Cellular oscillations and the regulation of growth: the pollen tube paradigm

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Summary

The occurrence of oscillatory behaviours in living cells can be viewed as a visible consequence of stable, regulatory homeostatic cycles. Therefore, they may be used as experimental windows on the underlying physiological mechanisms. Recent studies show that growing pollen tubes are an excellent biological model for these purposes. They unite experimental simplicity with clear oscillatory patterns of both structural and temporal features, most being measurable during real-time in live cells. There is evidence that these cellular oscillators involve an integrated input of plasma membrane ion fluxes, and a cytosolic choreography of protons, calcium and, most likely, potassium and chloride. In turn, these can create positive feedback regulation loops that are able to generate and self-sustain a number of spatial and temporal patterns. Other features, including cell wall assembly and rheology, turgor, and the cytoskeleton, play important roles and are targets or modulators of ion dynamics. Many of these features have similarities with other cell types, notably with apical-growing cells. Pollen tubes may thus serve as a powerful model for exploring the basis of cell growth and morphogenesis. ***BioEssays*** **23:86–94, 2001.**

Introduction

Pollen grain germination and tube growth are excellent models for the study of growth and morphogenesis at the cellular level. Being the carriers of male gametes in all higher plants, evolution has shaped these cells in such a way that they have to elongate dramatically, sometimes over centimeters within the female tissues without division or further differentiation. Since pollen tubes grow quickly, are easy to maintain and experimentally manipulate, they have become popular systems (reviewed in Refs. 1–5) and indeed represent a paradigmatic example of tip growth. As such, they may be used to highlight mechanisms applicable to many other cells, including fungal hyphae, root hairs and other plants cells, as well as certain specialised animal cells. The growing pollen tube also appears to be well suited for the study of various aspects of non-linear dynamics in biological systems, since a considerable body of evidence shows that the growth process is driven by different non-linear phenomena, such as pulses and sustained oscillations. Indeed, from current theory and experimentation, it is reasonable to expect the emergence of complex spatial and temporal patterns of biological relevance when this kind of non-linear dynamics results from activity in spatially extended systems, like the cytosol.[6–9] Given the favourable properties of the pollen tube and its potential to become a cellular model, it seems justified to review the available data about its oscillatory characteristics, with the hope of gaining insight into basic, regulatory aspects of cell growth and morphogenesis. So what is our view of non-linearity in biology?

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The importance of being "oscillatory"!

Considerable effort has been devoted in the past to characterise biological processes in terms of equilibrium states. When describing a continuous phenomenon, it is common to represent time as discrete intervals, to which statistical analysis can be applied in order to obtain average values. The latter are commonly interpreted as reflecting the natural evolution of the system to an equilibrium point. Frequently time intervals are chosen to eliminate "random variations" of the system, globally rejected as "noise". This situation is changing, however, owing to an appreciation of non-equilibrium as a common feature of a number of highly regulated biological processes.[10,11] Underlying this change is the combination of a new theoretical framework—deterministic chaos and the mathematical tools that were developed to study non-linear phenomenon.[12,13] and the refining of methods that allow monitoring of biological features in vivo, on a nearly-continuous timescale. Biological rhythms, which are among the most conspicuous properties of living systems, have now been described at the cellular and molecular levels.[14–16]

The biological relevance of oscillatory dynamics can be related to the general physical principles of self-organising processes.[6,7,17] In strict biological terms, the attributes of these non-linear dynamics are their robust, homeostatic properties, which are expressed, for instance, in oscillations

and other more complex ''attractors''. A common term in non-linear jargon, an ''attractor'' can be defined as a stable dynamic state that works as a homeostatic structure towards which all possible functional regimens of a system will converge (see Fig. 1). Working under these conditions, systems will react homeostatically to fortuitous perturbations, restoring the previous dynamic state, in spite of transient changes. Oscillations also promote the global synchronisation of independent molecular processes through the action of diffusible molecules, leading to the formation of coherent spatial patterns, mainly chemical waves.[18–21] The reason for this apparent ''uncertainty'' in homeostasis is easier to accept if one envisages the molecular and functional complexity of cells, where genes and molecules are organised in complex pathways around complex structural arrays, with a number of regulatory feedback loops. For example, the expression of this complexity along signal transduction cascades has been proposed to have important roles on the epigenetic regulation of the phenotype in plants,[22,23] with consequent distributed regulation and higher plasticity, stability and robustness, leading to a more effective response to unpredictable natural stresses.[18–20] The problem remains, though, of how these networks of information interact leading to the emergence of self-organising processes, from basic biophysical and biochemical mechanisms, to complex spatial patterns?

