Gerhard Obermeyer
José Feijó *Editors*

Pollen Tip Growth

From Biophysical Aspects to Systems Biology

Springer

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Editors
Gerhard Obermeyer
Department of Molecular Biology
University of Salzburg
Salzburg, Austria

José Feijó
Cell Biology Molecular Genetics
University of Maryland
College Park
Maryland, USA

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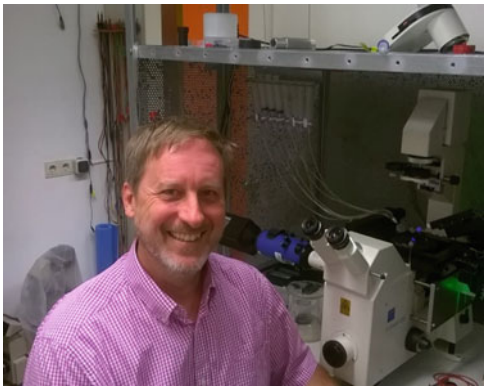

Gerhard Obermeyer born in 1961, studied biology at the University of Konstanz, Germany, with majors in membrane and cell biophysics. His Ph.D. thesis at the Karlsruhe Institute of Technology (Germany) included the imaging of tip-localized Ca^{2+} gradients in pollen tubes and first patch-clamp experiments to characterize pollen ion channels. As a postdoc, he worked at Wye College (now part of Imperial College, London, UK) on ion channels from guard cells and pollen grains and continued pollen research at the Institute of Plant Physiology (University of Salzburg, Austria) with intermediate fellowships to visit labs at the University of Adelaide (South Australia), in Cuernavaca (Mexico), and in Oxford (UK) to work on symbiosome membranes and intracellular pH measurements. Finally, he became an associate professor at the University of Salzburg where he established molecular plant physiology in research and teaching.

Prof. Obermeyer's research focuses mainly on pollen physiology. The growth of pollen tubes through the style tissue is a prerequisite for a successful fertilization which guarantees high crop yields for human nutrition. Problems caused by global warming like drought and temperature stress, can disturb pollen function and are studied using several single-molecule/single-cell techniques in combination with -omics approaches to reveal functional protein complexes in the plasma membrane and their role in osmosensing and osmoregulation as well as in tip growth.

José Feijó studied biology at the University of Lisbon, Portugal, specializing in cell biology of orchid pollen, and obtained his master's in plant biotechnology. During his Ph.D. he enlarged his focus into development, progressively introducing electrophysiology and mathematical modelling as routine approaches to the study of pollen tubes. A Fulbright fellowship brought him to Peter Hepler's lab at the University of Massachusetts in Amherst to further deepen his skills on ion imaging. His return to the University of Lisbon in 1996 marked the beginning of his professorship and independent research career; from 1999 to 2013, he ran in parallel with an independent lab at the Gulbenkian Institute for Science. Along this path, he has served as a director of the Imaging Unit, organized over a dozen EMBO practical courses on plant development and imaging, acted as a curator, and collaborated with numerous educational projects; he was also responsible for a number of initiatives to commemorate Darwin's bicentenary in 2009. In late 2013, he moved to the University of Maryland, College Park, and lives in Washington, DC.

The research of Feijó's group is focused on the development of integrated models of apical cell growth and morphogenesis, using the pollen tube as a biological model, ion dynamics as an experimental paradigm, and theoretical modeling as an integrative tool. The group uses Arabidopsis, lily, tobacco, and tomato as model species for higher plants and the moss Physcomitrella as an evolutionary correlate of apical growth evolution. On the path to develop models by which ion dynamics choreographies integrate spatial and temporal cues to coordinate cell biology, the group contributed to novel ion channels and sensors involved in pollen tube biology and their regulation mechanisms. Most results involve a combination of imaging, electrophysiology, genetics, and molecular biology. Feijó's group further pioneered transcriptomics of plant male gametes and its consequences for plant reproduction and improvement, namely, at the epigenetics level. These activities were routinely grounded in collaborations with groups in over 12 countries in the 4 continents.

The authors met at the Sexual Plant Reproduction meeting in Vienna, Austria, in 1998, and collaborated; they became good friends ever since.

