How does a hypha grow? The biophysics of pressurized growth in fungi.

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Published in Nature Reviews Microbiology: http://dx.doi.org/10.1038/nrmicro2591

Abstract

The underlying mechanisms for the growth of fungal hyphae are rooted in the physical property of cell pressure. The internal hydrostatic pressure (turgor) is one of the major forces that drives the localized expansion at the hyphal tip, which is the cause of the characteristic filamentous shape of the hypha. Calcium gradients regulate tip growth, secretory vesicles that contribute to this process are actively transported to the growing tip by molecular motors along cytoskeletal structures. Turgor is controlled by an osmotic MAP kinase cascade that causes de novo synthesis of osmolytes and ion uptake from the external medium. However, as discussed in this Review, turgor and pressure have additional roles in hyphal growth, for example, by causing the mass flow of cytoplasm from the basal mycelial network towards the expanding hyphal tips at the colony edge.

Introduction

At the edge of a fungal colony, leading hyphae grow into new territory in search of nutrients and food. Behind the colony edge, the hyphae will interconnect to form a three dimensional network optimized to extract nutrients from the surrounding medium in order to fuel continued exploration (Figure 1). Colony growth can be very fast, about 10–100 μm per minute, depending on the organism, nutrient availability and temperature, and involves the continual synthesis of all the cellular constituents necessary for rapid cell expansion. A major driving force for cell expansion is pressure.

Pressure is a thermodynamic state property that affects the life of all organisms. In cells that lack a cell wall, excessive pressure can result in cell lysis and death. In cells that do have a wall (most bacteria, algae, fungi and plants), an internal hydrostatic pressure (turgor) provides mechanical support for freestanding structures and a force that drives cellular expansion, penetration of substrates and other processes. Extreme examples from fungi are the projectile release of spores at >100,000 × g in Ascomycota[1, 2] and Zygomycota[2], and the application of force sufficient for pathogenic fungi to penetrate and ramify throughout the host tissue[3]. The turgor is created by osmosis that occurs in response to differences in the internal concentrations of osmotically active substances (osmolytes) that are higher than those in the surrounding milieu. During cell growth (expansion and division), remodeling of the cell wall is required to increase cellular volume. Much of our understanding of the mechanisms underlying cellular expansion has been revealed from studies on tip-growing cells —which expand by tubular extension. Examples of tip-growing model systems are found in many phylogenetic clades (including plant pollen tubes and root hairs, algae and fungi and fungal allies). Tip growth is orthogonal, exhibiting a gradient of expansion that is maximal at the tip and declining as the full width

of the cell is attained behind the tip[4, 5], as suggested by Reinhardt in 1892[6]. Such a pattern of expansion is consistent with turgor-driven growth.

In this review, I will describe the roles of turgor and pressure in fungal growth (for previous reviews on the roles of turgor in mycelial fungi see Refs. [7, 8]). How is turgor regulated? How does it affect tip growth? Do intra-hyphal pressure gradients play a role in fungal growth (e.g. in the transport of new materials to the growing tip)? These areas of active research reveal the mechanisms of growth by members of the Fungal Kingdom, and are relevant to applied research on pathogenicity and the control of fungal diseases.

The microbiological physics of pressure

The nature of turgor and its relation to cellular expansion are founded on the Ideal Gas Law (since molecules in the gaseous phase will be in equilibrium with the same molecular species in the liquid phase), $PV = nRT$ (where P is pressure, V is the cell volume, n is the number of moles of osmotically active solutes, R is the gas constant and T the temperature). Thus, pressure, volume and solutes are all inter-related properties (temperature is usually assumed to remain constant) that are most easily explored in thermodynamics by invoking a closed system in which all but one of the properties is unchanging. However, in a growing cell *all* these properties will change. For example, increases in volume affect both pressure and solute concentration (n/V), whereas increases in pressure will increase volume and decrease concentration. Complicating matters even more, these changes are affected by water flow into or out of the cell and the extensibility of the wall (Figure 2). To explore how these properties of the cell are inter-related during cellular expansion, the simplest approach is to consider them separately.

Pressure and volume

Changes in pressure affect volume, but the extent of the change depends upon the resistance of the wall to deformation. This is described by the equation $(dP/dV) = (\varepsilon/V)$; changing either pressure (dP) or volume (dV) will cause the other to change. By how much depends on the initial volume (V) and the elasticity of the wall (described by ε, the volumetric modulus of elasticity). In walled cells, the modulus of elasticity of the wall increases with pressure[9, 10, 11, 12, 13, 14]. That is, at lower pressures, volume changes are relatively large. But as the pressure increases, the wall is no longer able to stretch, so the volume change is smaller. From an experimental perspective, optical measurements of hyphal diameter (from which relative hyphal volume can be calculated) offer an easy way to assess turgor changes (see Box 1 for a summary of techniques that can be used to measure turgor). However, the relation between relative hyphal volume and turgor is not linear, due to the pressure dependence of wall elasticity. Furthermore, any maintenance of turgor during cell expansion is affected by water flow into the cell.

Water flow

The dynamics of pressure and volume changes will depend upon the rate of water flow into (or out of) the cell. The rate of change in pressure or volume depends upon the hydraulic conductivity of the cell membrane and wall; fast water flow through a highly conductive membrane results in fast volume changes, whereas at a low enough hydraulic conductivity, the volume will not change at all. From the viewpoint of membrane biophysics, hydraulic conductivities are seldom thought to be limiting, because of the high permeation of water through membranes. But there must be instances where water flow

through membranes can be limiting, as indicated by the discovery of aquaporins[15] (protein channels that transport water across cellular membranes), and their presence and expression in walled cells[16]. However, many fungal genomes lack aquaporin-encoding genes[17]. Furthermore, strains of *Saccharomyces cerevisiae* and *Candida albicans* in which the aquaporin genes have been mutated or deleted exhibit no discernible growth differences under normal conditions[17]. Only under the extreme condition of freeze-thaw are the aquaporins found to be beneficial to yeasts[17]. When rapid freezing occurs, extracellular water freezes first while the cellular water becomes super-cooled. Aquaporins enhance the rapid outflow of water from the cell and thereby inhibit the formation of lethal ice within the yeast cell. In the filamentous fungus *Neurospora crassa*, comparisons of hyperosmotic-induced hyphal volume shrinkage and hypoosmotic-induced hyphal tip lysis revealed no differences between wildtype and an aquaporin knockout strain (Sheng, McClure and Lew, unpublished observations). Thus, during tip expansion under normal hyphal growth conditions, water will flow freely into the expansion zone without the assistance of aquaporins.