Oscillations as emerging properties of self-regulated systems

The complex array of interactions within a cell is likely to spontaneously produce complex phenomena, such as oscillatory or chaotic behaviours.[17] Theoretically, it requires only two or three independent dynamic variables (e.g. concentration, gene expression, channel activity) and a set of non-linear terms in the equations that describe their interaction (e.g. a feedback loop, membrane depolarisation or transport, co-factor or transcription factor binding) to generate these kind of behaviours.[12] While these conditions do not necessarily imply oscillations, they do make their existence possible. This is an important point, since it has been shown, mainly by researchers in ecology and population dynamics, that the same equation describing the interactions in a dynamic system may produce dramatically different results with subtle changes in their parameters.[24] In other words, the same regulatory events, usually expressed by a set of differential equations, can produce a steady-state, oscillations and even chaos just because very subtle changes in the system properties occurred at a point. While some systems tend to have an intrinsic homeostasis and, when perturbed or subjected to external stresses, evolve to homeostatic structures (so called dynamic attractors), others undergo transitions to other functional regimens (or move to a different attractor). This general framework has received confirmation in various cellular and chemical systems.[14,21] Oscillations can be represented as ''limit cycle'' attractors (Fig. 1), which exhibit dynamic trajectories such that arbitary initial conditions nearby will tend toward a periodic orbit, the attractor, as time moves forward. While the mathematical and physical characterisation of such aspects are beyond the scope of this paper, it should be noted that there is wide agreement that oscillations are widely used by biological systems to convey and propagate information efficiently within a spatially extended system. In other words, oscillations can effectively coordinate spatial and temporal relationships, and through reaction-diffusion mechanisms (e.g., where ions diffuse from a channel in the plasma membrane, and then participate in reactions in the cytoplasm), generate a number of basic patterns of morphogenesis.[8] To what extent do these oscillations happen in pollen tubes?

Oscillatory patterns in pollen tubes: cytosolic calcium and protons

Growing pollen tubes possess a steep ''tip focused'' gradient of cytosolic calcium $e[Ca^{2+}]$, which is essential for growth.[25–27] The high point of the gradient is adjacent to the plasma membrane at the extreme apex of the growing tube, and appears to be derived largely, if not entirely, from influx of extracellular Ca^{2+}.[28–30] Of particular interest, this gradient oscillates in magnitude over time.[29] In long pollen tubes of *Lilium* (>1 mm), the gradient displays a four-fold change in the $e[Ca^{2+}]$, from 750 nM to over 3000 nM. A series of independent studies, using video analysis of pollen tubes, showed that the growth rate also oscillated[29] reaching 3–4 fold peak-to-trough variations and a period of 15–60 seconds. Further analysis, in which the growth rate and cytosolic free $[Ca^{2+}]$ were carefully correlated, revealed that they were in phase.[30,31] Thus when the $e[Ca^{2+}]$ is elevated, the growth rate is fast and conversely when the $e[Ca^{2+}]$ is depressed, the growth rate is slow (Fig. 2). So far no apparent secondary waves or gradients have been found to propagate from this primary oscillating gradient.[30] Its magnitude appears to be governed by influx of extracellular Ca^{2+}, presumably through channels in the apical plasma membrane. The possibility has been raised that the channels are stretch activated, and thus only those at the extreme apex, where wall deformation is greatest, will have a high probability of being open.[2,29] Cytosolic pH (pH_c) oscillations have also recently been reported in *Lilium*.[32,33] Messerli and Robinson, while failing to detect any standing gradient, did observe an oscillating, basipetal wave of protons (H^+), which lagged behind the growth peak by 7.5 sec.[33] Feijó et al.,[32] by contrast, working under conditions of higher sensitivity, revealed a standing gradient consisting of an acidic apical domain and an alkaline band or ring located at the base of the clear zone (Fig. 3).[32] These oscillated and although the temporal resolution (ca. 10–15 sec.) is too low for correlational analysis, there appears to be a positive relation with growth consistent with previous

observations.[33] Thus low pH, or high H^+ concentration, in the tip, roughly correlates with the most rapid growth rate.[32]

Oscillations in extracellular currents

Pollen tubes are true polarised electric dipoles in which the grain acts as the source and the tube as the sink of a large cationic current that traverses the tube cytosol. Well before it was evident that both growth and the intracellular gradients exhibited oscillatory behaviour, Weisenseel et al.[34] had already noted that when lily pollen tubes reached a certain size the total inward current of the tube changed from a statistically steady current to pulsatile monophasic bursts with periods ranging from 30 to 50 seconds. Once this oscillatory behaviour starts, it continues until the tube dies, but there is no apparent change in the morphology or dramatic alterations on the average growth rate when compared to other tubes with steady currents. More recently, the ionic nature of the currents has been determined using ion-specific vibrating probes, which showed clearly that Ca^{2+} is one of the major current carrier during this oscillatory phase,[30,35] but H^+,[32] K^+[36] and chloride (Cl^-)[37] are also important components.

