Tip growth in plant cells may be amoeboid and not generated by turgor pressure

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Cellular growth in higher plants is generated (powered) by internal turgor pressure. Basic physics shows that the pressure required to deform a plastic tube by elongation is inversely proportional to the tube's diameter. Accordingly, the turgor required to drive tip growth of very narrow cylindrical plant cells becomes very high, probably too high to be realized in living cells. The non-involvement of turgor in tip growth is demonstrated directly in living diatoms secreting fine tubular spines of silica. In some species, the membrane at the tip of the rigid tube is deformed inwards into its lumen during normal extension, whereas in other species, many cells are partly plasmolysed during normal, active spine ('seta') extension. Evidence from other cells is consistent with the general conclusion that turgor is not significant in tip growth. We support the alternative hypothesis proposed by M. Harold and colleagues that extension in tip cells can be amoeboid, driven by cycling of the actin cytoskeleton. Actively growing setae display an internal, fibrous, collar-like sleeve, probably of actin at the tip; it is visualized as a molecular treadmill ('nanomachine') that uses as its support-base the rigid tube that has just been secreted. This scenario can thereby explain how the perfectly even diameter of very long, fine setae is maintained throughout their extension, even when their tips are far distant from the cell body.

Keywords: actin; hyphae; microtubule; spine; tip growth; turgor

1. INTRODUCTION

(a) *Turgor pressure and cell growth: the paradigm*

Cells of higher plants are contained within a tough cell wall. Turgor pressure keeps non-growing cells turgid and can drive cellular expansion when the wall is sufficiently plastic to allow deformation. Regulation of wall deformation is exerted by the cytoskeleton: transversely orientated cytoplasmic microtubules close to the cell membrane control the orientation of cellulose fibres being deposited in the adjacent wall (Williamson 1991; Cyr 1994). In expanding cells, the transverse reinforcement thus engendered constrains expansion so as to generate their cylindrical morphology. Such cells display 'intercalary growth' because stretching and deposition of the wall take place evenly along its length.

In contrast, other plant cells extend by 'tip growth' (or 'apical growth') during which the wall expands exclusively at one end where concurrent secretion of wall material takes place. In higher plants, tip growth is characteristic of pollen tubes, protonema and root hairs. Elsewhere, fungal hyphae exhibit tip growth, as do many algae, when extending hairs, rhizoids, spines and other appendages. Tip-growing cells are usually generating outgrowths whose cylindrical wall has a very narrow bore. The vectorial properties of wall deformability become critically important under such circumstances. It is widely assumed that as in intercalary growth, turgor pressure drives tip extension (for reviews,

see Picton & Steer (1982); Steer & Steer (1989); Koch (1994); Harold *et al.* (1995); and Benkert *et al.* (1997)). Contrary to this assumption, we show examples from living cells and simple physical principles which both refute the paradigm. The idea is not new: Harold *et al.* (1995) cite Reinhardt who, as early as 1892, rejected a role for turgor in tip growth.

(b) *Previous observations on living diatoms*

Our attention was directed at this issue when studying morphogenesis of the new siliceous cell wall ('valve') of dividing diatoms. As part of valve morphogenesis, some genera form various types of spines. One of these is called the 'labiate process' and we followed its extension in the diatom *Ditylum* (Pickett-Heaps *et al.* 1988). The name labiate process is given to a particular type of small pore or slit in the valve of certain species and it derives from the characteristic internal lips or flanges of silica that flank the slit on the inside of the wall. Externally, this slit often is not elaborated upon, although in some species, a single, long, perfectly straight or curved, tubular spine extends from the slit in the valve face. The labiate process may be involved in secretion of mucilage (reviewed in Pickett-Heaps *et al.* (1986, 1988)). In all diatoms, the new wall is secreted within a 'silica deposition vesicle' (SDV), an extensible membrane whose form (including all types of spines) is continuously moulded during silica deposition (reviewed in Pickett-Heaps *et al.* (1990)). Extrusion of labiate processes is quite rapid and invariably associated with an unusual ultrastructural feature, the multilayered 'labiate process apparatus',