During normal growth, it is expected that water inflow should be maximal at the site of expansion, and taper off exponentially behind the growing tip (Figure 2) (because water inflow at the expanding tip attenuates the volume changes further behind the tip). Expansion at the tip may be regulated by wall extensibility.

Extensibility

Extensibility is a measure of how much the cell wall will expand as the pressure inside the cell is increased. In walled cells, extensibility varies with pressure: at low pressure, the wall is able to expand to a considerable extent, but at higher pressure it becomes stiff and inextensible, a prelude to structural failure and bursting. At the tip, although pressure is the driving force for tip extension, the *rate of growth* depends upon the *extensibility* of the wall (and crucially, the supply of cell wall[18] and membrane material for the expanding tip). Expansion of the tip wall can be either elastic and/or plastic. Elastic expansion is reversible (the stretched wall can revert to its original state, if turgor declines). Plastic expansion is irreversible (the wall shape has changed and cannot revert to its original dimensions). Cell growth requires plastic deformation, which is due to the incorporation of new wall and membrane material at the expanding tip. The relationship between growth rate and wall softness (measured by the pressure at which the tip will burst) was explored in the hyphal (non-fungal) oomycete *Achlya bisexualis*[19]. These studies supported the classic relation proposed by Lockhart[20] that growth rate depends upon extensibility and a pressure above a threshold level (a minimum pressure below which cell expansion will not occur). That is, plastic deformation of the wall requires some minimum turgor, below which cell expansion will not occur. The interplay between turgor and extensibility has also been explored by researchers working on cell growth in algae, and to a lesser extent higher plants. Experimentally, it is possible to clamp the pressure inside the cell to a specified value by impaling the cell with a pressure probe and modifying the turgor (Box 1). Increasing the turgor results in an increase in elongation rate that soon returns to the original rate even though pressure is maintained at an elevated level; thus, changes in wall extensibility are used to maintain a constant growth rate.

But how does extensibility change? The best experimental evidence on changes in wall extensibility comes from research on the giant Characean algae of the genus *Chara*. Because of their large cell size, measurement and manipulation of turgor is fairly easy[21]. Normal turgor in *Chara* is about 600 kPa. When turgor is increased using the pressure probe, growth accelerates, but only transiently before

reverting to its original rate[22]. The extensibility is regulated by pectin and calcium pectate in the wall[23] as well as the tension created by turgor[24]. In the wall, calcium cross-links the pectin, stiffening the wall. When new pectin is supplied during cell expansion, it competes for the calcium, causing the wall to loosen to allow continued expansion. The ability for calcium-linked pectin to release calcium, thereby loosening the wall, requires a threshold pressure. Thus, pectin, calcium and turgor coordinately maintain the growth rate through wall extensibility in Characean algae[25]. In hyphal oomycetes *A. bisexualis*, *A. ambisexualis* and *Saprolegnia ferax*, secretion of endoglucanase at high external osmolarities may play a similar role in regulating wall extensibility to maintain normal growth rates when turgor is low[26]. In lily pollen tubes, growth rate and turgor are not correlated[27, 28], and direct measurements of cell wall 'stiffness' using a micro-indentation technique underscores the role of extensibility as the primary regulator of extension rates[29]. Micro-indentation measurements use a very fine glass fiber that touches the wall; the deflection of the fiber is used to determine the force required to indent the wall. This technique could also be used to explore the viscoelastic properties of the fungal wall, in concert with detailed explorations of the relation between its wall composition and hyphal tip growth, to determine whether regulation of extensibility plays a role in tip growth similar to the situation in Characean algae.

During hyphal growth in fungi and oomycetes, elevated Ca^{2+} at the tip regulates hyphal tip growth. The mechanisms creating the Ca^{2+} gradient vary depending on the organism, but in all the studied cases it involves a signal transduction pathway that detects changes in wall tension. In the hyphal oomycete *S. ferax*, calcium is transported into the cell by stretch-activated Ca^{2+} channels at the tip[30] (this is also true for plant pollen tubes[31]). However, in the fungus *N. crassa*, Ca^{2+} influx is not preferentially located at the tip[32], and stretch-activated Ca^{2+} channels do not control cell expansion since their inhibition does not impair growth[33, 34]. Instead, the Ca^{2+} gradient is generated and maintained internally[35]: inositol-1,4,5-trisphosphate (IP_3) mediates the release of Ca^{2+} from internal stores at the tip, which then causes fusion of vesicles at the expanding tip. The Ca^{2+} is sequestered behind the tip by the endoplasmic reticulum (because of the action of a Ca^{2+}-ATPase pump)[36, 37]and tip-localized mitochondria[38]. The IP_3 appears to be produced by a stretch-activated phospholipase C[39]. Knockout mutants of Ca^{2+} transporters (*nca–1*, *nca–2*, *nca–3* and *cax*) exhibit normal growth morphology, and thus are not obligatory for hyphal tip growth[40].