In recent work, the period and phase relationship of the extracellular oscillations in Ca^{2+} flux were analysed.[30,38] A novel discovery was made that while the extracellular oscillations in Ca^{2+} flux exhibited the same periodicity as growth and intracellular Ca^{2+}, they are delayed in phase by about 11–15 sec (Fig. 5), i.e., the growth profile defines the behaviour of the extracellular current profile. These data present a puzzle because it was expected that the extracellular current, which presumably supports the influx for the intracellular gradient, would be in phase with the oscillation in magnitude of that gradient. A possible explanation was set forth by admitting a role for the cell wall matrix in buffering Ca^{2+}, resulting in an apparent delay in extracellular

Figure 1. Phase-space reconstruction using a time series of H^+ influx at the tip of a growing pollen tube (series initially similar to Fig. 4). In a simplified definition, "phase-space" representation is a graphic tool for analyzing the dynamics of a complex nonlinear system. It is aimed at representing the dynamic relations between the independent variables of a system, expressed by trajectories toward a stable dynamic state. When the measured variables are not independent, each variable can be simply represented in a delay map. In a time series with a constant temporal shift (Δt), every value in a series is plotted against the next one in the series; the resulting plot denotes the evolution of the system. Note the aggregation of cycle trajectories in a restricted area of the phase-space, defining an attraction domain, which in this specific example is a limit-cycle attractor. The convergence of trajectories in time represents a homeostatic behavior of this system, and is by itself a graphic representation of its dynamic regulation characteristics. The homeostatic characteristic of pollen tube limit-cycle attractor is well expressed in the experiment depicted, in which readings were acquired at 3 sec intervals. Starting from its initial condition (red trace) a perturbation was imposed by using a micropipette to double the concentration of calcium close to the tip. Immediately after the injection, the pollen tube underwent a series of undershoot/overshoot fluctuations (yellow line starting in the black arrowhead), likely to correspond to membrane de/hyperpolarisation, transiently resulting in reverse, mixed or null fluxes. After 3 cycles, however, the system entered the limit cycle area (black double arrow) and became engaged on the original limit cycle again (blue line). (Blue and red arrows represent time-course direction).