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which surmounts the lips of silica on the inner face of the valve (Li & Volcani 1985; Pickett-Heaps *et al.* 1988). Of immediate relevance to this paper is the mechanism of spine extension. From video recordings of live cells extruding spines, we noted that the membrane at the growing tip of the spine was curved inwards, into the lumen of the forming tube. Clearly, the spine could not have been extruded as a result of internal pressure. Thus, we discounted a role for turgor in driving tip extension, although at that stage we could not suggest an alternative mechanism.

More recently, we followed extrusion of other types of diatom spines. Each cell of the planktonic genus *Chaetoceros* has four spines, the 'setae', at each corner; their morphology is characteristic of each species. Frequently, setae are very long; in *Ch. peruvianus* (Pickett-Heaps *et al.* 1994), cells measuring about $20\ \mu\text{m} \times 30\ \mu\text{m}$ can have spines well in excess of $200\ \mu\text{m}$; they are elegantly curved and finely tapered along their length. Although setae are superficially similar to the labiate process of *Ditylum*, their morphogenesis and function are entirely different. From video recordings, we recorded setae growing at a rate of about $0.4\ \mu\text{m min}^{-1}$. Under the electron microscope, we discovered a complex of three fibrous sleeves lining the inside of the SDV at the very tip of growing spines. Because the tip of the spines stained weakly with rhodamine-phalloidin, we believe that these contained actin. The nearby microtubules were sparse and their disposition was irregular and did not suggest that they played any role in tip morphogenesis. As with *Ditylum*, we doubted that turgor pressure was responsible for seta growth, and control over morphogenesis was an enigma, for it was difficult to imagine how extension of such long, fine spines could be driven and/or controlled from the distant, tiny cell body.

2. MATERIALS AND METHODS

The methods used are set out in detail in Sampson *et al.* (1996) and Pickett-Heaps *et al.* (1994). Briefly, actively growing cells were sealed into chambers of glass slides several hours before division. They were then recorded using high-resolution colour TV cameras coupled to a Sony or Panasonic video disc recorder that was controlled by a timing device which recorded single frames at desired intervals, and which also controlled a shutter that cut off microscope illumination between exposures. The latter was vital in ensuring the cells were not killed by illumination needed for high-resolution microscopy using differential interference imaging.

For electron microscopy, cells were collected by gentle filtration. Specimens for scanning electron microscopy were fixed on the filter pad in a few drops of 1% osmium tetroxide in seawater, washed after 10 min, and then the pad was slowly dehydrated in acetone, critical-point dried and mounted on a stub. Cells were coated in gold and examined as normal. Specimens prepared for transmission microscopy were handled as set out in Pickett-Heaps *et al.* (1994), being fixed in glutaraldehyde and osmium, dehydrated in acetone, embedded in Spurr's resin and flat-embedded between Teflon-coated glass slides. Selected cells forming setae were excised from the resin wafer, mounted on a stub, serially sectioned, stained with lead and uranium and examined on formvar-coated slot grids.

3. RESULTS

(a) *Seta extrusion in Ch. decipiens*

Following the work on *Ch. peruvianus* (see § 1), one of the authors (J.D.P.-H.) has further investigated seta extension in *Ch. decipiens*, another planktonic species, which measures between $16\text{--}30\ \mu\text{m}$ by $60\text{--}70\ \mu\text{m}$ before division. Each cell bears four elegantly curved setae, often over $120\ \mu\text{m}$ long and between 0.5 and $0.8\ \mu\text{m}$ in width (figure 1). They grow at around $0.7\ \mu\text{m min}^{-1}$ and are finer than setae of *Ch. peruvianus*. Two points were apparent from observation of dividing cells. First, many living cells rapidly extruding setae were partly plasmolysed within their rigid walls (a normal attribute at this stage) and so these partly deflated cells could not have had significant turgor (figure 2*a-c*). Second, the morphogenetic system at the tip of the cell is simpler than that of *Ch. peruvianus*, consisting of a short, single fibrous sleeve running from the inside of the just-secreted wall to just past it, on to the inside of the cell membrane at the tip (figures 3 and 4). The disposition of this feature indicated strongly that it had a critical role in morphogenesis of the growing tip.

