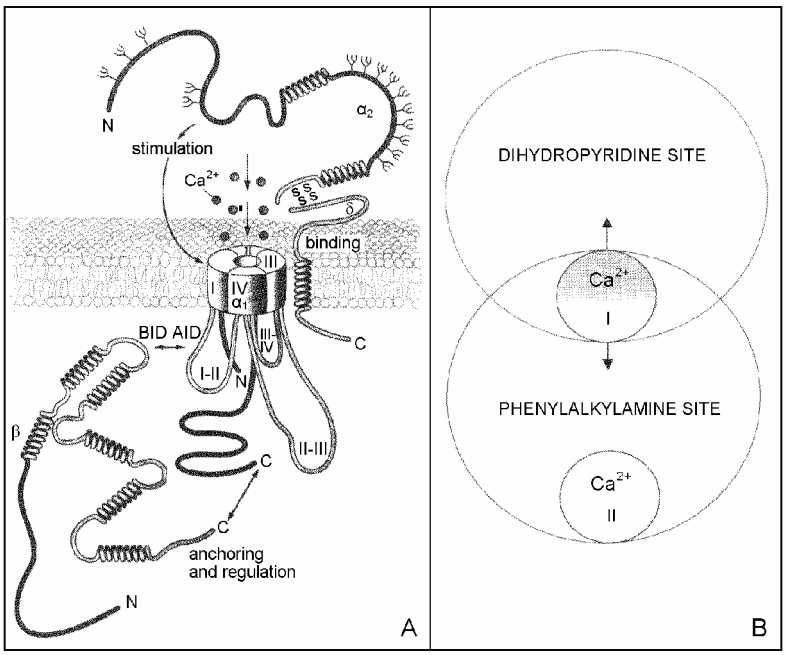

Fig. 1.*A.* A model representing the structural organization of a voltage-gated calcium channel. The calcium channels regulated by membrane potential share common features among different organisms. Adapted from Trewavas (2000). *B.* Model presenting calcium channel antagonists phenylalkylamine- and dihydropyridine-binding domains on the α_1-subunit of the Ca^{2+} channel, depicted as overlapping circles, enclosing Ca^{2+} binding site I. Another Ca^{2+} binding site (Ca^{2+} II) is enclosed by the phenylalkylamine-binding domain to symbolize a coupled metal ion-binding site, which can inhibit phenylalkylamine binding. *Arrows* indicate the reciprocal allosteric interaction between the two drug binding sites (according to Knaus *et al*. 1992).

Regulation of L type calcium channel activity

Based on their biophysical properties, including conductance and voltage sensitivity, calcium current is attributed to movement of ions through T-, N-, L- and P-type of Ca^{2+} channels. L-type calcium channels mediate voltage-controlled Ca^{2+} entry into a variety of excitable cells. Activity of these channels is modulated by different classes of Ca^{2+} channel antagonists. There is strong evidence for three Ca^{2+}-antagonist receptor sites

on the Ca^{2+} channels in animal cells (for dihydropyridines, phenylalkyl-amines and benzothiazepines). Effects of these drugs are exerted by their binding to allosterically coupled domains on the pore forming α_1-subunit. A state-dependent binding of Ca^{2+} and antagonists explains Ca^{2+} movement through these channels (Knaus *et al.* 1992). Dihydropyridine- and phenylalkylamine-binding domains share a common high affinity Ca^{2+} binding site (Ca^{2+} site 1) (Fig. 1*B*). Ca^{2+} at this site is necessary for the stability of high affinity drug-binding domains. If Ca^{2+} bound to this site is removed by chelators, the α_1 subunit suffers a complete loss of the most characteristic feature of L-type Ca^{2+} channels (voltage sensors), *i.e.* high affinity Ca^{2+} antagonist drug binding. If Ca^{2+} is reintroduced, the binding domains convert back into high affinity state. Another Ca^{2+} binding site (Ca^{2+} site II) is coupled in a negative fashion to the phenylalkylamine-binding site. Ca^{2+} site I is the priming site of the voltage sensor, oriented to the extracellular face of the Ca^{2+} channel, whereas Ca^{2+} site II is oriented to the cytosol.