Thus, the tip-localized Ca^{2+} gradient is maintained during cell expansion by a variety of mechanisms. In all cases, stretching of the membrane and wall results in elevated Ca^{2+}, which would then mediate vesicle fusion to allow continued cell expansion and cell wall synthesis[41, 42]. The mechanism underlying Ca^{2+}-mediated vesicle fusion in fungi is not understood, but in animals it relies upon several proteins, such as those belonging to the SNARE (SNAP receptor proteins involved in membrane fusion) family[43]. SNARE proteins have been identified in *N. crassa*, and are localized to hyphal tips[44]. Gene inactivation of two of these SNARE members (*nsyn1* and *nsyn2*) impair hyphal tip growth[45]. A complete picture of how elevated Ca^{2+} could mediate this process remains unknown, awaiting further research.

With successful expansion, turgor will decline, so it must be maintained to provide a constant driving force during hyphal growth.

Turgor regulation

In the natural environment, fungi may face wide extremes in extracellular osmolarity. These can result from changes in water availability as substrates dry out, or the sudden osmotic shock of rain, a hypoosmotic stress that could cause the internal hydrostatic pressure to rise to point of causing cell wall rupture and cell lysis. To sustain growth (in the presence or absence of osmotic shock), fungi must be able to adjust the concentrations of solutes internally to keep turgor at an acceptable level, or modify cell wall extensibility (see above).

Osmoresponses

Turgor provides a driving force for cell expansion in many organisms, and therefore it may need to be regulated. However, it should be noted that some hyphal organisms (e.g. the oomycete *A. bisexualis*) are not active turgor regulators[46] (see below). Turgor regulation can be explored directly by challenging the cells to an osmotic shock that causes a change in turgor. Normally, this is done by using an impermeant substance, that is, an osmolyte that cannot enter the cell and, thus, cannot contribute to turgor recovery. When a permeant solute is added to the extracellular medium, the cell initially loses water (and pressure declines), but uptake of the solute causes the intracellular osmolarity to increase, re-balancing the turgor. In the case of an impermeant solute, the cell must regulate turgor on its own, by taking up ions and synthesizing internal osmolytes such as glycerol. In *N. crassa*, turgor recovery takes about 10 minutes after treatment with permeant NaCl, compared to 60–90 minutes when the impermeant sucrose is used[47]. After sucrose treatment in *N. crassa*, turgor declines and a decrease in hyphal volume occurs simultaneously with a transient depolarization of the membrane potential (all within about 2 minutes), followed by a rapid cessation of hyphal growth[48] (Figure 3). Then the membrane potential recovers, and hyperpolarizes after 4 minutes. The sustained hyperpolarization to a potential significantly more negative than the initial potential occurs concomitantly with increased net outward flux of H^+, consistent with activation of the plasma membrane H^+-ATPase[47]. The hyperpolarized potential is presumably the driving force for subsequent net ion uptake (K^+ and Cl^-) followed by turgor recovery[47]. The synthesis of glycerol occurs after about 20 minutes[49].

The osmotic MAP kinase cascade

The two mechanisms for turgor regulation in fungi (ion accumulation and osmolyte synthesis) are controlled by an osmotic MAP kinase pathway[47]. In general, MAP (mitogen activated protein) pathways are comprised of a cascade of kinases that are sequentially activated by phosphorylation to amplify a transduction signal. The kinases are usually abbreviated as MAP kinase, MAPK kinase and MAPKK kinase to indicate their position in the sequential cascade. The MAP pathway is activated by external stimuli acting on an upstream receptor, and the most studied consequence of this activation is the expression of pathway-specific genes. In fungi, MAP kinase cascades have roles in different processes, such as the responses to external pheromones in mating and the responses to various stresses. The osmotic MAP kinase cascade was initially characterized in *S. cerevisiae*[50] and appears to be ubiquitous in fungi[51], although variants exist in the upstream cascade components[52, 53, 54, 55, 56].

Members of the osmotic MAP kinase pathway from a number of fungal species were initially identified because mutations in the encoding genes resulted in osmo-sensitive mutants. In *N. crassa*, the mutants of the osmotic MAP kinase pathway (*os–1* [sensor], *os–4* [MAPKK kinase], *os–5* [MAPK kinase] and *os–2* [MAP kinase]) are osmo-sensitive, and at least *os–1*and *os–2* mutants have lower turgor than wildtype and exhibit incomplete turgor recovery in response to hyperosmotic shock[47]. That these mutants possess

lower turgor than wildtype even under normal conditions suggests that the cascade operates even under non-stressed conditions.

The cascade controls ion transport (that is, H^+ pump activity and K^+ uptake) as part of the cell's osmotic response, and does so even in the absence of *de novo* protein synthesis[47] (Figure 4). The electrical response to hyperosmotic treatment of *N. crassa* —hyperpolarization— and subsequent ion uptake are not observed in mutants of the osmotic MAP kinase cascade. The common description of the cascade as the HOG pathway (High Osmotic Glycerol), referring to glycerol synthesis after high salt treatment in *S. cerevisiae*, is unfortunate. Certainly, activation of the osmotic MAP kinase cascade by osmotic shock causes the synthesis of osmolytes (glycerol in *N. crassa*[57]), but turgor is regulated even in the absence of glycerol synthesis by activation of ion uptake – as demonstrated for the osmo-sensitive *cut* mutant of *N. crassa*, which harbours a mutation unrelated to the osmotic MAP kinase cascade[49]. The central role of the osmotic MAP kinase cascade in regulation of ion transport is supported by the observation that ectopic activation of the cascade with the phenylpyrrolle fludioxonil (which activates the OS–1 sensor[58, 59]) causes not only glycerol synthesis[60], but also activation of the H^+-ATPase and ion uptake[61].

Besides the osmotic MAP kinase cascade, other proteins play a role in turgor regulation, probably as part of separate transduction pathways. *N. crassa* knockout mutants[62] of the genes *mid*–1 and *ptk2*, encoding respectively a putative stretch-activated channel and a kinase regulator of H^+-ATPase activity, exhibit lower turgor than wildtype under standard growing conditions[63, 64] and lower H^+-ATPase activity (Figure 4).