It is now appropriate to briefly consider whether turgor pressure could, even in principle, drive growth of setae.

(b) *Pressure and stress in highly curved surfaces*

Consider a thin-walled narrow tube of radius R and wall thickness τ . An internal hydrostatic (turgor) pressure p is resisted by a tensile stress S in the tube wall (see figure 5). By considering a transverse section of the tube, as in figure 5, it follows that, in equilibrium, the force caused by the hydrostatic pressure is resisted by an equal and opposite force due to the stress in the tube wall. Thus, we have

$$p(\pi R^2) = S(2\pi R\tau), \quad (1)$$

from which it follows that

$$p = 2\tau S/T, \quad (2)$$

i.e. the pressure required to produce a given wall stress is inversely proportional to the radius. Hence, for a given wall thickness, the narrower the tube, the higher the pressure required to reach the values of wall stress needed to cause plastic deformation and therefore, growth. For very narrow tubes, the required turgor pressure becomes very high indeed. (A corollary of this is the well-known fact that for a given wall thickness, thin tubes can withstand greater pressures than tubes of larger radius.) Exactly the same argument holds for the case of a tip modelled as a hemisphere, as in figure 6.

A similar argument based on a longitudinal bisection of a tube of length l , as in figure 7, leads to the relations

$$p(2Rl) = S'(2\tau l) \quad (3)$$

and

$$p = \tau S'/R. \quad (4)$$

A comparison with equation (2) shows that the longitudinal stress S produced by a given pressure is one-half as great as the circumferential stress S' ; hence the tendency for growth in tube diameter before elongation, and hence

Figure 1. Living cells of *Ch. decipiens*, from colour video recordings. Seven of the eight cells have just divided and would soon have grown new setae intercalated between the older pairs.

Figure 2. Living cells of *Ch. decipiens*, from colour video recordings. Early stages of seta extrusion, *ca.* 5 min apart. At seta initiation (*a*), the protoplasts fill their silica wall. Soon after extrusion starts, the protoplasts become slightly plasmolysed (arrows in (*b*)), a condition that may last throughout extension (*c*).

Figure 3. Tip of actively elongating setae under the electron microscope. The cytoplasm, containing a mitochondrion (m), is naked at the tip. Bar, 500 nm.

Figure 4. Tip of actively elongating setae under the electron microscope. The tube of just-secreted silica within the silica deposition vesicle ends at the large arrowheads. Immediately inside this tube and extending just outside it, defining the diameter of the cytoplasm, is the fibrous 'sleeve' (joined arrows). Inside the sleeve are two microtubules. Bar, 200 nm.

Figure 5. See text, and equations (1) and (2).

also the need for transverse reinforcement in large cylindrical plant cells.

In summary, the pressure required to extend the tip of a tube goes up inversely as the radius of curvature. Two examples from common experience illustrate this issue. Cylinders that burst under pressure always fail along their side walls, never at their hemispherical ends. Likewise, upon inflation, long, narrow balloons never start growing at their tip.

Figure 6. See text, and equations (1) and (2).

Figure 7. See text, and equation (7).

4. DISCUSSION

Using living cells, the observations above and in §1 demonstrate that turgor cannot be involved in growth of some, perhaps all, siliceous spines in diatoms. Theory confirms that in general principle, a role for turgor is unlikely in extension of any protrusions of very narrow diameter, i.e. other types of tip growth.

