CHAPTER 15

MS Channels in Tip-Growing Systems

Mark A. Messerli* **and Kenneth R. Robinson**†

*BioCurrents Research Center, Program in Molecular Physiology,
Marine Biological Laboratory, Woods Hole, Massachusetts 02543
†Department of Biological Sciences, Purdue University,
West Lafayette, Indiana 47907

I. OVERVIEW

Wall-enclosed cells expanding by tip growth face the problem of delivering new wall and membrane material to the growing tip, and regulating the turgor pressure so that an appropriate force for overcoming wall resistance is applied, without inducing bursting. We have selected four diverse organisms for review, based on electrophysiological data showing the existence of mechanosensitive (MS) channels in the plasma membranes of these organisms and imaging data showing ion gradients at the tip. The four organisms are pollen tubes of the lily, *Lilium longiflorum*, hyphae of the water mold, *Saprolegnia ferax*, rhizoids of the fucoid embryo, and hyphae of the bread mold, *Neurospora crassa.* We conclude that the MS channels play an essential role as the sensors of turgor pressure in these organisms and act through feedback mechanisms to regulate various aspects of growth and the

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response to osmotic challenge. However, there is striking divergence in the roles played by the MS channels in the four cases. The available data indicate that current methods for measuring turgor pressure in these cells are sufficiently imprecise to the extent that the relatively small variations in turgor that can affect the open/closed state of MS channels have not been yet detected. Clearly, more sensitive methods for measuring turgor are needed to detect these subtle changes in turgor variation.

II. INTRODUCTION

Tip growth is a form of extension in which cellular expansion occurs at a single restricted site. It typically results in the formation of cylindrical- or conical-shaped cells. Tip growth is a common feature of expansion in plants, fungi, and algae, leading to the production of root hairs, pollen tubes, fungal hyphae, and filamentous growth of algae and lower plants. In most cases, tip growth appears to be used by nonmotile organisms in order to maximize the space over which an organism can collect nutrients while minimizing the amount of growth necessary to cover the distance between the organism and the fresh supply of nutrients. In the case of pollen, tip growth is used as a vehicle to transport the sperm cells long distances through the pistil, as the sperm cells by themselves are nonmotile.

The tip-growing organisms mentioned above share common features at both the cellular level and the tip growth regions as well with the physiology associated with tip growth. The cells of these organisms are enclosed within cell walls and maintain turgor pressure. The tip-growing regions contain dense vesicular pools at the fusion zones where new cell membrane and cell wall are elaborated, building on the tips from the inside out. As Frank Harold (2002) has pointed out, ''It is no trivial matter to enlarge the cellular container, working entirely with forces generated internally, while preserving the cell's integrity.'' Turgor within the cell is everywhere the same, yet growth must be focused on a restricted region. The secretory vesicles must supply new cell wall materials as well as new membrane and there is no easy solution to providing just the right amount of both, so excess membrane must be removed via endocytosis in a highly regulated manner. In addition, a number of the tip-growing organisms must cope with a variable external osmotic environment, so turgor can undergo rapid fluctuations to which the cell must respond.

An important, perhaps universal, feature of tip growth is the necessity of an intracellular, tip-high gradient of Ca^{2+}. In the case of pollen tubes, the correlation of the gradient with growth is especially strong and oscillations of growth rate occur at the same frequency as oscillations of tip Ca^{2+}

(Holdaway-Clarke *et al.*, 1997; Messerli and Robinson, 1997; Messerli *et al.*, 2000). A Ca^{2+} gradient is a form of directional information, which seems to regulate the location and rate of vesicle fusion. It has long been an attractive hypothesis that MS channels might be localized or locally activated at the tip with the role of maintaining and regulating the Ca^{2+} gradient. Plasma membrane MS channels are a common feature of tip-growing systems. In addition, tip-growing organisms must maintain osmotic and ionic homeostasis against a massive influx of water that is necessary for volume expansion. K^+ is generally a significant component of the cytoplasm and is the major inorganic ion contributing to cytosolic osmolarity; thus, it is reasonable to expect that tip-localized MS K^+ channels may contribute to K^+ homeostasis.

In this chapter, we focus on four of the most well-characterized systems that elongate via tip growth: the lily pollen tube, hyphae of the water mold, *Saprolegnia ferax*, rhizoids of the fucoid embryo, and hyphae of the bread mold, *N. crassa*. Examples of each of these organisms are shown in Fig. 1. Our choice is determined by the fact that there is direct evidence for MS channels in these organisms as well as information about intracellular ion gradients from imaging of fluorescent ion indicators. A similar abundance of information is not available for other tip-growing systems including plant root hairs and germ tubes of *Uromyces*, even though *Uromyces* was the first tip-growing system in which an MS channel was characterized (Zhou *et al.*, 1991). Each of the four systems to be discussed contains plasma membrane MS channels that are permeable to Ca^{2+} and maintain tip-high Ca^{2+} gradients during growth but only lily pollen tubes and hyphae of *S. ferax* appear to use MS channels to generate that Ca^{2+} gradient.