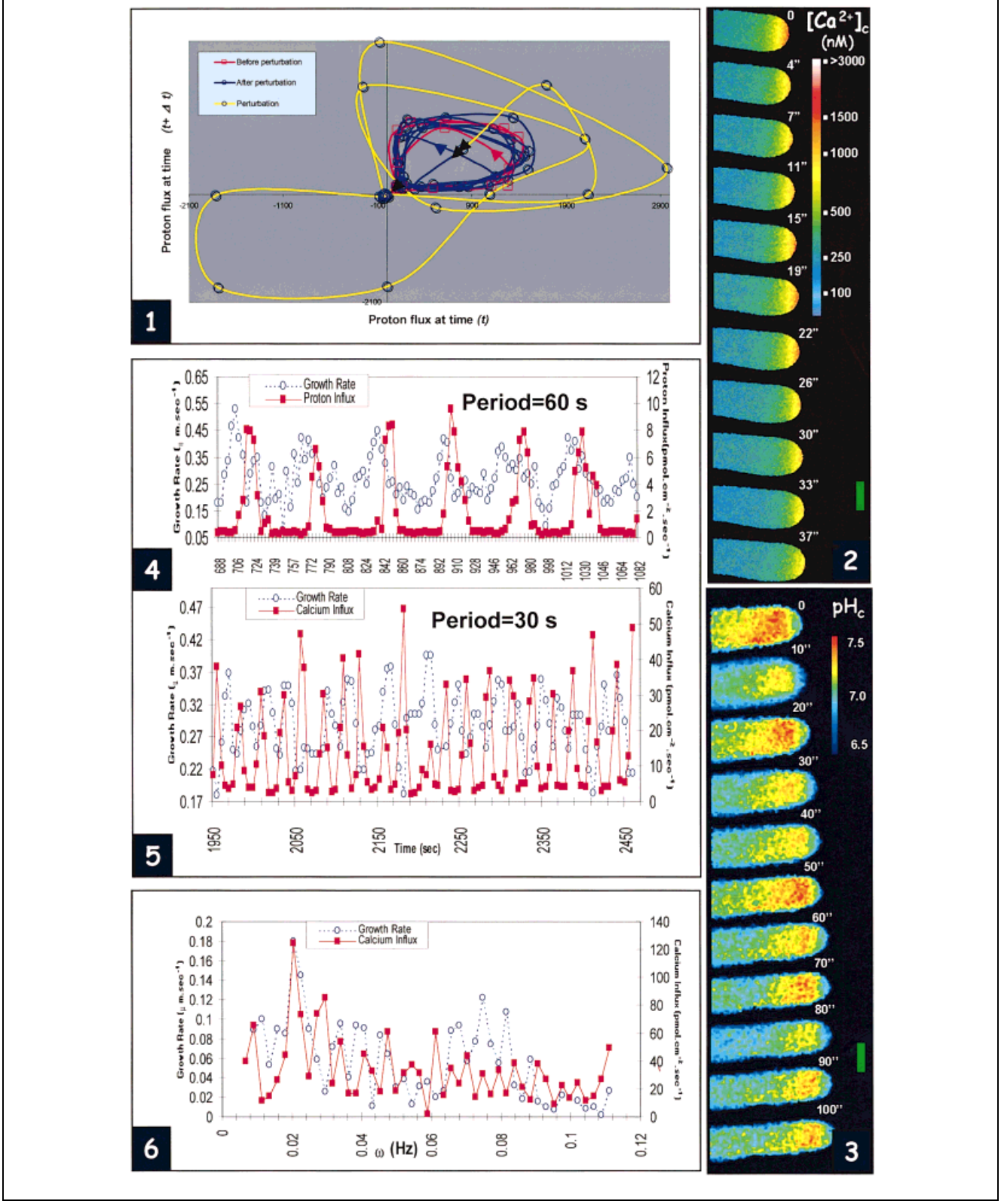
Figure 2. Typical pattern of cytosolic calcium concentration ($[Ca^{2+}]_c$) in a growing pollen tube. From a resting level of about 400 nM, the concentration rises to over 2–3 μM in the apical sub-membranar domain. This tip-focused gradient seems to originate from a restricted volume adjacent to the membrane at the very apex. The magnitude of the $[Ca^{2+}]_c$ gradient oscillates in phase with the growth rate, with an amplitude that can approach 2.0 μM (see Ref. 30 for details).

Figure 3. Typical pattern of cytosolic proton (H^+) concentration (pH_c) in a growing pollen tube. The pollen tube shank shows a resting level around 7.0, but a slightly acidic gradient is detectable at the apex of growing tubes, dropping as low as 6.5. More conspicuously, an alkaline "band" (ratio-imaging does not provide sufficient resolution to decide if it is a cylinder, or a torus) is a constitutive characteristic of living tubes, even when growth is arrested, with pH values over 7.5. Oscillations occur in the alkaline band with magnitudes ranging within 0.3 to 0.5 pH units; fine image analysis also reveals them in the acidic tip. Both $[Ca^{2+}]_c$ and pH_c patterns and changes therein are closely matched by the corresponding extracellular fluxes (see Ref. 31 for details) (green vertical bar represents 15 μm).

Figure 4. Double plot of extracellular H^+ flux, as measured with an ion-specific vibrating probe, and growth-rate, as measured by time-lapse video analysis. The growth rate change scribes a quasi sinusoidal curve while the extracellular H^+ influx exhibits more of a spiking behaviour; there is a matching number of cycles between these events. Note that the two series are not in phase; rather growth seems to slightly precede H^+ influx. This trend has been confirmed by various numerical methods and is computed to be in the range of 5–9 sec (each data point averages about 3 sec; see Refs. 27 and 23 for details).

Figure 5. Double plot of extracellular Ca^{2+} influx and growth rate, measured as in Fig. 4. Again, a matching number of cycles between Ca^{2+} influx at the tip and growth rate is evident and indicates a correlation between them (21 cycles, in this specific series). The period depicted (30 sec) is close to the average values for *Lilium*. Growth rate and Ca^{2+} influx, while exhibiting the same period, are not in phase, with peak influx following peak growth rate by about 11–15 sec. (each data point averages about 4.3 sec; see Ref.15 for details)

Figure 6. Fourier transform plot of a calcium time series like the one in Fig. 5. Two prominent peaks in growth rate and calcium influx totally overlap, showing that the molecular oscillator behind both has exactly the same period (in this case 0.2 Hz = 50 sec. period) and, presumably, could be the same. The other smaller peaks correspond to other components of the wave function. Some, specially the short period ones, are likely to reflect inherent noise associated with the techniques employed. Some, however, may reveal encoded information that may be used experimentally on a non-disruptive way.