Two more categories of Ca^{2+} channels that must be mentioned here: IP_3-dependent and cyclic ADP ribose activated channels. The application of IP_3 releases Ca^{2+} from vacuoles and tonoplast vesicles isolated from many plant species and tissues (White 2000, Malho *et al.* 2000). The IP_3 dependent Ca^{2+} channels are electrically silent in the absence of IP_3 and can be activated half-maximally at IP_3 concentrations as low as 200 nM (Trewavas 1999). Their absolute requirement for IP_3 strongly suggests that these channels are involved in Ca^{2+}-mediated signal transduction. The IP_3 dependent Ca^{2+} channels activate at physiological tonoplast membrane potentials. They mediate Ca^{2+} influx to the cytoplasm and excessive cytoplasmic Ca^{2+} loading might be prevented because Ca^{2+} influx will depolarize the tonoplast and shut the channels. The opening of IP_3-dependent Ca^{2+} channels has been implicated in regulation of pressure potential in beet root in response to hyperosmotic stress, closing of stomata in response to ABA and the self-incompatibility and reorientation responses of pollen tube (White 2000). Cyclic ADP ribose (cADPR) activates inward rectifying currents in vacuoles from red beet taproots (K_m of 24 nM) and guard cells. The channels underlying this current appear to be fairly selective for Ca^{2+} over K^+, and will mediate Ca^{2+} efflux from the vacuole. A role for cADPR-dependent Ca^{2+} channels in two ABA-dependent signaling pathways has been proposed. A link between the induction of *kin 2* (cold responsive) and *rd29A* (desiccation responsive) genes by ABA in tomato hypocotyl cells and cADPR-dependent Ca^{2+} release is suggested by the observations that cADPR is increased in response to ABA and microinjection of cADPR or Ca^{2+} induced gene expression. Finally inhibition of cADPR production and buffering $[Ca^{2+}]$ cyt to low concentrations prevented gene expression.

Probes for Ca^{2+} channels and their visualization by confocal laser scanning microscopy (CLSM)

Two classes of fluorescent probes are commercially available for the localization of L-type Ca^{2+} channels in viable cells (*e.g.* Richard 1996). These include fluorescent dihydrophyridines (DHP) and fluorescent phenylalkylamines (PAA). Conjugated dihydropyridines (BODIPY) bind to L-type Ca^{2+} channels with high affinity and inhibit Ca^{2+} influx. Upon binding to α_1 subunit, this ligand exhibits an increase in fluorescence quantum yield and fluorescence energy transfer between its fluorophore and one or more of the channel tryptophan residues (Knaus *et al.* 1982). Spatial distribution and density of L-type calcium channels is determined by CLSM. Using fluorescently labelled phenylalkylamine (DM-BODIPY-PAA), Knaus *et al.* (1992) have obtained compelling evidence for the coupling between Ca^{2+} binding sites and phenyl-alkylamine receptors in purified L-type Ca^{2+} channels. DM-BODIPY-PAA binds to same sites as PAA (such as verapamil) and emits a green fluorescence signal. Fluorescence due to this probe is strongly reduced in the presence of a non-fluorescent competitor of PAA-bepridil. Thus, DM-BODIPY-PAA appears to be suitable for labelling L-type Ca^{2+} channels which bear a receptor for PAA and are included in lipidic membranes.

Applications

Protoplast culture: Distribution of PAA-binding sites alters during protoplast culture and differs according to the type of division. This suggests a redistribution of ion channels during protoplast culture. Vallee *et al.* (1997, 1999) investigated the *in vivo* localization of calcium channel antagonist (PAA)-binding sites in cultured protoplasts of sun-flower. Freshly isolated protoplasts show random distribution of fluorescence due to DM-BODIPY-PAA on the plasma membrane (Valee *et al.* 1999). After a few days of culture, PAA-binding sites are no longer restricted to the plasma membrane but are present inside the cell, along the cytoplasmic strands,