Non-turgid growth

The central role of turgor in hyphal growth is, however, not universal. Some hyphal organisms such as the oomycetes *S. ferax* and *A. bisexualis* do not regulate turgor[19, 46, 65] and therefore may not rely solely on turgor to drive growth. In fact, tip growth in *S. ferax* can occur in the absence of measurable turgor[66]. Since the life cycles of *S. ferax* and *A. bisexualis* have motile cell stages with contractile vacuoles for osmoregulation, their osmoregulatory mechanisms may be different from those of fungi. In *A. bisexualis*, invasively and non-invasively growing hypha have similar turgor and burst pressures[67] but different distributions of actin, which suggests that the cytoskeleton plays a role in invasive growth in this organism.

Furthermore, even in a turgor-regulating organism like *N. crassa*, only a few mutations are required to create an ameboidal cell (a mutant called *slime*[68, 69]), with pseudopodial-like extensions presumably driven by the cytoskeleton. The *slime* mutant is wall-less[70] and can be lysed with osmotic treatment[71], so turgor, if any, should be nil to very low. Clearly, growth and substrate penetration may not depend solely upon turgor, and alternative tip growth patterns and processes can exist[72].

Mass flow of cytoplasm

Microscopic observation of mycelial colonies often reveals the active movement of cytoplasm through the hyphal network, usually towards growing hyphae at the colony edge. However, the nature of the apparent cytoplasmic flow cannot be discerned by microscopic observation, since movements mediated by the cytoskeleton and associated molecular motors might give the optical impression of mass movement of cytoplasm. Mass (or cytoplasmic) flow is defined as the movement of the internal volume

within the hypha, similar to water flow in a pipe. Direct evidence of mass flow comes from experiments injecting silicon oil (which would not provide any binding sites for the molecular motors) into hyphae; the oil globules move similarly to larger organelles, such as vacuoles, indicating that mass flow is occurring[73].

How is the mass movement of cytoplasm produced? Early research on *N. crassa* reported important apical-basal differences in osmotic pressures measured using an incipient plasmolysis technique (Box 1) that would more than explain a pressure-driven migration of cytoplasm towards the growing tips at the colony edge: apical pressures of 1.26 MPa *versus* basal pressures of 1.77 MPa[74]. However, such pressure differentials have not been observed using the more direct pressure probe methods, which report much lower turgor (*ca* 500 kPa) and no measurable pressure gradient. Reasons for the discrepancy are not clear, although scoring for incipient plasmolysis is subjective, and it is difficult to correct for volume changes of the hyphae and differing cell wall extensibility of apical and basal cells[74, 75]. However, small pressure gradients (0.05 to 10 kPa cm^{-1}) would be, in theory, enough to explain the mass flow of cytoplasm if the flow is laminar (see Box 2). Such small pressure differences cannot be measured directly with the pressure probe.

An alternative approach to demonstrating the role of a pressure differential in cytoplasm flow is to modify the intra-hyphal pressure gradient by local hyperosmotic treatment to pull water out of the hyphae at a localized region, thereby generating a low turgor region in the hyphal network. Placed *in front* of the colony edge, the hyperosmotic treatment causes a pressure drop at the colony edge, and mass flow rates towards the colony edge increase. Placed *behind* the observation point, so that the pressure drop occurs behind the colony edge, mass flow rates towards the colony edge slow (Figure 5)[73]. Basically, experimental modification of the intra-hyphal pressure gradient modifies flow in a manner consistent with mass flow. Therefore, these studies indicate that hyphal networks are micro-hydraulic networks. Regulation of flow involves closure of septa, which become plugged by Woronin bodies in filamentous ascomycetes. Modifications of the protein that tethers the Woronin body at the septum affect the ability of the hypha to plug the septa after wounding[76]. It has been proposed that, besides a role protecting the hyphae from wounding, septa may regulate rates of cytoplasmic mass flow that in turn regulate rates of hyphal growth at the colony edge[77]. The concept of growth-induced mass flow in mycelial networks[78] is a promising perspective from which to build future experimental explorations.

Molecular motors and mass flow: The Great Divide?

The roles of mass flow and active transport via molecular motors in the delivery of building materials to the hyphal tip are difficult to reconcile because of the different biophysical and biochemical regimes involved, which require the use of different experimental approaches. Mass flow operates over long distances; molecular motors do not. How they relate to hyphal growth is also quite different.

Models of hyphal growth that focus exclusively on either molecular motors or turgor have been devised. Sugden et al.[79] presented a physical model of vesicles carried by kinesin molecular motors along microtubule tracks that extend into the tip. Such a model of vesicles hopping along multiple one-dimensional lattices could supply the necessary vesicles, and could be self-regulating (a traffic 'jam' would ensue should tip growth be halted for whatever reason). In contrast, Boudaoud[80] described a model that was focused on how turgor and tension interact to 'shape' the growing cell. Thus, both

molecular motors and turgor are essential, but integrating the two into a cohesive growth mechanism is challenging.

With the availability of mutants and fast methods for gene knockout in *N. crassa*[62], and tools for live cell fluorescence imaging[81, 82, 83], it has become possible to explore hyphal growth and the cytoskeleton roles in greater detail. For instance, an actin mutant of *N. crassa* exhibits aberrant morphology (apical branching), but the general tip-growing form of hyphae is retained[84]. The knockout is available only as a heterokaryon and thus likely to be essential. Mutations in the genes encoding the microtubule-related motors dynactin and dynein in *N. crassa* affect nuclei distribution (nuclei are basally located but still migrate with the growing tip) and affect the 'straightness' of hyphal growth (the hyphae zig-zag as they grow)[85 86 87]. In these mutants, nuclei transport along with the growing tip still occurs, even in the absence of a functional microtubular system, presumably due to mass flow[88]. Nuclei movement through hyphal fusions between conidial germlings (newly germinated conidia) is still observed in microtubular mutants of *N. crassa*, but whether this can be ascribed to mass flow is unclear[89].