Before perturbation
After perturbation
Perturbation
Proton flux at time (t+ Δ t)
Proton flux at time (t)
1
[Ca2+]c (nM)
>3000
1500
1000
500
250
100
2
Growth Rate
Proton Influx
Period=60 s
4
Growth Rate
Calcium Influx
Period=30 s
Time (sec)
5
Growth Rate
Calcium Influx
ω (Hz)
6
pHc
7.5
7.0
6.5
3

Ca^{2+} crossing the plasma membrane and affect cytosolic concentration. Considerably more data are needed, however, to decipher which chemical reactions are occurring to account for the Ca^{2+} influx and what the rheological consequences are, since elevations in Ca^{2+} in the wall have been shown to increase mechanical stiffness[39,30] and there is little evidence that this is occurring.

Very recently[36,38] H^+ fluxes at the tip have been shown to behave similarly to those of Ca^{2+}, i.e., with the extracellular influx lagging the growth peak, although with a slightly smaller delay, about 6–9 sec (Fig. 4). Again, changes in cell wall chemistry could contribute to these fluxes, since H^+ are involved. The observation, however, that H^+ fluxes remain relatively high even when tube growth is totally arrested, argues against the idea that cell wall build-up accounts for a major H^+ absorption.[41] Clearly, further clarification of the molecular nature of these apparent delays between extracellular and intracellular Ca^{2+} and H^+ fluxes and growth is needed.

While the oscillations described above have focused on pollen tubes longer than 1 mm, there are other oscillatory behaviours that occur during the early germination of *Hemerocallis* and *Lilium* pollen.[37,42] After tube emergence from the pollen grain, there is a refractory slow-growing period before it proceeds to the final growth rate. During this period (roughly when tubes are shorter than 75–100 μm) a series of 3 to 5 pulses of inward extracellular current at the tip occur and seem to establish the onset of the mechanisms that will condition the subsequent tube growth. These pulses were also measured with a Ca^{2+} probe, and, again, this ion seems to be the main carrier of the current. A distinction should be established between these highly localised transitory pulses which resemble more closely the pulsatile behaviour of animal eggs after fertilisation,[43] and the previously described oscillations which are sustained and may proceed for hours with the same pattern.

Oscillations in growth rates

Finally, in our discussion of oscillatory behaviours of pollen tubes we draw attention to periodic depositions of cell wall material. Using immuno-cytochemistry, Li et al.[44,45] showed that arabinogalactans and pectins were deposited in a ring-like pattern with remarkable periodicity along the length of tobacco/petunia pollen tubes. The frequency of these rings was roughly correlated with the periodic changes in the growth rate. In these species, slow growth phases are interrupted at regular intervals (3–8 min) by fast elongation phases (5–20 sec), sometimes reaching 50 fold the basal growth rates.[45–47] Although lily pollen tubes show an even oscillatory pattern of growth and do not generate wall rings, they can be experimentally induced to undergo marked growth fluctuations, and to deposit thickened rings of pectin during the slow phases of growth.[48] These observations seem consistent with the idea that wall deposition occurs at a uniform rate, and thus is uncoupled, at least partially, from the processes of cell elongation. The process of ring formation is especially odd for the arabinogalactans since, in contrast to the pectins, they are deposited at the tip of *Lilium* but not in *Nicotiana* or *Petunia* pollen tubes. In this instance, there may be some sort of spatial coupling between the regulatory events at the tip, where growth takes place, and the tube shank, where arabinogalactans are incorporated in the cell wall matrix. While a functional "continuum" has been proposed involving the endomembrane system, cytoskeleton and cell wall,[49] the nature of this coupling remains obscure.

The periodic nature of the pulsatile growth rate in *Nicotiana* and *Petunia* has been further investigated using pharmacology.[47,50,51] With the view that growth is fundamentally underpinned by Ca^{2+} channels, Golgi secretion, and cytoskeletal activity, the authors show that the pulsatile behaviour is dependent on gadolinium and lanthanum sensitive Ca^{2+}-channels (presumably, stretch-activated), but not affected by the organic blockers verapamil and nifedipine. While it is now widely recognised that distinctions on the channel type that are based on pharmacology alone are not entirely reliable, it seems likely that this striking difference in the inhibition reaction is a consequence of the specificity of the channels. In addition, while Golgi activity appears not to be relevant to the pulsatory nature of growth, cytoskeleton inhibitors had a striking effect, both in eliminating pulses, and/or shaping their behaviour to different periods.