Part I
Introduction

Chapter 1
Pollen Tubes and Tip Growth: of Biophysics and Tipomics

Gerhard Obermeyer and José Feijó

Keywords Biophysics • Molecular mechanisms • Omics techniques • Pollen • Systems pollen biology • Tip growth • Tipomics

1.1 Introduction

Pollen is the male gametophyte of higher plants and is responsible for the successful transport of the sperm cells to the ovules. After landing on a stigma, pollen grains will germinate and grow a pollen tube through the stigma and style tissue towards the ovules, where fertilization takes place. In terms of cell biology, the elongation of the pollen tube is characterized by a dramatically polarized growth process, tip growth, which is common to root hairs, fungal hyphae and some developing neurites. Due to their simple morphology and function, growing pollen tubes became the most well-established model system to study tip growth. Both the editors of this book have been enthusiastic paladins of this trend, even since they first met in 1994 during the 13th International Congress on Sexual Plant Reproduction in Vienna. Our intellectual enthusiasm was first materialized in an essay in which a naive and simple yet forward theoretical model was set forth, implying a set of electrochemistry rules to be at the core of a minimal set of mechanism underlying cell polarity establishment and maintenance during pollen tube growth (Feijó et al. 1995). In short, subcellular biophysical processes, like ion transport, endogenous electrical fields and a tip-focussed Ca^{2+} gradient, would regulate the shape and growth rate in an essentially self-organizing process. Ever since then, the multitude of nuts and bolts and genes and pathways and their biological consequences have amounted to a vast literature in practically every aspect of the biology of pollen.

G. Obermeyer (✉)
Molecular Plant Biophysics and Biochemistry, Department of Molecular Biology, University of Salzburg, Billrothstrasse 11, 5020 Salzburg, Austria
e-mail: gerhard.obermeyer@sbg.ac.at

J. Feijó
Department of Cell Biology and Molecular Genetics, University of Maryland, Bioscience Research Building, College Park, MD 20742, USA
e-mail: jfeijo@umd.edu

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And yet, some of the essential parts of this naive biophysical view of the pollen tube remained elusive, namely the existence and features of the channels responsible for the unique ion biology of pollen (Michard et al. 2017).

By necessity, the views on tip growth in pollen tubes have changed immensely ever since this fortunate encounter of the editors, and a multitude of functional and theoretical models have been proposed, reflecting as many facets as the expertise area of the different groups that since then have been pushing the field forward, be it cytoskeleton, biomechanics, membrane trafficking, signalling, protein sorting, etc. [reviewed by Feijó et al. (2001), Holdaway-Clarke and Hepler (2003), Bibikova et al. (2004) and Certal et al. (2008)]. And from a systems biological view, the emergence of transcriptomics had a tremendous impact, with pollen and fertilization emerging as some of the best described biological processes in terms of specific transcriptional profiles, with databases in practically every facet of its biology.

And yet a generalized accepted model for cell apical growth is lacking. Of notice, from our initial biophysical perspective of the pollen tube, the relationships between the activity of ion channels and pumps in the plasma membrane of the growing pollen tube, the ion fluxes and cytosolic gradients of K^+, Cl^-, Ca^{2+} and H^+ they generate and growth rate, determinants of growth direction and oscillatory growth of pollen tubes are still elusive and subject to multiple interpretation. Even a simple hypothesis, like the heterogeneous distribution of different ion channel types and pumps is responsible for the localized, specific ion fluxes along a growing pollen tube (Feijó et al. 1995) that could be easily tested by following expression chimeras of GFP, is largely missing for practically every transporter identified either by physiology, by genetics or even less by transcriptomics (Michard et al. 2017). Usually, the spatial distribution of these ion fluxes around a germinating pollen grain or along a growing pollen tube can be measured with ion-sensitive vibrating electrodes (Kunkel et al. 2006). Although this technique allows a characterization of the currents and current pattern, it cannot identify the transport protein responsible for the fluxes. In addition, a number of signal transduction pathways are already known as components of a regulatory network that contribute to activation or inactivation of cellular processes involved in tip growth. The signal transduction pathways include the cytosolic free Ca^{2+} concentration, the cytosolic pH, protein phosphorylation and 14-3-3 proteins, G-proteins, Ca^{2+} activated kinases, CBL/CIPK pairs and phospholipids (Kost et al. 1999; Pertl et al. 2001; Potocky et al. 2003; Monteiro et al. 2005; Michard et al. 2008; Steinhorst et al. 2015). All mentioned components were found to be essential for the growth process of pollen tubes, and one may assume that they also interact with each other thus forming a complex, spatially and temporally regulated network coordinating the dynamic events necessary for pollen tube growth. In accordance with these results, the molecular and cellular processes do not form a linear causality chain, but pollen tube growth can be seen as a complex system of interacting networks formed by nodes connected with edges (see Klipp et al. (2009) and Coruzzi and Gutiérrez (2009) for an introduction to "Systems Biology"). Such type of systems studies for pollen tube tip growth could plausibly be achieved by combining information coming from the different omic levels, if properly framed with specific hypothesis-driven approaches. While any such model