What should be emphasized is the intrinsic mechanical difference between transport mediated by molecular motors and mass flow. In the first case, only the bound cargo is transported: due to the low Reynolds number environment, the cargo will not 'drag' surrounding cytoplasm with it (Box 2). Evidence in support of this assertion comes from experiments in which inhibition of cytoplasmic particle streaming has no effect on movement of cytoplasm-located fluorescent dyes through coupled cells[90]. In the case of mass flow, there is no discrimination: anything that is not anchored will be transported.

Macro-scale polarity

There is no doubt that there is an intrinsic polar distribution of cytological elements and ion transporters at the growing hyphal apex: a micro-scale polarity of tip-enriched actin[91, 92], tip-localized mitochondria[38] and the Spitzenkörper[93]. But macro-scale polarity may also exist. Unlike molecular motors, mass flow operates over long distances (cm). In other words, small osmotic imbalances might create slight intra-hyphal pressure gradients, effecting mass movement over long distances. This raises the question of whether mass flow has a role in creating a macro-polarity in the hyphal colony (Figure 6). One can envision the leading hypha exploring new territory, while behind, in the mycelial network, maximal uptake of nutrients will occur, leading to increased water inflow, increased pressure, and a driving force that 'pushes' cytoplasm towards the colony edge. If this is so, then one would expect to see evidence of long-distance polarity in other aspects, especially in ion transport —a generator of osmotic gradients that cause changes in turgor and that could also create electrical currents.

It is possible to measure electrical currents that surround organisms by using a vibrating probe. Indeed, electrical currents surrounding hyphae have been reported for several fungi; the electrical current is inward at the tip and outward basally[94]. In *N. crassa*, the electrical current is carried principally by H^+ [95, 96]. Indirect support for the role of polar currents in normal growth comes from experiments documenting the effect of extracellular voltage fields on the direction of growth[97, 98]. However, it is unlikely that there is a single electrogenic transporter responsible for the polar currents. Net currents will depend upon the ion species available for uptake, which can include NH_4^+ and NO_3^- that will affect other fluxes due to their impact on pH regulation, as will transport of small metabolites[99]. With respect to fluxes of specific ions (Ca^{2+} and H^+) measured with ion-selective probes, some hyphal organisms exhibit tip-localized ion currents (the oomycete *S. ferax*), but others do not (the ascomycete *N. crassa*)[32], due in part to

asymmetric localization of ion channels in *S. ferax* which does not occur in *N. crassa*[33]. The distally-located plasma membrane H^+-ATPase[100] may play a key role in generating the polar current patterns, in the creation of the trans-hyphal pressure gradient, and is the likely cause of a depolarized potential commonly reported at the growing edge of the colony[35, 101, 102].

There is no *universal* model of micro- and macro-scale polarity in hyphae, but the evidence does support the possibility of variations on a central theme of polarity. It should not be surprising that multiple disparate mechanisms —pressure and molecular motors— contribute to the growth of the fungi. The use of proteins labeled with fluorescent tags may assist greatly in revealing the extent of micro- and macro-scale polarity *in vivo*, extending previous cytochemical studies[103].

Concluding remarks

How does a hypha grow? So far, we know parts of the answer. Localized incorporation of vesicles and wall material create the characteristic tip-growing form. Cytoskeletal structures (actin, microtubules and associated molecular motors) organize the polar architecture of the growing tip. But microbiological physics is also important. Pressure is a driving force for cellular expansion and growth. We are beginning to understand elements of the regulation of pressure and growth, revealed through the juxtaposition of mutant analysis and physical measurements of turgor. Regulation of wall extensibility is a key element that controls the rates of cell expansion – but much more needs to be done. Direct experimental measurements of hyphae extensibility would be the first step in a quantitative description that would assist the development of models of growth. The nature of fungal micro-fluidics has been established, but we do not know how it integrates with other aspects of the cellular growth, requiring new methods of inquiry. Radioisotope and/or fluorescent mapping of cytoplasm flow are two techniques that may reveal how patterns of flow contribute to colony growth.

Box 1: Measuring cell pressure

There are a variety of techniques for measuring turgor in walled cells[75]. Simplest is the addition of a hyperosmotic solution outside the cells. As water leaves the cell, it shrinks, and eventually the protoplast pulls away from the wall, which is known as plasmolysis. The process is observed through a microscope, and the percentage of plasmolyzed cells is plotted versus the osmolarity of the osmotically active substance, to yield a measure of the threshold osmolarity causing plasmolysis. The technique is fairly easy to do, but there are some caveats. The first is that the osmolyte must not be able to penetrate the cell, otherwise it will change the internal osmolarity. Moreover, scoring for plasmolysis is subjective, so careful researchers will use a 'blind' evaluation, scoring plates not knowing what treatment has been applied.

Another technique, osmometry, involves the isolation of the internal cell sap (both cytoplasm and vacuolar contents) and measurement of its osmolarity relative to that of the external milieu. This is normally done using commercially available osmometers (which measure osmolality, osmotically active moles per kg of solvent). At equilibrium —when the water potential inside and outside of the cell is the same— the sap osmolality is equal to the turgor. However, it is often difficult to ensure that the cell sap is not contaminated by the external medium (which can 'cling' to the cell walls), so this is not a popular technique.

A third method, the pressure probe, uses a fine glass 'needle' filled with an immiscible liquid (low viscosity silicon oil is common). The needle is attached to a holder containing a pressure transducer. When the cell is impaled with the needle, turgor will force the oil interface into the needle. The amount of pressure that must be applied to 'push' the oil interface back to the tip is a measure of the cell turgor. The pressure probe measures the cell turgor directly, but may cause damage to the cell as a consequence of impalement. When the pressure probe is used to measure turgor of filamentous fungi, it is helpful to monitor the cell under the microscope to ensure that there is no cell damage (see, for example, Figure 3).