Different kinds of dynamics ?

Taken together, these results point to three classes of pollen tube growth behaviours, as follows: (1) spiking, which may occur in the onset of tube growth, (2) continuous growth, which should better be described as statistically stable but with random small fluctuations around a trend line (e.g. *Lilium* and *Hemerocallis* with tubes <1 mm), and (3) sustained quasi-sinusoidal oscillations, with variable periods between 10 seconds and a few minutes (e.g. *Lilium* with tubes <1 mm, *Nicotiana*, *Petunia* all the time). This categorisation implies that oscillations are not necessary for growth to occur in certain species, but even in those, the system seems to evolve spontaneously to an oscillating condition. Such a variety of dynamic behaviours could suggest basic differences in the molecular mechanisms underlying the growth process. Yet, the outcome of growth and morphogenesis, i.e. polarised tip growth with the typical dome shape and underlying cytoplasmic structure, are strikingly robust, even in species in which the three types of growth behaviour seem to occur sequentially during elongation (e.g. *Lilium*). Thus, it can be argued that these apparent differences in growth behaviour can be generated by the same molecular mechanism, if one assumes that this mechanism is governed by a set of simple non-linear regulation steps. Different dynamic patterns would then cor-

respond to different points of homeostatic stability of the system, namely the dynamical attractors (see Fig. 1).

Could this be the case for pollen tubes? The evolutionary success of their growth mechanism is overwhelming: while the higher plant female gametophyte follows a number of different development algorithms,[52] the male gametophyte is universally expressed as a pollen tube with the same structure. Observed differences in growth behaviour could then be a consequence of growth-derived variations in the conditions of dynamic stability of basic regulatory molecular mechanisms (i.e. tube length and/or increased volume), and the consequent existence of more than one possible stable dynamic set of regulation parameters (more than one attractor). It should be noted that the transition from an apparently stable system to oscillatory growth occurs spontaneously without major changes in any of the structural and growth parameters .[29,34,50,51] Together with the confirmation that, once set on a limit-cycle attractor, it reacts to perturbations by returning to its original trajectory (Fig. 1), the available data calls for specific experimentation to test other ways to disrupt functional relationships and, ideally, to determine all possible atractors of the system and ways to jump from one to another. This, however, requires a careful evaluation of the techniques available.

Experimental criteria to look at cellular non-linear dynamics

At this point, it is important to ask if we can gain insight into the regulatory mechanisms of a living system by looking at its continuous temporal evolution. The answer should be "yes" if certain criteria are met, namely: (1) the system should be examined with minimal interference in its normal physiology; (2) a maximal number of independent variables (with a minimum of two) relevant to the description of the system's biology should be studied with continuous recording and (3) the variables studied must have some sort of temporal synchronisation. These criteria have different practical implications. For example, criterion (1) implies the exclusive use of non-invasive, real-time techniques, where real-time is satisfied by a measuring technique that is at least one order of magnitude faster than the phenomenon. Gross manipulation of the system dynamics should be avoided. While pharamacological inhibitors can yield some initially useful indications, they can also eventually trigger a number of unpredictable effects at different levels, which are likely to be amplified throughout the non-linear regulatory cascade. Criterion (2) ideally should allow for the establishment of groups of variables with the same dynamic behaviour, and for the possibility of deciding which variables are regulatory. Criterion (3), which is perhaps the most difficult to achieve, emphasises the importance of stable oscillatory patterns. When a system evolves around a stable monotonic condition, there is no possible way for correlating its variables without serious experimental perturbation (e.g. inhibitors, blockers, mutations, etc.), and thus the question always remains if the correlation is real or the result of the perturbation. At the other extreme, chaotic conditions are also not ideal, since a correlation between variables is difficult to establish without extensive numerical and analytical methods. In between these two extremes are sustained oscillations, which provide the most accessible dynamic pattern for analysis. Correlations are easy to make by analysis of the period and amplitude and, within certain limits, allow non-dimensional, qualitative comparison of data from different techniques and different organisational levels. Wave function analysis, including Fourier transforms (Fig. 6), power spectra or wavelet analysis,[30,36] provide clear, quantitative data. Phase shifts and phase-space analysis (Fig. 1) provide clues about the regulatory sequence of events. Utilisation of these methods[30,36,38] was instrumental in disproving earlier estimations of the event sequence in pollen tubes, which had been wrongly inferred from time-course analysis only.[29,51] In a prototype experimental system, mild perturbations could then be analysed in terms of period change, phase shift or wave and power spectral analysis,[53] and these pertubations could eventually be increased until there is a disruption of the correlation between variables, which reveals functional links.