(a) *Turgor in other tip-growing cells*

Harold and colleagues (Money & Harold 1992; Harold *et al.* 1995, 1996) observed that the hyphae of some fungi grow well when placed into an osmoticum that matches that of the cell. In other species, turgor could not be detected in actively growing hyphae (Money & Harold 1993). In pollen tubes, Benkert *et al.* (1997), although not ruling out turgor as having a role in tip extension, could detect no correlation between the two. Harold *et al.* (1995) suggested an alternative (see later) that does not require turgor as the driving force.

(b) *The cytoskeleton of tip-growing cells*

The mechanical strength of the wall, and the means by which the cell prevents it bursting, are obviously relevant to this issue. In the large vacuolated plant cells that display intercalary growth driven by turgor pressure, the wall is transversely reinforced by cellulose microfibrils whose deposition is controlled by the adjacent transverse cortical microtubules (see §1). As shown above (equations (3) and (4)), the greatest stress these walls are subject to by pressure is circumferential and so this reinforcement is exactly what one would expect.

The cytoplasmic organization of tip-growing cells is significantly different. Transverse microtubules are not present in tip-growing cells, hardly surprising given the impossibly tight curvature required of them. Instead, longitudinally orientated microtubules near the walls of root hairs (Newcomb & Bonnett 1965) match the orientation of adjacent wall microfibrils, except at the tip where the microfibrils are random (for exceptions, see Trass *et al.* (1985)). Apparently, root hairs do not require the reinforcement provided by transverse microfibrils, yet the physics of stress would tend to split these walls under the high turgor needed to push out the tip. The lumen of growing fungal hyphae, pollen tubes (see references in Heath & Kaminski (1989) and Pierson & Cresti (1992)) and diatom setae (Pickett-Heaps *et al.* 1994) also contain axial (longitudinal) microtubules which may become rare at the actively expanding tip region and which often are tightly associated with actin filaments (e.g. Lancelle *et al.* 1987; Lancelle & Hepler 1992).

The actin cytoskeleton is prominent in the tip protoneura (Doonan *et al.* 1988) and fungal hyphae (Heath 1987, 1990; Heath & Kaminski 1989; Jackson & Heath 1993), many of which display a complex called the 'Spitzenkörper' (Grove & Bracker 1970). There is evidence for similar actin at the tip of pollen tubes (e.g. Perdue & Parthasarathy 1985). In growing setae of diatoms, a simple or complex sleeve of fibrous material located immediately ahead of the growing SDV extends into the lumen of the seta at the forming edge and this sleeve probably contains actin (Pickett-Heaps *et al.* 1994).

(c) *Amoeboid extension involving actin cycling*

Many animal cells display the phenomenon known as 'ruffling' at their flattened membranous margin, owing to steady cycling of the peripheral actin cytoskeleton immediately underneath the membrane. If ruffling is coupled with adhesion of the actin cytoskeleton through the membrane to an external substrate, force is exerted and cycling generates amoeboid movement (e.g. Alberts *et al.* 1994, p. 845). Very fine, straight filopodia can be extended from the cell's margin; these often contain a core of actin filaments and may cycle, growing at their tip while being continuously displaced inward. For those who find this cycling difficult to envisage, the two types have been vividly recorded *in vivo* by Kenneth Edds and reproduced in our video disc (Pickett-Heaps & Pickett-Heaps 1993).