III. *LILIUM LONGIFLORUM* POLLEN TUBES

Fertilization in seed plants is dependent on tip growth by the male gametophyte, the pollen grain. When the pollen grain lands on a compatible stigma, it germinates a tube that penetrates the stigma, grows through the length of the style and into the ovary in order to release two sperm, effecting double fertilization of the egg and central cell. The tube acts as a conduit through which the sperms are transported through the pistil. *L. longiflorum* pollen tubes traverse the 11 cm of style in about 2.5 days (Jauh and Lord, 1995) to reach the ovary at the base. Tubes then have to grow up to an additional 4 cm to reach eggs at the base of the ovary. While tubes grow at an average rate of 30 μm/min for the entire trip down the style, they grow at about 14 μ and 12.5 μm/min for the first and last 2 cm of the stylar trip, respectively (Jauh and Lord, 1995). Average growth rates in culture for tubes

shorter than 2 cm are between 10 and 15 μm/min (reviewed in Messerli *et al.*, 2000), thereby closely matching growth rates down the style for the same length of tube. Lily pollen tubes only need Ca^{2+}, K^{+}, boric acid, an impermeant osmoticum, and a slightly acidic pH to maintain these growth rates in culture. Growth is driven by bulk exocytosis of plasma membrane and cell wall material at the tip. The "clear zone" at the tip (Fig. 1A) contains an inverted

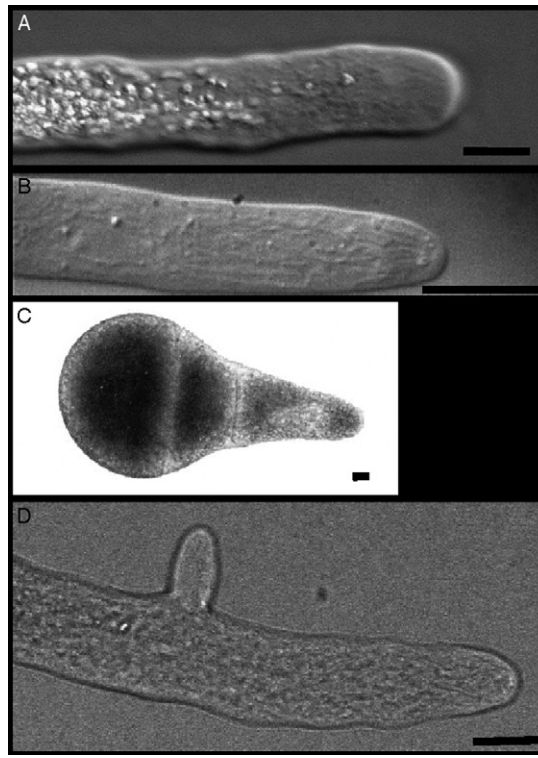

FIGURE 1 Pictures of four tip-growing systems. (A) Differential interference contrast (DIC) picture of a growing *L. longiflorum* pollen tube. The relatively smooth zone near the tip is referred to as the "clear zone" containing secretory vesicles. Larger organelles are seen more distal to the tip. (B) A DIC picture of the tip of a growing *S. ferax* hypha kindly provided by Roger R. Lew. This picture is an example of those used to analyze pulsatile growth in hyphal organisms (Sampson *et al.*, 2003). (C) *Silvetia compressa* after second cell division in the tip-growing rhizoid. Only first cell division has taken place in the thallus. (D) Transmitted light picture of a growing *N. crassa* hypha. This picture is an example of those used to study the role of mitochondria in fungal tip growth (Levina and Lew, 2006), and kindly provided by the authors. A second smaller hypha has branched about 50 μm behind the tip of the primary hypha. Scale bars in the lower right corner of each picture are 10 μm.

cone of secretory vesicles enclosing cell wall precursors (VanDerWoude *et al.*, 1971; Lancelle and Hepler, 1992).

In culture, lily pollen tubes grow in a relatively steady manner until they reach between 0.7 and 1.0 mm in length after which they begin to grow in an oscillating manner with peak growth rates—two- to fivefold greater than the basal growth rates and periods ranging between 20 and 60 s (Pierson *et al.*, 1996; Messerli and Robinson, 1997). A number of other species of pollen tubes have been shown to grow in an oscillating or pulsatile manner as well (Pierson *et al.*, 1995). *Arabidopsis* pollen tubes have been shown to grow in an oscillating manner down the cut style, supporting the hypothesis that oscillating growth is the predominant manner of growth in the style (Iwano *et al.*, 2004).

In early studies, Ca^{2+} was shown to be an essential nutrient for growth as pollen tubes did not germinate or grow in the absence of Ca^{2+} (Brewbaker and Kwack, 1963). The first attempt to map cytoplasmic distribution of Ca^{2+} was performed by long- and short-term incubation of lily pollen tubes in $^{45}Ca^{2+}$ (Jaffe *et al.*, 1975). Lily tubes that were incubated in $^{45}Ca^{2+}$ for 2–5 h and rinsed for 1 h as well as tubes that were incubated for only 1–3 min showed tip-high accumulation of $^{45}Ca^{2+}$. The accumulation of $^{45}Ca^{2+}$ after long-term exposure and rinse may reflect tip localized cytoplasmic Ca^{2+} stores, while the accumulation after brief exposure led to the inference that rapid Ca^{2+} uptake through the plasma membrane occurred at the tips compared to the shafts of the tubes. Imaging the intracellular free $[Ca^{2+}]$ with Indo-1 (Rathore *et al.*, 1991) or with Fura-2 10-kDa dextran (Miller *et al.*, 1992) showed a tip-high cytosolic Ca^{2+} gradient that was only present in growing tubes. Abolishing the Ca^{2+} gradient by various means disrupted or stopped growth (Rathore *et al.*, 1991; Miller *et al.*, 1992; Pierson *et al.*, 1994; Messerli and Robinson, 1997) providing evidence that growth, with emphasis on exocytosis, was dependent on the tip-high Ca^{2+} gradient. During oscillating growth in lily pollen tubes, an oscillating rise in the cytosolic tip-high Ca^{2+} gradient occurs but lags growth by about 4 s (Messerli and Robinson, 1997; Messerli *et al.*, 2000). If the tip-high Ca^{2+} gradient is correlated with exocytosis, then a small amount of exocytosis is always occurring in growing tubes and a large bulk exocytotic event occurs about 4 s after the surge in cellular expansion. This phase shift between cellular expansion and the rise in cytosolic Ca^{2+} leads to the idea that the sensor for cellular expansion was an MS Ca^{2+} channel. Consistent with this idea is that the tip Ca^{2+} is increased in response to hypotonic medium (Messerli and Robinson, 2003) and eliminated in response to hypertonic medium (Pierson *et al.*, 1994; Messerli and Robinson, 2003).