Box 2: Micro-fluidic mechanics

Mass flow of a liquid through a tube can be either laminar (smoothly transitioning from no flow at the edges to maximal flow at the center) or turbulent (chaotic, with vortices). The type of flow can be predicted by calculation of the Reynolds number (R_e), which is defined by the equation $R_e = (\varrho \upsilon d)/\eta$ (where ϱ is the density, υ is the velocity, d the tube diameter, and η is the viscosity). For flow through a tube, the flow will be laminar when $R_e < 10^3$, and the pressure gradients required for flow can be calculated from the following equation (known as the Poiseuille equation): $dP/dx = 8\upsilon\eta/r^2$ (where υ is the velocity of flow, η is the viscosity and r is the radius of the tube). In hyphae of *Neurospora crassa*, the R_e is about 10^{-4}, so turbulent flow is not occurring. In this case, the pressure gradient required for flow (calculated from the Poiseuille equation) is in the range 0.0005-0.1 bars cm^{-1} (0.05 to 10 kPa cm^{-1})[73], which is similar to the values observed for microfluidic devices[104]. These pressure gradients are very low compared to the normal turgor of the hyphae (430-570 kPa[46, 47, 49, 64]) and, due to experimental variability of turgor measurements, cannot be demonstrated with the pressure probe technique.

Because the flow of the hyphal cytoplasm is laminar, there is no mixing due to turbulence. Instead, mixing must occur through diffusion[104]. To give a quantitative explanation of the intertwining of mass flow and diffusive flow, another dimensionless number, the Peclet (P_e) number, or the ratio of velocity-driven mixing to diffusive mixing, provides a basic test of the importance of the two types of mixing[105, 106, 107, 108]. The Peclet number is defined by the equation $P_e = \upsilon d/D$ (where υ is the velocity, d the tube diameter and D the diffusion coefficient). For a protein in a fungal hypha, the diffusion coefficient would be about 7×10^{-11} m^2 s^{-1}, the flow velocity is about 5×10^{-6} m s^{-1}, and the tube diameter about 15×10^{-6} m [73]. So, P_e is about 1 and, therefore, both flow and diffusion contribute equally. For metabolites smaller than a protein, diffusion is more important since they diffuse more quickly. Diffusion of organelles would have a higher P_e (because of their smaller diffusion coefficient); that is, flow of organelles is more important than diffusive mixing in a fungal hypha. Besides, organelles are often transported with the assistance of molecular motors.

Glossary

Kinesin: a molecular motor that normally moves towards the plus end of microtubules, energized by ATP hydrolysis.

Heterokaryon: hyphae which contain multiple nuclei that are genetically distinct.

Pectin: a carbohydrate polymer (polysaccharide) that is part of the cell wall composition in walled cells of many organisms, such as algae and plants; pectin crosslinks can play a role in controlling wall strength and extensibility.

Oomycetes: hyphal organisms that are morphologically similar to fungi, but belonging to the phylogentically distinct Kingdom Straminipila.

Spitzenkörper: an organized assembly of secretory vesicles located at the tip of the growing hypha.

Woronin bodies: proteinaceous granules located at septal pores that can seal the pore in response to cellular damage.

Figures

Figure 1. Architecture of a fungal colony. From an initial inoculation with mycelium or spores, a fungal colony will radiate out, seeking to maximize its ability to obtain nutrients from the substrate. At the colony edge, leading hyphae are the first cells to invade new territory. Behind the colony edge, the hyphae branch and connect with other hyphae, thereby creating a network that optimizes the ability of the colony to extract nutrients from the substrate.

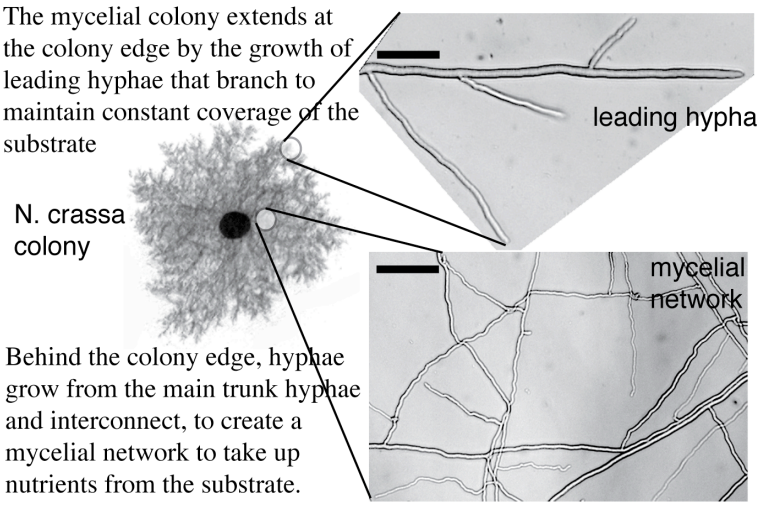


Figure 2. Pressure, volume, water flow and hyphal growth. Embedded in the figure are the relations that govern the various aspects of water relations in a growing hypha (a far more rigorous analysis is provided in Ref.[109]). The hydrostatic force that expands the extensible tip is governed by the difference in osmotically active solutes inside and outside of the cell, as given by the equation $\Delta P = RT(c_i - c_o)$ (where R is the gas constant, T the temperature and '$c_i - c_o$' the difference in concentration inside [c_i] and outside [c_o]). Water flow into the cell during expansion depends upon the change in volume over time (dV/dt), and the area (A) through which the water can flow, as described by the formula $J_v = -(1/A)(dV/dt)$. The water flow can also be described on the basis of the hydraulic conductivity (L_p) of the membrane and the pressure difference caused by the volume increase: $J_v = L_p\Delta P$. It is likely that water inflow will be greatest at the expanding tip, declining exponentially behind the tip. With water inflow, c_i will decline (and thus ΔP); so, steady-state growth requires maintenance of the turgor through uptake of solutes and biosynthesis of osmotically active solutes within the cell. Migration of cytoplasm along with the growing tip can depend upon the small intra-hyphal pressure difference created by tip expansion. The volume flow is described by the Poiseuille function $J_v = (\Delta P/l)(\pi/8\eta)R^4$, where $\Delta P/l$ is the intrahyphal gradient (pressure/length), η is the viscosity, and R the hyphal radius.. Organellar movements may also be mediated by the coordinated action of the cytoskeleton and molecular motors.