will by necessity face enormous challenges for validation because of elevated degree of spatial and temporal dynamics characteristic of pollen tube growth, even coarse approaches could be tremendously influential to characterize the information flow carried by different networks that collectively contribute to the major outcome of pollen tubes: growth and delivery of the sperm.

This was essentially the mind-set of the authors while embarking the edition of this book. The invited authors contributed chapters that highlight many important aspects of pollen physiology and tube tip growth and other conceptual fields which are needed to better understand it. Hopefully our book will provide an updated and balanced overview of the current knowledge and present future research perspectives and will contribute to fill the many gaps obviously still existing in our fundamental understanding on how pollen tubes growth is driven and regulated by molecular interactions underlying the cellular processes. Chapters range from molecular biophysical concepts to comprehensive omic studies and computational modelling of the tip growth process. In addition, a chapter on root hair cells is included to provide, although almost similar in many aspects, an alternative view on the underlying molecular principles of the tip growth process.

1.2 Some Unsolved (Bio)physical Aspects of Tip Growth

It's very simple: the pollen tube and all other tip-growing cells grow by increasing their volume and, in the case of plant cells, mostly through the uptake and accumulation of water in the vacuole. Although simple, this process causes many difficulties and problems for pollen tubes. For example, net water uptake will cause an increase in turgor pressure and, thus, will exert pressures yet to be precisely determined at the nascent cell wall at the tube tip. This means that the stability of the newly formed cell wall has to be adapted to the growth speed and to the water uptake rates, and one might expect receptors for turgor pressure and/or cell wall strain, which sense the actual osmotic state and the stability of the cell wall. On a different perspective, in growing pollen tubes, the water potential inside the tube has to be lower than in the surrounding tissue to enable an influx of water. This can be achieved by regulating the cytosolic osmotic and turgor pressure of the pollen, but also of the surrounding style tissue. Of relevance, these two issues bring to the board two major aspects of an essential biophysics approach, biomechanics and electrochemistry, that can and should be properly quantifiable and modelled.

Although these considerations are prerequisites for every fast growing plant cell, not much is known about the molecules, processes and signalling pathways involved in the regulation of water transport. Biophysical concepts, recent results and future perspectives are summarized in the first chapters of this book with Chap. 2 describing aspects of water transport in pollen tubes and Chaps. 3, 4 and 5 covering subjects of cell wall stability and mechanical forces during tube expansion. Readers will notice that open questions are in most cases related to still unknown physical parameters. Other than just turgor pressure, also the viscosity and elastic modulus

of cell walls are important for the stability and flexibility of the cell wall to adapt to new micro-environments along the path to the ovule.

1.3 Technical Improvements and a Wish List for the Future

To answer some of these unsolved biophysical questions, new techniques must be developed or adapted to the fast growing pollen tube. For instance, our understanding will immensely increase if one could monitor the dynamic changes of cell wall elasticity in an elongating tube. In a dramatic turn of page into nano-techniques, Chaps. 4 and 5 propose original approaches based on micromachined mazes, which incorporate microfluidics capacity allowing the use of non-invasive cellular force microscopy to reveal changes in cell wall elasticity, live cell imaging and the challenge of the growing tubes with a number of microfluidic treatments that can physically alter the forces involved in growth. The promise that these approaches can incorporate higher level of resolution in terms of live cell imaging could be of paramount importance to understand the flexibility of the pollen tube growth process.

In fact, starting from the early 1990s, which have seen some, still coarse, videos of tip-localized Ca^{2+} gradients determining the pollen tube growth direction, the techniques of fluorescent reporters are becoming more and more sophisticated. By adapting the use of genetically engineered nanosensors almost every ion (Ca^{2+}, pH, phosphate), metabolite (glucose, sucrose, ATP) or physical parameter (membrane voltage) can be monitored in living plant cells and, therefore, also in pollen tubes (Swanson et al. 2011; Okumoto et al. 2012). So far, only Ca^{2+}, pH and chloride sensors have been used in pollen tubes, but looking at the still increasing availability of nanosensor constructs (Hamers et al. 2014), we might be able to watch combinations of parameters simultaneously in the future, maybe in real time and under physiological conditions, namely, in pollen tubes growing through the style tissue.