Additional ionic oscillations have been detected during oscillating tip growth of lily pollen tubes, specifically, tip restricted, oscillating influx of

H^+ and K^+ (Messerli *et al.*, 1999). These oscillating influxes were coincident, lagging growth by 11–13 s, and were of equal magnitude. Intracellular oscillating acidification was shown earlier to lag growth by 7.5–12.5 s (Messerli and Robinson, 1998) supporting the idea that at least H^+ and maybe K^+ is crossing the plasma membrane. The coincidence of H^+/K^+ influx along with the equal peak magnitude leads to the postulation of oscillating activity of an H^+/K^+ symporter that may be necessary to drive K^+ uptake to maintain ionic or osmotic balance after the surge in growth. Under external conditions that are expected to decrease the activity of an H^+/K^+ symporter, weaker oscillating influx of H^+ has been reported (Feijó *et al.*, 1999), indicating that under special growth conditions, H^+/K^+ symport may not be necessary to maintain lily pollen tube growth.

Two types of plasma membrane MS channels have been identified in lily pollen, an MS Ca^{2+} channel and an MS K^+ channel (Dutta and Robinson, 2004). Single channels were characterized in isolated patches, so some degree of cytoplasmic regulation may be missing. Pollen grains did not express MS channels at 10 or 30 min after hydration but expressed the two MS channels within an hour after hydration, approximately the time when pollen tube germination begins. This may indicate that the two MS channels are not involved with pollen grain hydration but are necessary for tube germination and growth. The MS Ca^{2+} channel was localized to the pregermination fold on pollen grains and to protoplasts generated from the tips of pollen tubes. The pregermination fold is a thin slit along the pollen grain from which the pollen tube can germinate. The fold causes a groove on grain protoplasts. There was a 97% chance of finding the MS Ca^{2+} channel at that groove and only a 2% chance when looking over the entire surface of the grain. MS Ca^{2+} channels on the grain protoplast only opened in response to pressure, had an instantaneous conductance of about 15 pS, and had a permeability ratio $P_{Ca^{2+}}/P_{K^+}$ of 100 indicating high selectivity for Ca^{2+} over K^+. Channels were blocked by 10-μM Gd^{3+} and a 1/3000 dilution of crude spider venom (*Grammostola spatulata*) which is known to block MS channels from pituitary cells at a final dilution of 1/1000 (Chen *et al.*, 1996). Lily pollen tube growth was also stopped as soon as tubes could be viewed when introduced to 1/3000 dilution of spider venom (Dutta and Robinson, 2004) or when exposed to 20-μM Gd^{3+} (unpublished observations) indicating a requirement for MS channel activity even at the basal rate of growth. For comparison, neither this dilution of spider venom nor 50-μM Gd^{3+} affected the activity of a spontaneous K^+ that was also identified on the pollen grains. At the pollen tube tip, the MS Ca^{2+} channels occurred in higher density and possessed higher conductance than the MS Ca^{2+} channels on the grain. No other MS, voltage-gated, or spontaneous cation or anion channels were found in the protoplasts generated from the pollen tube tip

(Dutta and Robinson, 2004). Channel characterization was not performed along the shaft of the tubes.

Our current model for lily pollen tube growth assumes a low level of activation of MS Ca^{2+} channels at the tip as a tip-high Ca^{2+} gradient is always present in growing tubes (Rathore *et al.*, 1991; Miller *et al.*, 1992) even at the low rates of growth during oscillating growth (Messerli *et al.*, 2000). A surge in expansion during oscillating growth leads to increased tension on the plasma membrane that opens additional MS Ca^{2+} channels leading to Ca^{2+} influx across the plasma membrane and generating a bulk exocytotic event, releasing plasma membrane and cell wall at the tip. There is reason to think that only a small triggering amount of Ca^{2+} enters through the MS channels and that Ca^{2+} or IP3 induces additional release of Ca^{2+} from intracellular stores to generate a bulk of the rise in cytosolic [Ca^{2+}] (Messerli and Robinson, 1997, 2003; Messerli *et al.*, 1999). The bulk exocytosis would then be expected to lead to a ruffled/slackened plasma membrane and a thicker cell wall that will decrease the activity of MS Ca^{2+} channels and impede further extension, respectively. The events are repeated during each oscillating surge in growth. The mechanism that generates the surge in expansion may involve controlled weakening of the cell wall or increased turgor pressure that overcomes the strength of the cell wall.

Unlike the MS Ca^{2+} channel, the MS K^+ channel identified on the lily pollen was not found at the pregermination fold or at the pollen tube tip but was found over the rest of the surface of the grain (Dutta and Robinson, 2004). Similar to the MS Ca^{2+} channel, the MS K^+ channel was only activated during applied pressure and was blocked by 10-μM Gd^{3+} and 1/3000 dilution of the crude *G. spatulata* venom. The MS K^+ channel possessed an average conductance of 33 pS in 24-mM K^+_{ext} and did not show fatigue like the MS Ca^{2+} channels.