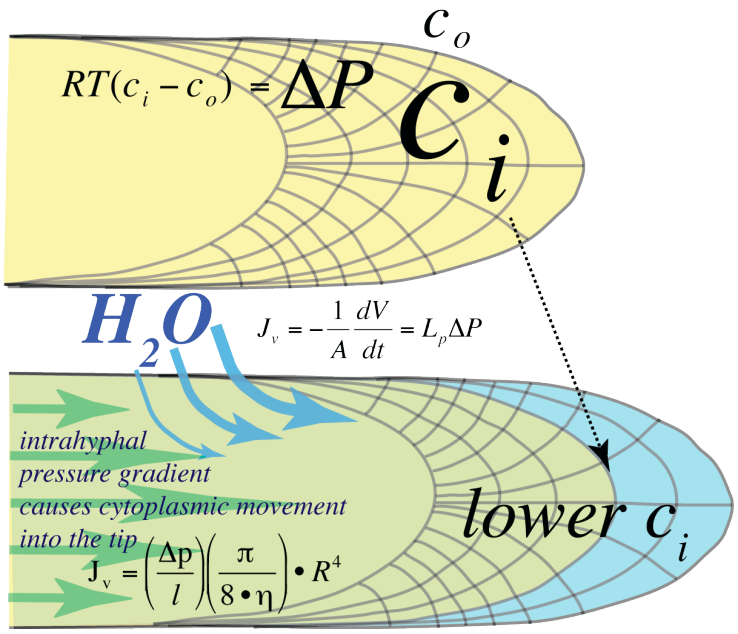


Figure 3. **Pressure, volume and its regulation.** The data shown here were obtained from time courses in which hyperosmotic treatment was used to decrease turgor in *Neurospora crassa*, and the process of turgor recovery monitored over 70 minutes [9]. Turgor was measured with a pressure probe. The photographic inserts (with time in minutes) show an example of a hypha impaled with a pressure probe, and the changes in hyphal diameter caused by hyperosmotic treatment. The spherical structures inside the impaled hypha are droplets of silicon oil, which move away from the impalement site as a consequence of cytoplasmic flow. Turgor (and relative hyphal volume) decreases immediately after hyperosmotic treatment, soon followed by growth arrest. The electrical potential becomes more negative as the H^+-ATPase is activated, followed by ion uptake into the hypha. As internal osmolarity increases, turgor recovery is observed, as well as resumption of hyphal growth. Glycerol synthesis also occurs, but lags turgor recovery.

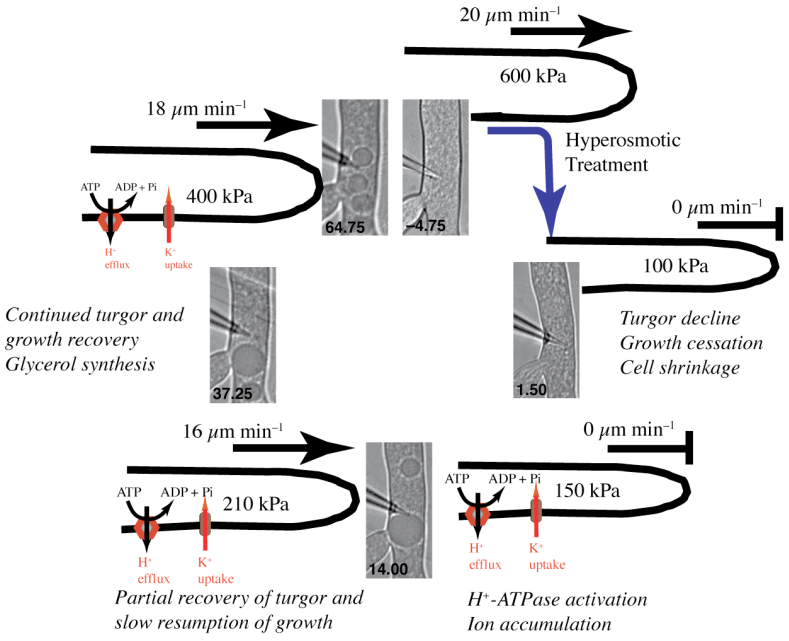


Figure 4. Turgor recovery mechanisms in *Neurospora crassa*. Shown is a model of the roles of the osmotic MAP kinase cascade, *cut* (an osmosensitive mutant that does not synthesize glycerol), MID–1 (a stretch-activated channel) and PTK2 (a kinase regulator of the H^+-ATPase). The MAP kinase cascade is a central control point for turgor recovery, activating glycerol synthesis and ion uptake (which, alone, is sufficient for turgor recovery). The other gene products (PTK–2 and MID–1) function in separate pathway(s) that contribute to turgor under normal growing conditions.