So far, most experiments have been performed with in vitro growing pollen tubes in liquid or on solidified media. An increasing number of studies have shown that pollen tubes are conditioned during their journey through the stigma and style tissue (see Chap. 8). Using microfluidic chambers or lab-on-chip devices for pollen tube growth, the physiological conditions of a surrounding tissue can be mimicked, and, furthermore, semi-in vivo pollen cultures can be set up that allow pollen grains to germinate on the stigma and grow a few microns through the style tissue before entering a microchannel, which then allows a detailed and high-resolution view of the growing tip. The use of various microfluidic devices is described and discussed in Chaps. 4, 5 and 8.

Also in the front of better and improved imaging methods, Chap. 6 pushes the envelope in terms of microscopic observations of membrane trafficking and referred to a number of essential publications on state-of-the-art microscopy, for instance, light sheet microscopy (Maizel et al. 2011; Kumar et al. 2014), fluorescence

lifetime FRET (Schleifenbaum et al. 2009), 2-photon confocal microscopy (Feijó and Moreno 2004) or TIRF (Wang et al. 2016).

1.4 Tipomics: Pollen Systems Biology and Modelling Tip Growth

A major step towards a comprehensive view of the tip growth process in pollen tubes was the application of various omics techniques to reveal the molecular details of as many cellular processes as possible. In addition to the pollen transcriptome, the data from proteome and metabolome allow the modelling of metabolic pathways during different states of the pollen's life, whereas studies of the protein interactome and the phosphoproteome show the dynamics of signalling events and signal transduction pathways in pollen (see Chaps. 10, 11 and 12). Hereby, the system can be restricted to a single-cell type like the pollen grain, but may also span different tissue and organs of the whole organism. By considering the time dependence of signal propagation, a time-resolved map of information flux inside a cell or inside an organism can be studied. The spatial resolution of cellular dynamics is getting more and more refined as e.g. with the studies of distribution of proteins and metabolites in cell organelles. Integration can be achieved by information on protein localization derived from databases, literature or immunolocalization experiments and be used to reveal the dynamics of protein re-localization during biological processes in regular proteomics approaches (Dunkley et al. 2006; Pertl-Obermeyer et al. 2016). Excellent examples of the technical feasibility with omic methods are given in recent studies that investigated the phosphoproteome dynamics of in vivo and in vitro insulin signalling with a highly time-resolved, spatial distribution of signalling information (Humphrey et al. 2015), showed a high spatial resolution of cellular organelles (Christoforou et al. 2016) or gave a comprehensive protein interaction map for human cells (Liu et al. 2015) and enabled the detection of the spatial distribution of metabolites in cell organelles (Krueger et al. 2011; Arrivault et al. 2014). In pollen, the experimental challenge will be to apply the recently developed omics techniques to the fast growing pollen tube with a suitable time and spatial resolution. A major limitation will be the material that can be collected at each time point and the preparation methods to avoid loss of material and to extract all components of interest. These problems are addressed by the authors of Chaps. 10, 11 and 12. However, recent improvements in mass spectrometry allow an optimistic view that the amount of experimental material can be decreased even further. Due to the development of more sensitive and faster mass spectrometers (Michalski et al. 2011, 2012) and open source analysis software, e.g. MaxQuant (Tyanova et al. 2016) or PatternLab (Carvalho et al. 2016), proteome data can be sampled with only low amounts of starting material and analysed in a flexible way, thus enabling also proteome analyses with *Arabidopsis* pollen during signalling events at higher time resolution than just ungerminated and germinated. While the transcriptome can be

studied already in single cells (Ntranos et al. 2016), the single pollen proteomics might be possible in the near future, too.