The characterization of the MS channels in lily pollen tubes has placed necessary constraints on the system that should help to elucidate the physiological control of this tip-growing system. For example, it has been postulated that the H^+ influx at the tip of the tubes may occur through a nonspecific MS or other nonspecific cation channel (Feijó *et al.*, 1999; Holdaway-Clarke and Hepler, 2003). The MS Ca^{2+} channel at the tips of pollen tubes has higher permeability for Ca^{2+} over K^+ than other commonly known nonselective cation channels (Hille, 2001). No other MS, voltage-gated, or spontaneous channels were identified at the tip (Dutta and Robinson, 2004). The strongest indication that H^+ does not enter through the MS Ca^{2+} channels comes from the temporal offset of the two events. The oscillating tip-high Ca^{2+} gradient follows growth by 4.1 s (Messerli *et al.*, 2000), while oscillating H^+ influx and cytosolic acidification follow growth by 11 s (Messerli *et al.*, 1999) and 7.5–12.5 s (Messerli and Robinson, 1998), respectively. H^+ conduction through

the MS Ca^{2+} channel should be at the same time as the rise in intracellular Ca^{2+} not 7 s later. The ~7 s offset between intracellular Ca^{2+} and H^+/K^+ influx also indicates that intracellular Ca^{2+} is probably not activating H^+/K^+ influx. The lack of K^+ channels in the tip-derived protoplasts supports the idea that the large oscillating K^+ influx at the tip is not due to K^+ uptake through ion channels but may be due to uptake via transporters such as the putative H^+/K^+ symporter discussed previously. However, the presence of an MS K^+ channel on lily pollen grains seems peculiar. Passive uptake of K^+ through ion channels requires a sufficiently negative membrane potential, which is driven negative by H^+ efflux via the P-type H^+ ATPase. The membrane potential of lily pollen tubes is very close to, that is, 10 mV more negative than the predicted K^+ equilibrium potential (Messerli *et al.*, 1999) providing a weak driving force for K^+ uptake. Activation of the MS K^+ channels would have very little impact on passive K^+ uptake in the grain where the greater density of spontaneous K^+ channels with greater conductance are located (Dutta and Robinson, 2004). MS K^+ channels could help clamp the resting potential closer to the K^+ equilibrium potential or may be a mechanism to help control turgor. While *Silvetia* zygotes respond to hypotonic medium by releasing K^+ and Cl^- (more below), lily pollen tubes have no or very few anion channels (Dutta and Robinson, 2004). In response to hypotonic conditions, activation of an MS K^+ channel may help to release K^+ and reduce turgor.

IV. *SAPROLEGNIA FERAX* HYPHAE

S. ferax is a freshwater mold (oomycete) resembling fungi but classified as protist. It feeds on dead or decaying matter as well as on living organisms that have been compromised. The primary site for nutrient uptake in *S. ferax* is the branching, threadlike, hyphae. Hyphae are generally cylinders of 10-μm diameter with tips shaped more like a hemiellipsoid rather than a hemisphere. The tips of the hyphae have an abundance of vesicles containing cell wall material (Heath *et al.*, 1985) that are located within the tip-high Ca^{2+} zone (Hyde and Heath, 1997). Increased growth rate is correlated with increased cytosolic [Ca^{2+}] and steeper Ca^{2+} gradients. Nongrowing hyphae, in most cases, do not show any gradient or have reduced Ca^{2+} at the tip compared to more distal regions (Hyde and Heath, 1997). Despite the presence of an intracellular, tip-high Ca^{2+} gradient, hyphae can elongate in medium with nominally zero free Ca^{2+}, at least for a short time, before they eventually stop growing (Jackson and Heath, 1989). Under such conditions, they exhibit abnormal morphology with increased hyphal diameter. Peak growth rates, ~10 μm/min, are achieved in the presence of 1- to 50-mM Ca^{2+}, which is about five times greater than in nominally zero Ca^{2+} medium at least for the

first 12 h in varying Ca^{2+} medium (Jackson and Heath, 1989). This indicates that while growth is not necessarily dependent on Ca^{2+} availability from the medium, it is stimulated in the presence of external Ca^{2+}. Despite the fact that Ca^{2+} availability from the medium is not necessary to maintain reduced rates of growth, Ca^{2+} influx through tip Ca^{2+} channels was proposed to be a fundamental requirement for growth based on the fact that addition of 100-μM Gd^{3+} reversibly stops tip growth and abolishes the tip-high Ca_i^{2+}gradient (Garrill *et al.*, 1993). Hyphae resume growth about 30 min after removal of Gd^{3+}. It was later postulated that *S. ferax* hyphae may use a Ca^{2+} recycling scheme where Ca^{2+} release from secretory vesicles raises the $[Ca^{2+}]$ in the periplasmic space, between the plasma membrane and the cell wall. Ca^{2+} from the periplasmic space can then enter the cytosol through plasma membrane ion channels and generate the tip-high Ca^{2+} gradient that promotes exocytosis (Torralba and Heath, 2001). The reduced rate of growth in low Ca^{2+} was indicated as evidence that this mode of Ca^{2+} recycling was less efficient to promote growth than when Ca^{2+} was available from the surrounding medium.