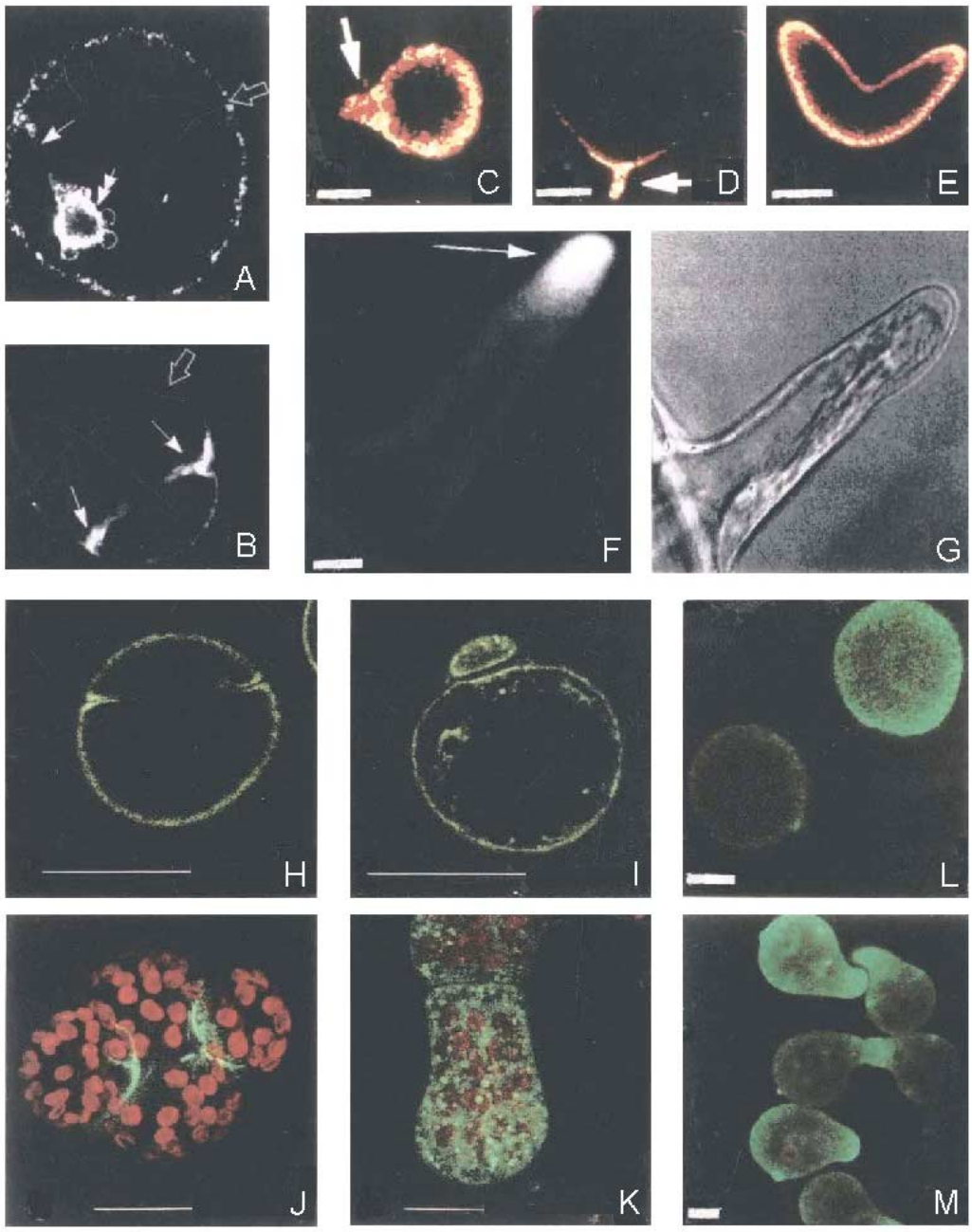

Fig. 2. CLSM images (median confocal sections) showing the distribution of L-type Ca^{2+} channels in different plant groups at specific stages of development. *A*,*B* - DM-BODIPY PAA labelled protoplasts of sunflower 2 d after culture but still undivided, showing strong fluorescence from the plasma membrane and the nuclear membrane (*A*), and protoplasts undergoing unequal division in agarose medium showing strong fluorescence from the newly formed cross wall (*B*). DM-BODIPY-PAA labelled globular embryos showing fluorescence in the outer cell layers (*C*) and suspensor; heart-shaped embryos showing labelling in the suspensor (*D*) and outer cell layers (*E*). *F*,*G* - gradient of fluorescence from a DM-BODIPY-DHP labelled root hair (*F*) and its bright-field image (*G*). *H*,*I*,*J*,*K* - distribution of DM-BODIPY-PAA binding sites in the protoplasts of the moss *Physcomitrella patens* showing unequal division (*H*,*I*), germ tube (*J*) and polar tube formation (*K*). Note high accumulation of the PAA-binding sites along the margins of the cross wall (*H*), plasma membrane and cytoplasmic contents of the smaller daughter cell (*I*), nuclear membrane in the parent cell (*I*), base of the septum separating the germ tube from the parent cell (*J*) and cytosolic contents of the polar tube (*K*). *L*,*M* - ST-BODIPY-DHP labelled zygotes of *Fucus* showing uniformity of gradient of fluorescence in non-polar zygotes (*L*) and gradient of tip high fuorescence in the polar tube (*M*). *Bar* = 30 μm (*A*,*B*); 100 μm (*C*); 200 μm (*D*,*E*); 10 μm (*F*,*G*); 20 μm (*H*,*I*,*J*,*K*); 25 μm (*L*,*M*). Illustrations redrawn from Vallee *et al.* (1999) (*A*,*B*); XuHan *et al.* (1999) (*C*,*D*,*E*); Bibikova *et al.* (2000) (*F*,*G*); Bhatla *et al.* (2002) (*H*,*I*,*J*,*K*); Shaw and Quatrano (1996) (*L*,*M*).

on the membranes of vesicles and vacuoles, and are highly concentrated on or near the nuclear envelope. Just after cell division, channel activity is restricted to a zone close to the new cell wall. There is no or only weak channels localization along the periphery of cell wall. Thus, intracellular distribution of PAA binding sites in sunflower protoplasts appears to be linked in the cell division (Fig. 2*A*,*B*).