(d) *Actin cycling as the mechanism of tip growth*

Harold *et al.* (1995) propose that hyphal extension in fungi is driven by a dynamic, actin-based system equivalent to that involved in filopodial extension or ruffling. This idea 'has been germinating in mycologists' minds for well over a decade. Its central thesis is that an advancing tip is analogous in many ways to a crawling amoeba' (Harold *et al.* 1995) and it is discussed, for example, by Steer (1990) without (in our reading) endorsing it over the turgor model. This scenario provides a plausible basis to explain extension of setae where the cytology is much simpler and easier to interpret than in other tip-growing cells. In *Chaetoceros*, the sleeve always keeps about the same dimensions in actively elongating setae. Thus, the sleeve either (i) moves forward intact as the tip extends, or (ii) it is continuously assembling at its distal (tip) end while being disassembled at its proximal end. Because actin cycling is a well-known activity of living cells, we suggest that cycling in the fibrous band is accompanied by its attachment internally to the rigid, just-formed silica wall instead of the external attachment required for amoeboid movement. Extension could then be driven by a self-propelling, molecular treadmill whose support-base is the wall that has just been laid down. Alternatively, the sleeve could move forward as an integral unit via attachment to the wall, using motor molecules such as myosin. Either model explains how the diameter of the seta is precisely controlled along its length: the extending SDV acts as a template determining the diameter of the just-formed silica wall. Furthermore, the morphogenetic system is located at the tip and could be independent from the distant cell body. Steer (1990), although in general holding to an osmotically driven hyphal extension, also notes the possible involvement of actin adhesion points on the hyphal wall, which could be involved in extension via the actin cytoskeleton.

One final point reinforces this argument. Diatoms display exquisite control over turgor pressure during their life cycle. During normal vegetative growth, diatoms slide their siliceous wall segments apart; sliding is complex and requires the concurrent addition of a series of siliceous 'girdle bands' to the valve (for details, see Round *et al.* (1990)). Some sliding is necessary prior to initiation of setae, as it exposes two slots in the girdle bands through which the setae grow. Turgor pressure does drive this expansion, and (as in figure 2a) the protoplasts are fully turgid at this moment. However, once expansion is

over, the protoplasts can shrink from the valve during seta growth (figure 2c). Delicate control over turgor is even more evident with centric diatoms about to release tiny sperm cells from the valves in which they are formed. At a given stage in differentiation, all the spermatogenous cells within one cell inflate together, becoming compressed within the valves, which are then forced apart. Immediately the halves separate, the spermatogenous protoplasts shrink back to normal size, divide several more times and differentiate sperm which can swim off. Thus, diatoms do use turgor pressure for cellular expansion, but not for tip growth.

(e) *The evolution of intercalary growth*

If the proposition of Harold *et al.* (1995) is valid, tip growth is simply one specialization of an ancient cytoskeletal system present in all cells and displayed elsewhere as various forms of amoeboid movement. We suggest that the morphogenetic systems that control intercalary growth of higher plant cells evolved as walled cells became vacuolated and too large for actin cycling to drive expansion. Perhaps extant transitional forms are already available in the form of larger hyphae, protonema, pollen tubes, etc., for which turgor becomes efficient as a mechanism for driving growth. We suggest that during this evolutionary transition from very narrow to larger diameter cells, the primitive axial microtubule system became increasingly helical (for examples, see Trass *et al.* (1985)) and finally transversely orientated. The microtubule cytoskeleton would thereby affect microfibril deposition so as to reinforce walls whose expansion was increasingly generated by pressure. One reviewer (John Raven) also pointed out to us that the microenvironment of tip-growing cells could have become important during evolution. Many tip-growing cells have to mechanically push their way through soil particles (e.g. root hairs, rhizoids) or host tissues (e.g. pollen tubes, fungal hyphae). There would presumably be advantages in harnessing turgor pressure for growth in such circumstances, particularly as the cellular extensions become larger.

(f) *Biological nanofabrication*

There is currently much interest in nanofabrication, the manufacturing of minute structures with potential as vehicles for micromachines. The diatoms are virtuosos of biological nanofabrication (Pickett-Heaps *et al.* 1990), creating geometrically precise walls with intricately patterned features from that most refractory of materials, silica. *Chaetoceros* appears to use a tiny biological machine for creating a beautifully sculpted silica tube of the finest bore.

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