Plasma membrane ion channels were identified at the tips and along the shafts of *Saprolegnia* hyphae. Protoplasts were generated by treating hyphae with a hypertonic medium and cell wall degrading enzymes to liberate plasma membrane in a "string of pearls" arrangement (Garrill *et al.*, 1992). This enabled relative localization of channels to the tip protoplast or protoplasts comprising regions behind the tip. Cell-attached patch configuration was used, as seal resistance greater than 0.5 GΩ could not be obtained despite optimizing conditions specifically to obtain higher seal resistance. MS channels were found more often in tip protoplasts than in protoplasts distal to the tip, while the distribution of a Ca^{2+}-activated K^+ channel was more evenly distributed (Garrill *et al.*, 1992, 1993). The chance of finding a channel decreased from 92% at tip protoplasts to 54% in posterior protoplasts indicating a decrease in channel density behind the tip (Garrill *et al.*, 1992). Two MS channels were characterized, a larger conductance channel that was permeable to both K^+ and Ca^{2+} but not Cl^- and a smaller conductance channel that may be permeable to Mg^{2+}. Activation of the MS channels should have greater impact on the intracellular free $[Ca^{2+}]$ than $[Mg^{2+}]$ considering equal external concentrations of the two ions, but a greatly reduced intracellular $[Ca^{2+}]$ 0.2–2 μM compared to intracellular $[Mg^{2+}]$, 200 μM. MS channels were inhibited by 100-μM Gd^{3+} that reversibly blocked growth and decreased the tip-high cytosolic Ca^{2+} gradient (Garrill *et al.*, 1993). Treatment of hyphae with the actin depolymerizing factor, cytochalasin E, stopped growth and when applied prior to protoplast generation, it significantly reduced the density of MS channels at the tip without significantly altering the density in protoplasts behind the tip

(Levina *et al*., 1994). Cytochalasin did not affect the aggregation of MS channels but did increase the number of patches in which no MS channels were found. These data provide evidence that the F-actin caps at the tips of hyphae may be necessary for maintaining the tip-high density of Ca^{2+}-permeable MS channels that are necessary for generating the tip-high Ca^{2+} and tip growth.

V. *SILVETIA COMPRESSA* RHIZOIDS

The zygotes of the Fucaceae, especially species of *Fucus* and *Silvetia* [formerly *Pelvetia* (Serrão *et al*., 1999)] have been invaluable in the study of the emergence of developmental polarity. When released from the fronds into intertidal seawater, the eggs are radially symmetric, with a central nucleus. After fertilization by motile sperm, the gravitationally and optically dense zygotes settle to the bottom, attach to the substrate, and select an embryonic axis in response to environmental cues, chiefly unidirectional blue light (Robinson *et al*., 1999). Some hours later, the symmetry is visibly broken by germination, which is characterized by the localized emergence of a tip-growing rhizoid on the side of the cell away from the light source. Ca^{2+} is centrally involved in the symmetry breaking operation. Tracer flux studies showed that in response to light, the Ca^{2+} influx on the shaded side became fivefold greater than on the illuminated side (Robinson and Jaffe, 1975). If the cells are grown in the dark, the orientation of the axis can be directed by an external gradient of Ca^{2+} ionophore, with the rhizoid emerging from the region exposed to the high concentration of the ionophore (Robinson and Cone, 1980). Finally, direct visualization of a cytoplasmic Ca^{2+} gradient in response to unilateral light and prior to germination has been reported (Pu and Robinson, 1998). First cell division follows about a day after fertilization and the plane of the division is perpendicular to the long axis of the embryo, resulting in two highly unequal cells with different developmental fates. The rhizoidal tip continues to elongate as development proceeds and continues to be sensitive to blue light, as the tip will grow away from a light source. Water relations are especially important in these organisms as the unfertilized eggs lack a cell wall and thus cannot support turgor pressure. Fertilization leads to the secretion of a wall and the cells increase their cytoplasmic osmolarity by actively taking up K^+ and Cl^- (Allen *et al*., 1972; Robinson and Jaffe, 1973) so that they develop turgor pressure of about 6 atm.

In addition to unilateral light, a number of other external asymmetries can polarize a population of dark-grown zygotes, including external osmotic gradients. According to Jaffe (1968), cells exposed to a gradient of 50 mM in the osmolarity of the surrounding seawater are effectively polarized so that the rhizoids later emerge from the region of high water potential.

While the effects of such a gradient are complex and would create polarized water flow through the cell, the possible involvement of MS channels was suggested by this result, especially in view of later findings that showed the essential role of Ca^{2+} gradients in formation of a polar axis (discussed below). It should also be noted that the fucoid embryos develop in the intertidal zone and are thus naturally exposed to variations in external osmolarity, necessitating a physiological mechanism for dealing with osmotic stress.

The first direct evidence of plasma membrane MS channels in fucoid zygotes came from the initial application of the so-called "vibrating probe" to detect localized net current movements across cell membranes. The vibrating probe is a platinum electrode used in the self-referencing mode; that is, it is vibrated at audio frequencies between two points near a cell and measures the voltage gradients produced by any net ionic currents moving through the cell. Nuccitelli and Jaffe (1974) observed large pulses of current entering the tip-growing rhizoids of *Silvetia* zygotes. The pulses were as large as 30 $\mu A/cm^2$, typically lasted 60–90 s, and often occurred in groups of two or three nearly identical pulses. Ion substitution experiments indicated that the outward movement of both Cl^- and K^+ occurred at the tip, but that Cl^- flux was larger, producing net inward current (Nuccitelli and Jaffe, 1976b). K^+ efflux elsewhere from the embryonic membrane completed the current loop. These experiments also indicated that Ca^{2+} entry triggered the pulses. Subsequently, it was found that the pulses could be stimulated by lowering the osmolarity of the seawater and thus transiently increasing turgor pressure (Nuccitelli and Jaffe, 1976a). Raising external osmolarity partially suppressed the pulses. The total current carried by the pulses in a 20-min period was roughly proportional to the decrease in osmotic pressure. In order to verify that Cl^- efflux was an important component of the current pulse, the cells were loaded with $^{36}Cl^-$ and the release of the tracer monitored in a laminar flow system. They found that Cl^- efflux increased 33-fold during the first 10 min of exposure to seawater with 17% lower osmolarity, thus directly demonstrating the involvement of Cl^- efflux in the inward current pulses. They concluded that a current pulse results from the opening of a patch of Cl^- channels by Ca^{2+} entry at the tip. Perhaps the Ca^{2+} channels are very sensitive to membrane stretching.