Morphogenesis: DM-BODIPY-PAA differentially labels embryonic and non-embryonic tissues of sunflower (XuHan *et al.* 1999). Labelling in embryonic tissues is strongly reduced by bepridil. Strong DM-BODIPY-PAA labelling signals have been recorded in the suspensor, the protoderm and epidermis of both zygotic and somatic embryos, but a weak fluorescence is observed in the inner embryonic tissues and also in non-embryonic tissues with the exception of root tips (Fig. 2*C*,*D*,*E*). The similarities in the labelling pattern found in zygotic and somatic embryos point to similar properties and functions of their epidermis. Strong labelling of the suspensor of sunflower zygotic embryos can be related to their critical role during early embryogenesis, *e.g.*, transport of nutrients.

Root hairs: Clustering of Ca^{2+} channels at the apex of growing root hairs has been observed by loading the root hairs of *Arabidopsis* with BODIPY-DHP and image analysis using confocal microscope (Bibikova and Gilroy 2000). Bright fluorescence due to Ca^{2+} channels is thus observed throughout the volume of the apical cytoplasm (Fig. 2*F*,*G*). Thus, it does not simply reflect Ca^{2+} channel accumulation along the root hair plasma membrane. Such gradient in fluorescence may well reflect intracellular Ca^{2+} channels on their way to insertion into the apical plasma membrane, as part of the secretory machinery. In a growing root hair, the vacuole is excluded from the site where the Ca^{2+} gradient is formed. Thus, the vacuole is an unlikely candidate for maintaining the apical gradient.

Moss protoplasts: Upon culture, isolated protoplasts of mosses regenerate into cell filaments. This process is affected by different physical (*e.g.* light), hormonal (*e.g.* auxins) and nonhormonal chemical factors, such as calcium (Bhatla *et al* 1995). The phytohormone signal may be mediated by changes in cytosolic Ca^{2+} concentrations, involving Ca^{2+} channels. In the moss *Physcomitrella patens*, protoplasts undergo unequal divisions, leading to germ tube formation from the smaller cell. These divisions are efficiently blocked by naphthylphthalamic acid (NPA; a specific blocker of auxin effux channels) and the calcium channel antagonist, bepridil, thus linking auxin and Ca^{2+} fluxes during polarity induction in the protoplasts (Bhatla *et al.* 2000). In freshly isolated protoplasts of *Physcomitrella patens*, the DM-DOBIPY-PAA binding sites are distributed on or close to the plasma membrane and are organized into clusters. Beginning of cell plate formation is marked with a clustering of the Ca^{2+} binding sites along the margins of the new cell plates (Fig. 2*H*,*I*,*J*,*K*) .