Within limits, growing pollen tubes satisfy several of the conditions noted above. They exhibit oscillations with remarkable periodicity and synchronisation in a number of parameters, and these oscillations are accessible by continuous recording with minimally invasive methods (growth rate with time lapse video, cytosolic ion concentration with ratiometric or other imaging methods and extracellular fluxes with vibrating probes). The growing pollen tube is thus a system in which the three criteria outlined above may be met. So what molecular links have already been described and what more do we need?

The molecular basis of pollen tube oscillations

In order to dissect and model the oscillator, a number of assumptions must be made. Firstly, the molecular basis of the oscillator should be grounded in the nature of the growth mechanism itself. It is widely accepted that pollen tubes grow by exocytosis of wall precursors contained in Golgi-derived vesicles. Furthermore, this growth takes place exclusively in the extreme apex of the tube.[4,5] It thus seems reasonable to assume (1) that the molecular mechanisms that shapes and control growth exist exclusively in the tip (i.e. the terminal apex devoided of large organelles) and their temporal interplay is governed by feed-back loops that function on about the timescale of the reported periods. Given the large number of individual events involved, such as vesicular exocytosis, it seems reasonable to assume also that (2) the nature of the regulatory events is governed by biophysical phenomena or fast chemical or biochemical reactions. If these assumptions hold, it becomes apparent that the regulatory mechanism does

not involve any organelle or cytoplasmic structure that is not present in the tip area, and also cannot be accounted for by regulation through direct gene expression or enzyme induction. In conclusion, the mechanism should operate as a dynamic condition, well away from equilibrium and maintained by a localised enzyme-like process.

Among the structures that are situated most closely to the locus of growth are the Golgi vesicles, the cell wall, and the intervening plasma membrane. The Golgi vesicles, in different species, have been described as being organised in an "inverted cone", in which the base of the cone is adjacent to the apical plasma membrane, while the tip of the cone extends basally about 10 μm. Although it seems well established that these vesicles are transported by an acto-myosin system to the apical clear zone, there are, nevertheless, many questions concerning exactly how the vesicles are directed to the point of fusion, and how the events of docking and exocytosis are regulated. Examination of the clear zone in healthy growing pollen tubes using DIC or phase contrast microscopy reveals that organelle movement is no longer organised in linear streaming lanes, but exhibits random, chaotic motion.[3,4] Regardless of the lack of apparent organisation in the pattern of motion it is evident that vesicles do indeed move apically and fuse with the plasma membrane.[54,55] Exactly how the fusion events are regulated in pollen tubes remain to be characterised, however, and thus we are compelled to borrow from studies in other systems.[56,57]

It has seemed likely that physico-chemical forces present in the apical cytoplasm are instrumental in orchestrating the organisation of the aggregating vesicles and in regulating their fusion at specific foci on the plasma membrane. As an organising principle, large polarised ionic currents, which would act as an electric field and promote the movement of charged particles according to the electric attracting or repulsive forces of the vesicle membrane proteins, are known to exist.[58–60] Their possible importance is backed-up by the fact that isolated vesicles move anodically on an electrical field.[61] Polarised distribution of channels (or polarised activation of channels) would serve as membrane spatial markers and would not only define the growth plane, but also condition the growing shape.[62] An extension of these concepts to membrane electrophoresis leads to the proposal of a regulatory mechanism of pollen tube growth that is based on the spatial and temporal self-organisation of membrane ion carriers and pumps, and their spatial segregation on the grounds of putative surface charges.[2] Crucial components are (1) the existence of stretch-activated Ca^{2+} channels at the extreme apex, and (2) the mechanical interplay between turgor pressure and the yielding of the cell wall, also at the extreme apex.[22,30,51]