A possible mechanism for the link between Ca^{2+} influx and Cl^- efflux was provided by Gilkey and Staehelin (1989), using freeze-fracture electron microscopy of ultrarapidly frozen, unfixed *Silvetia* embryos. They identified a population of 0.5-μm disk-shaped vesicles near the plasma membrane, which appeared to form reversible attachments to the membrane. Such attachments were increased when external osmolarity was decreased, leading to the suggestion that the vesicles contained Cl^- channels that were induced to fuse with the plasma membrane as a result of osmotic stress-induced local Ca^{2+} entry.

Direct confirmation of the existence of plasma membrane MS channels with significant Ca^{2+} permeability was provided by Taylor *et al.* (1996). They applied laser microsurgery to locally ablate the cell wall of *Fucus* embryos in order to generate protoplasts from either the rhizoid or thallus cell of two-celled embryos. Cell-attached patch-clamp recordings were then made from the protoplasts. The patches contained a low-conductance channel with a high open probability of 0.96 in the absence of pressure that increased to 1.0 with applied pressure and a high-conductance channel with an open probability that increased from 0.05 to 0.48 during the application of negative pressures of 0.5–2.0 kPa. The low-conductance channel was observed less frequently, so most of the presented results involved characterization of the high-conductance channel. The channels were uniformly distributed in the membrane along the rhizoid–thallus axis of zygotes. The sensitivity to mechanical stimulus was lost in excised patches, but this configuration allowed the exploration of voltage dependence and ion selectivity. The channels were found to increase their open probability with depolarization and to have a $P_{Ca^{2+}}/P_{K^+}$ ratio of 0.35–0.5. They further showed that exposure of embryos to reduced external osmolarity induced an increase in cytosolic Ca^{2+} at the rhizoid tip, which moved as a wave toward the base of the rhizoid.

The data cited above establish convincingly that MS channels are involved in osmotic regulation of the fucoid rhizoid. What is not clear from the data is the degree to which MS channels are involved in normal tip growth of fucoid rhizoids, absent osmotic stress. It is well established that there is a tip-high gradient of Ca^{2+} in the rhizoid (Brownlee and Wood, 1986; Brownlee and Pulsford, 1988; Berger and Brownlee, 1993; Pu and Robinson, 1998). Germination and growth of the rhizoid is highly sensitive to both 150-μM Gd^{3+} and 100-μM verapamil (Robinson, 1996), yet 100-μM verapamil did not affect open probability or conductance when applied to either face of the large-conductance MS channel (Taylor *et al.*, 1996). Gd^{3+} has been commonly used to inhibit MS channels but is a potent blocker of voltage-gated Ca^{2+} channels as well (Mlinar and Enyeart, 1993). Tip growth is inhibited by a Ca^{2+} channel blocker that does not interfere with Ca^{2+}-permeable MS channels. These results could be explained if the lower conductance Ca^{2+} channel reported by Taylor *et al.* (1996) was more critical for normal tip growth and it was blocked by verapamil. Current pulses that could be induced by hypoosmotic shock were also a regular feature of nonshocked rhizoids, albeit at low frequency (Nuccitelli and Jaffe, 1974). As far as we know, there are no long-term studies of rhizoidal Ca^{2+}, so it is not known if there are periodic pulses of elevated tip Ca^{2+} or if tip growth of rhizoids is pulsatile. Given the slow growth rate of the rhizoids, compared to pollen tubes and hyphae, such experiments will not be trivial.

VI. *NEUROSPORA CRASSA* HYPHAE

N. crassa is commonly referred to as a bread mold and classified as a fungus. The branching, threadlike, hyphae comprise the primary vegetative structure, the site for nutrient uptake, indicating that tip growth is the dominant form of cellular growth. The hyphae are 8- to 15-μm diameter cylinders with hemiellipsoidal tips and maintain average growth rates between 5 and 22 μm/min under standard conditions (Levina *et al.*, 1995; Sampson *et al.*, 2003). Similar to systems discussed above, a tip-high $[Ca^{2+}]$ is required for growth. Hyphae that are elongating have tip-high Ca^{2+}, while nongrowing hyphae do not (Levina *et al.*, 1995). Growth rates are correlated with the tip-high $[Ca^{2+}]$ but not the steepness of the gradient (Silverman-Gavrila and Lew, 2003). Depletion of the tip-high $[Ca^{2+}]$ by injection of the Ca^{2+} chelator, BAPTA, inhibited growth (Silverman-Gavrila and Lew, 2000). However, the generation of the tip-high $[Ca^{2+}]$ may not be due to plasma membrane channels as large changes in membrane potential, −200 to +50 mV, did not affect tip growth rate (Silverman-Gavrila and Lew, 2000).