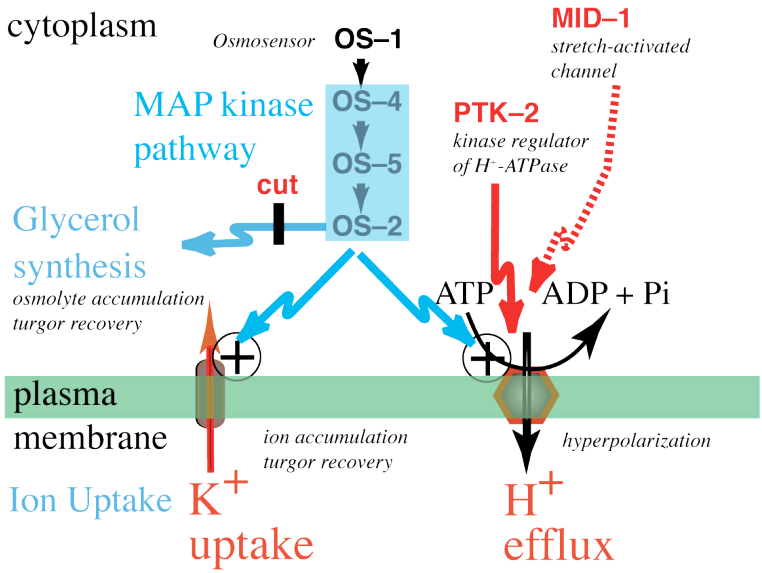


Figure 5. Mass flow of the hyphal cytoplasm. Evidence for intra-hyphal pressure-driven mass flow (measured 1 — 1.5 cm behind the colony edge) comes from experimental manipulation of intra-hyphal gradients by localized hyperosmotic treatment. At the tip, the treatment causes efflux of water from the tip, and acceleration of the normal mass flow velocity towards the tip (upper panel). Behind the tip, the treatment causes water efflux and retardation of the normal mass flow velocity towards the tip (lower panel). Thus, the hyphal network acts as a network of microhydraulic tubes.

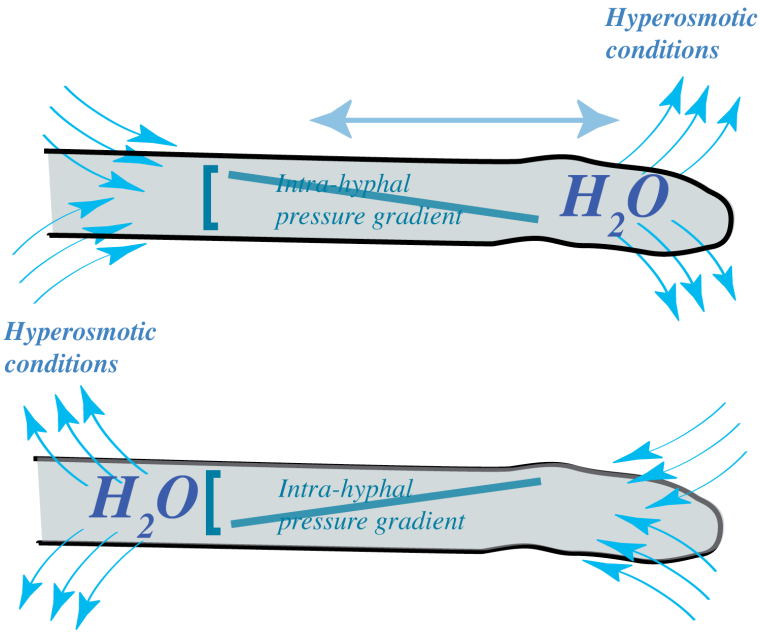
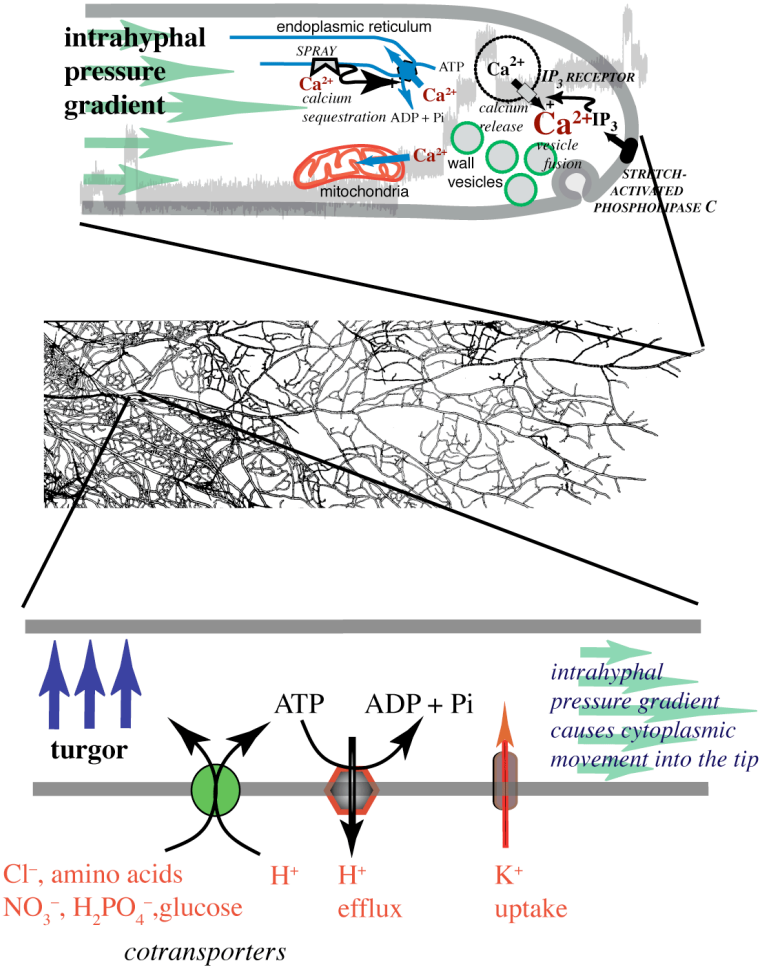


Figure 6. Model of polar distribution of transport in a mycelial colony. At the growing tip, the major transport system may function to maintain the tip-high Ca^{2+} gradient. Behind the tip, within the mycelial network, ion and nutrient uptake are driven by the activity of the plasma membrane H^+-ATPase. Some of the known (and assumed, such as Cl^-) H^+ co-transporters are shown, including those for amino acids[110], nitrate[111, 112], phosphate[113], and glucose[114]. SPRAY, a putative regulator of calcium sequestration, is also shown[115].



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