Polar axis formation in *Fucus* zygotes: Zygotes of *Pelvetia* and *Fucus* serve as excellent model systems to study establishment of polarity. An axis of polarity can be experimentally imposed upon the zygotes and continually reoriented during the first half of the zygotic cell cycle, using, *e.g.*, unilateral white light or electric current. After polar axis fixation (PAF), the polar axis can no longer be reoriented. Formation of a polar axis involves an actin-dependent localization of ion channels, some of which are calcium channels. Localized calcium ion influx is one of the earliest observable polar phenomena in zygotes and suggests that calcium channels might serve as molecular marker for the formation of the polar axis (Fig. 2*L*,*M*; Shaw and Quatrano 1996).

BODIPY-FL-DHP uniformly binds to newly fertilized zygotes. Zygotes in a population start exhibiting asymmetrical fluorescent labelling 6 h after fertilization in response to light. Addition of Bay-K 8644 (known to block FL-DHP binding) inhibits the binding of FL-DHP at the cell cortex, indicating that FL-DHP binds to dihydropyridine receptors and also blocks polar growth of rhizoids. Studies using electrophysiological and radiometric dye methods to measure calcium have demonstrated the presence of a high calcium gradient in the tips of *Fucus* rhizoids. These data, strongly suggest that FL-DHP receptors represent Ca^{2+} channels at the plasma membrane of zygotes. Thus, FL-DHP localization can be used as a visual assay to identify earlier intermediates in the process of polar axis formation. Time lapse video microscopy has shown physical translocation of ion channels to the plasma membrane. F-actin cytoskeleton is required for the proper localization or activation of Ca^{2+} channels in the plasma membrane. It is proposed from these studies that the ion channels which are translocated to specific locations of the plasma membrane, give rise to a cytoplasmic Ca^{2+} gradient. A role of Ca^{2+} is thus reported for polar growth in *Fucus* zygotes.

Pollen tubes: Pollen tubes exhibit extraordinarily pronounced polarized growth resulting from growth confined to the tip of the tubes. It involves localized secretion and cell wall synthesis. It is now well established that there is a tip to base gradient of cytosolic free calcium $[Ca^{2+}]_{cyt}$ in growing but not in non-growing pollen tubes. This gradient could be important in regulating the F-actin network which has a multifunctional role in coordinating different aspects of tip growth. Calcium channel activity in the growing tip of pollen tubes could be an important factor for maintaining the $[Ca^{2+}]_{cyt}$ gradient. Mahlo *et al.* (1995) employed a novel method to measure Ca^{2+} channel activity in living pollen tubes of *Agapanthus umbellatus*. It involves addition of extracellular Mn^{2+} to quench the fluorescence

of intercellular calcium probe, (Indo-1) at its Ca^{2+}-insensitive wavelength (isobestic point). The results presented in this work provide evidence for the pivotal role of Ca^{2+} channels in pollen tube growth and that they determine the size and site of the Ca^{2+} gradient. It has been suggested that ion channels activated by stretching of the plasma membrane act as sensors of cell pressure potential, external mechanical stresses, voltage gradients and chemical gradients.

Future scope

With the advent of technologies like CLSM and sensitive fluorescent probes for localizing calcium channels, it should now be possible to further understand the regulatory role of calcium channels in polarity induction, cell division and differentiation in plants. Whether the nature of cell division is at the origin of, or results from, the ion channel redistribution, remains to be elucidated. Since polar growth requires apical expansion of the cell wall, it would be interesting to monitor changes in the activity of Ca^{2+}- binding sites in the cell wall, such as pectin methylesterases, responsible for cross-linking the unesterified pectins. It is quite possible that communications between intracellular calcium stores, plasma membrane and cell wall may participate in the regulation of calcium channels. Activity of ion channels in tip-growing cells might be regulated by the degree of emptiness of intracellular calcium stores. Protein kinases might play a direct or indirect role in the release of Ca^{2+} from intracellular stores, thereby regulating the opening or closing of Ca^{2+} channels. Finally, it would be interesting to understand the basis of localized accumulation of ion channels during polarity induction through the movement of newly synthesized subunits of ion channels via intracellular vesicular traffic.

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