All current models accept that there is a breaking mechanical point based either in the yielding of the wall or in the increase of internal turgor, which leads to a stretching of the plasma membrane and consequent opening of the Ca^{2+} channels. In turn, this would allow Ca^{2+} to flow in and promote vesicle fusion.[57] From a formal standpoint, three variables (exocytosis, channel activity and wall rheology) and two positive feed-back loops (wall yielding increases channel activity, $[Ca^{2+}]$ increase promotes vesicle fusion) exist, and these generate sufficient conditions for oscillatory (or chaotic) patterns to occur. Furthermore, while negative feed-back loops are known to permit oscillations, positive feed-back mechanisms are more important, since they constitute a self-amplification mechanism (oscillations in cAMP in *Dictyostelium*, the glycolytic pathway and Ca^{2+} Refs. 14.63). All models for pollen tube growth remain speculative, however, since important aspects and components have not been properly characterised. While we are quite certain that Ca^{2+} channels must exist, in order to account for the presence of the tip focused gradient,[27–29,42,64] they have not been directly confirmed by patch-clamp or molecular isolation. Even less is known about their specificity, which is a crucial point since many stretch activated channels are relatively non-specific for cations, allowing the influx of not only Ca^{2+}, but H^+ and K^+.[65] More recently, plant mechanosensitive plasma membrane channels that allow influx of Ca^{2+} and efflux of K^+ have also been described.[66] In addition, the mechanical issue is not resolved. While recent data on cell wall extension favour the wall-yielding hypothesis,[67] most of these conclusions were drawn from typical pecto-cellulosic vegetative cell walls, which are quite different from the almost entirely pectinaceous wall of the pollen tube. Direct measurement of the internal turgor revealed elevated values but, unfortunately, experimental limitations precluded direct measurement during the oscillatory growth phase.[68] The most solid piece of evidence came from experimental manipulations of turgor that revealed that the growth rate can be markedly altered.[48]

As a component involved in the regulation of growth, the actin cytoskeleton deserves attention. Organelle streaming in the pollen tube is driven by an acto-myosin system, conspicuously present in these cells. It seems increasingly clear, however, that the actin cables do not extend into the region of the inverted cone of vesicles, where they will fuse. Most compelling are the recent observations showing that an endogenously expressed fusion of GFP with the actin binding domain of talin, while labelling the actin cables and fine filaments, does not reveal the presence of these structures in the extreme apex.[69] Nevertheless, we are faced with a puzzle, since agents such as profilin, DNAse and latrunculin, at levels that perturb actin turnover, but not streaming, are potent inhibitors of pollen tube elongation.[70] Even though actin filaments are not evident in the apex of the pollen tube, processes of assembly involving them apparently influence the fundamental aspects of growth.

While the leading "factors" have now been identified, the challenge is now to establish the functional ties between these

molecular entities to construct a cycle of events that corroborates the observable dynamics. Thus far, screening for mutants (in *Arabidopsis*) of pollen tube growth has been disappointing, with only a few of limited experimental value having been described.[71] The development of technology to measure more than two variables at the same time, combining different measuring techniques would be very promising. Also cloning and GFP-labelling of the proteins playing a role in this cycle would allow insight into their dynamics and a better description of the system's complexity. Lastly, an effort is needed to bring all the available kinetics and numerical data into a coherent numerical model that: (1) fully describes the system, (2) has a predictive output in terms of kinetics, and (3) ideally, has a useful output in terms of spatial organisation. One such effort has been made to describe H^+ dynamics.[32]

Conclusions

Evolution preserves robustness and effectiveness; these are clearly characteristics of the mechanism underlying pollen tube growth. The pollen tube is a highly regulated and homeostatically governed cell type, capable of growing on minimal requirements and of enduring differences in the concentration of outside solutes over three orders of magnitude. It faces the mechanical obstacles of the stigma and style, while maintaining the specificity of its target, namely to deliver the sperm cells into the ovule. From the above description and discussion, this apparent physiological complexity may be based on just a few basic features. The key point may thus be the self-organisation of cellular events revealed by the formation of coherent reaction-diffusion patterns, which are supported by non-linear kinetics mechanisms. The participation of prominent ions such as H^+, Ca^{2+}, K^+, and Cl^- ensures that these temporal patterns can be easily transmitted over the whole cell and expressed spatially, both by diffusion-reaction mechanisms and through signalling cascades. Furthermore, to a significant extent it involves inorganic ions and biomolecules that appeared early in evolution. Thus the pollen oscillator may represent a class of basic ion oscillator that underlies the spatial and temporal organisation of many other developing cells, at the least, those that expand or differentiate through tip growth. In this paper we have tried to emphasise that once a molecular mechanism is described and mathematically modelled, it can become a standard, applicable to other systems. Future research will show if our knowledge about pollen tube growth is enough to express one such molecular model reliably. Considering the importance of applying formal approaches to deal with complex cellular processes, we again emphasise the efficacy of the pollen grain and tube as an experimental model that is adequate to such a theoretical challenge. The generality of the biophysical and biochemical mechanisms discussed above encourages the prediction that such models could have general application to many different aspects of cellular physiology.

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