N. crassa hyphae were shown to have at least four plasma membrane ion channels, two MS channels and two spontaneous channels (Levina *et al.*, 1995). Channels were characterized in the cell-attached configuration due to the low seal resistance 0.1–0.2 GΩ. The lower conductance MS channel was ~65% of the conductance through the higher conductance MS channel. Currents increased through both MS channels on exposure to higher extracellular Ca^{2+}, indicating significant permeability to Ca^{2+} (Levina *et al.*, 1995). The Ca^{2+}-permeable MS channels were located uniformly over the surface of the hyphae with densities of 6.3, 5.9, and 7.6 channels/μm^2, comprising regions of the hyphae that were 0–116, 116–193, and >193 μm behind the growing tip. Addition of 100- and 500-μM Gd^{3+} decreased open time and the number of events of the MS channels but did not affect the spontaneous channels. Similar concentrations of Gd^{3+} only transiently decreased elongation of hyphae for less than 5 min, before growth returned to pretreatment rates and did not change the tip-high Ca^{2+} gradient after growth resumed to pretreatment rates (Levina *et al.*, 1995). Rinsing off the Gd^{3+} at the end of the experiment did nothing to change the average growth rate. Similar to the conclusion discussed above with *Silvetia* rhizoids, the two plasma membrane Ca^{2+}-permeable MS channels in *N. crassa* hyphae are not being used to generate the tip-high Ca^{2+} necessary for growth (Levina *et al.*, 1995). In fact an intracellular IP3-activated channel is thought to generate the intracellular tip-high Ca^{2+} gradient (Silverman-Gavrila and Lew, 2001, 2002). The Ca^{2+}-permeable MS channels in *N. crassa* are thought to be involved with turgor regulation or sensing of mechanical stress (Levina *et al.*, 1995).

VII. IS TURGOR NECESSARY FOR ACTIVATION OF MS CHANNELS?

Expansion and growth of these cell wall-enclosed, turgor maintaining systems is dependent on the interplay between the turgor and the restrictive mechanical barriers keeping turgor under control. The majority of the turgor measured under normal growth conditions could simply be offset by these mechanical barriers. Slight yielding of these barriers either by increasing turgor to overcome the barriers or by directly weakening the mechanical barriers is all that is necessary to open MS channels assuming that there is no slack in the plasma membrane. However, the idea of turgor generated expansion of tip-growing organisms has been brought into question by the inability to measure turgor in growing hyphae of two water molds, *S. ferax* and *Achlya bisexualis*, in increased external osmoticum (Money and Harold, 1993; Harold *et al*., 1996). We only include discussion of *S. ferax* as they have been shown to possess stretch-activated MS channels, while no evidence for MS channels in *A. bisexualis* exists yet. It was reported that 20 kPa is the smallest increment in oil pressure that could be controlled with confidence and indicating that "no measurable turgor" means that the turgor is between 0 and 20 kPa (Money and Harold, 1993; Harold *et al*., 1996). Is a reduction in turgor from 400 kPa to <20 kPa (Harold *et al*., 1996) sufficient to prevent the opening of MS channels? Despite the drop in turgor, *S. ferax* hyphae continued to grow as cylinders, although they appeared not to penetrate the agar (Money and Harold, 1993). A surface tension of only 2.5–5.0 mN/m will place MS channels from fungal germ tubes at their 50% open probability state (Zhou *et al*., 1991) and will place MS Ca^{2+} channels from lily pollen at their 40% open probability state (calculated from Dutta and Robinson, 2004). Assuming channels with similar characteristics in *S. ferax* hyphae with average diameters of 10 μm, a hydrostatic pressure difference of less than 2 kPa would be needed to open the channels to their 50% open probability state. As noted in Gustin *et al*. (1988), the larger the cell diameter, the greater the sensitivity to osmotic pressure changes. So it is perhaps not surprising that the cell diameter increased in *S. ferax* hyphae in response to hyperosmotic stress, thereby lowering the pressure necessary to activate the MS channels in the tip. This discussion is made to point out that a small, immeasurable amount of turgor could remain in the hyphae to drive opening of MS channels, even in very high external osmotic pressure.

With the data collected for MS channels in lily pollen tubes a similar point can be made. On the basis of the patch-clamp recordings of Dutta and Robinson (2004), we calculate that the MS Ca^{2+} channels have an open probability of 40% with 2.5- to 5.0-mN/m tension in the membrane. In the 15- to 18-μm diameter pollen tubes, a turgor change of only about 1 kPa is

necessary to take the channels from closed to their 40% open probability state. Benkert *et al.* (1997) reported that the average turgor pressure of lily pollen tubes is 209 kPa, which did not significantly change with large changes in external osmolarity, and that 5 kPa was the limit of detection for their pressure measuring system. While they reported that changes in turgor pressure did not occur during growth, their measuring system did not have adequate sensitivity to detect changes of which the MS Ca^{2+} channel is capable of responding.

VIII. CONCLUSIONS

MS channels with permeability to Ca^{2+} are common to each of the four tip-growing systems discussed. Specific permeability to Ca^{2+}, however, was not shared. The MS Ca^{2+} channel from lily tubes is 100 times more permeable to Ca^{2+} than K^+ (Dutta and Robinson, 2004), while the high-conductance MS channel from *Silvetia* was more permeable to K^+, $P_{Ca^{2+}}/P_{K^+} = 0.35 - 0.5$ (Taylor *et al.*, 1996). Hyphae of *S. ferax* possess an MS Ca^{2+}-permeable channel that is also permeable to K^+ (Garrill *et al.*, 1992), while no secondary permeability is listed for either of the two MS Ca^{2+}-permeable channels in *N. crassa* (Levina *et al.*, 1995). Despite these differences in permeability, each of the channels would have significant impact on changing the intracellular $[Ca^{2+}]$ due to the relatively high permeability to Ca^{2+}, the low intracellular $[Ca^{2+}]$, and the large electrochemical driving force on Ca^{2+}, four to six orders magnitude inward, enabling the transduction of membrane tension to intracellular Ca^{2+} signaling pathways. Therefore, the localized activation of the MS Ca^{2+} permeable channels could provide critical information regarding the use of these channels. The MS Ca^{2+}-permeable channels in lily pollen are localized to the pregermination fold and the growing tip, while the MS Ca^{2+}-permeable channels in *S. ferax* are localized to the tip of the hyphae, decreasing in density further behind the tip. Fluorescence imaging of Ca^{2+} indicators in both systems supports the idea of localized activation, creating tip-high $[Ca^{2+}]$ in the region where the channels are localized. The MS Ca^{2+}-permeable channels in *Silvetia* rhizoids and hyphae of *N. crassa*, on the other hand, were located evenly over the surface of the germinating zygote and along the shaft of the hyphae, respectively. However, despite the uniform distribution of the MS Ca^{2+}-permeable channels in *Silvetia*, channel activation, in response to hypotonic stress, leads to an increase in $[Ca^{2+}]$ only at the rhizoid and not over the entire organism (Taylor *et al.*, 1996). This indicates that other factors, such as barriers to turgor pressure, can influence activation of the channels and

that localization alone cannot be used to imply potential functions. Functional MS Ca^{2+}-permeable channels appear critical to maintaining the tip-high [Ca^{2+}] in growing lily pollen tubes and hyphae of *S. ferax* but not necessary for the tip-high [Ca^{2+}] in *Silvetia* rhizoids or *N. crassa* hyphae. It is thought that the Ca^{2+}-permeable MS channels in lily pollen and the *S. ferax* hyphae generate the tip-high [Ca^{2+}] in the region of the dense pools of secretory vesicles promoting exocytosis and tip growth. In *Silvetia* rhizoids, Ca^{2+} influx through MS Ca^{2+}-permeable channels may lead to additional secretion at the rhizoid, but it may be more critical for osmoregulation as it leads to large efflux of K^+ and Cl^- during hypoosomotic shock. The tip-high [Ca^{2+}] measured in *Silvetia* rhizoids under steady osmolarity may be maintained by the low-conductance plasma membrane Ca^{2+}-permeable channel at the tip that was nearly always open (Taylor *et al.*, 1996). The function of the Ca^{2+}-permeable MS channel in *N. crassa* hyphae was not identified but was postulated to regulate turgor or be involved with a signaling pathway generated by mechanical stress (Levina *et al.*, 1995).

Even though these MS Ca^{2+}-permeable channels appear to lead to different downstream events, the signal initiation event is the same: increased membrane tension. That signal may be generated by directly weakening the mechanical barriers to turgor or by turgor overcoming the strength of the barriers. In order for a cell to grow within an encasing wall, it must locally weaken the wall or overcome the strength of the wall and force it to expand in order for further growth to occur. The cell wall at the tips of lily pollen tubes only gets thicker when cell wall containing vesicles are released into a cell wall that has not expanded (Roy *et al.*, 1999). There is perhaps no better mechanism to determine whether sufficient cell wall yielding/expansion has occurred than to measure tension in the underlying plasma membrane with Ca^{2+}-permeable MS channels. This may work for rapidly growing cells like lily pollen tubes and hyphae of *S. ferax* but not for *Silvetia* rhizoids that grow about 100-fold slower. It may also not work for a terrestrial adapted fungus such as *N. crassa* that may not have readily available access to extracellular Ca^{2+} (Levina *et al.*, 1995). While *Silvetia* does have access to extracellular Ca^{2+}, the embryos survive in a much harsher environment, where large osmotic changes may occur. The MS Ca^{2+}-permeable channel is reserved to detect dramatic changes in external osmolarity and transduce a signal for the cell to reduce turgor to prevent bursting. Lily pollen tubes growing down the style of a compatible plant or *S. ferax* hyphae growing in freshwater conditions may never be subjected to such dramatically different osmotic conditions as *Silvetia*.

In three of the systems discussed, MS channels with K^+ permeability were reported. The large-conductance MS channel in *Silvetia* rhizoids was about — two to three times more permeable to K^+ than to Ca^{2+}. K^+-permeable MS channels were identified in lily pollen tubes and *S. ferax* hyphae but no

relative permeability was reported. In response to hypotonic stress, *Silvetia* rhizoids release both K^+ and Cl^-, identifying ionic efflux as a mechanism used to reduce turgor in this system. K^+ efflux through MS channels may be a common method for controlling turgor. If K^+-permeable MS channels pass water with K^+ as predicted for the KcsA channel (reviewed in MacKinnon, 2003), then they would be sensitive to osmotic gradients across the plasma membrane. K^+ ions have been dragged through HERG and Slo K^+ channels by imposition of transmembrane osmotic gradients indicating that K^+-permeable channels may be involved with osmotic balance (Alcayaga *et al.*, 1989; Ando *et al.*, 2005). There would be a problematic hyperpolarization if only K^+ efflux occurred, as it would increase the driving force for K^+ uptake. *Silvetia* rhizoids compensate for this problem by releasing Cl^- as well leading to a more electroneutral efflux of ions. K^+ efflux through K^+-permeable MS channels may help regulate turgor in the other systems as well.

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