Another experimental challenge for omics approaches is the application to in vivo situations. Pollen tubes growing through the style towards the egg cells are conditioned to respond to the female tissue signals (see Chap. 8). In addition, as most processes and molecules responsible for growth of pollen tubes can be observed in the tube tip and this tip responds fast to signals, a detailed view to the proteome, metabolome, ionome, interactome, etc. of the tube tip with a high time and spatial resolution might push our knowledge on tip growth dynamics further beyond the present horizon. In combination with physiological parameters, nonlinear statistics of the large omics data sets can be used in up-to-date mathematical modelling to generate unbiased computer models of tip growth (Klipp et al. 2009; Ingalls 2013). The last three Chaps. 13, 14 and 15 give an impression on how mathematical models can be derived from the present data and which considerations have to be made to generate a valuable model that allows testing our hypotheses and can make predictions that can be tested experimentally. In fact many such theoretical models have been proposed to explain the essential features of pollen tube, and a critical view of their essential features, potential and limitations is offered in Chap. 15.

1.5 Conclusion, Open Questions and Perspective

The last decades of pollen tip growth research have shown a vigorous increase in processes "important for tube growth". Almost every cellular process or molecule under investigation could be shown to be important, responsible, essential or crucial for pollen tubes. Have they all a similar importance or is there a master regulator? This will be a challenge to test mathematical models that describe pollen tip growth and also a challenge to design unbiased data collection in future experiments. Probably, we have to disband our own way of thinking in linear causation chains and have to imagine complex networks which provide numerous alternative ways to achieve an aim. This might be exactly the case for pollen tip growth. Why should such an important and robust but on the same hand delicate process, which creates the progeny of a population, be so vulnerably relying on a single event cascade or causality chain where a single failing chain link can cause the abortion of the process, namely, final growth stop? On the other hand, one may argue that pollen tube growth is highly competitive and selective, so that a single mistake is enough to kick this individual out of the gene pool ensuring a healthy next generation. As almost always in biology, a combination of both extremes is very likely: a complex network with a major route along all nodes but with several alternative ways and bypasses to achieve the goal.

All authors contributing to this book have pinpointed important gaps in the data of their research field. We are grateful for their generous effort in putting thorough texts of high quality that gain a special corpus we set together in the form of a reference book. We hope our collective effort as editors and authors may offer

other colleagues and young starting researchers solutions, either experimental or theoretical, to fill our perceived gaps of knowledge in this area or to solve some of the still many mysterious behaviour of pollen tube tips.

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Part II
Biophysics of Tip Growth

Chapter 2
Water Transport in Pollen

Gerhard Obermeyer

Abstract Pollen is the male gametophyte of plants, and its major function is to deliver sperm cells to the ovules to ensure a successful fertilisation. Sperm cells are carried inside the pollen tube, a growth extension of the vegetative cell of pollen that grows through the stigma and style tissue. The elongation/growth of pollen tubes is a highly regulated biological process, and the uptake of water is thought to account for the enormous increase in cell volume. Water transport into the pollen grain initiates the transition from the quiescent mature to the active, rehydrated pollen grain and subsequently allows the large increase in volume during tube growth. To maintain the water uptake, internal pollen water potential has to be lower than the external water potential which can be achieved by lowering the turgor pressure, by increasing the internal osmotic pressure or by decreasing the external osmotic pressure. Uptake or synthesis of osmotic active compounds may be necessary to adapt the internal osmotic pressure due to changes in osmotic conditions during the tube's journey through the stigma tissue. Especially in periods of severe drought stress, the water potential of the stigma cells may drop, and thus water uptake into the pollen tube is distorted. The ability to sense and to adapt to osmotic conditions is therefore an important feature of the pollen to warrant a successful fertilisation and in consequence to ensure high crop yields.

Keywords Aquaporin • Turgor pressure • Water potential • Water transport

Abbreviations

AQP	Aquaporin
L_P	Hydraulic conductivity
MIP	Major intrinsic protein

G. Obermeyer (✉)
Molecular Plant Biophysics and Biochemistry, Department of Molecular Biology, University of Salzburg, Billrothstrasse 11, 5020 Salzburg, Austria
e-mail: gerhard.obermeyer@sbg.ac.at

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PIP Plasma membrane intrinsic protein
PM Plasma membrane
P_{os} Osmotic permeability

2.1 Introduction

No water, no life. This simple statement counts for all biological organisms and life itself. Particularly in plants, water uptake and transport through the entire plant fuels a number of physiological processes that rely on constant water supply. For instance, uptake of nutrients by roots, transport of ions and sugars by the xylem and phloem system, respectively, and finally plant growth which among other factors is driven by elongation of single cells, which is supported by taking up water. Fast-growing plant organs need a high water uptake rate to maintain their turgor pressure despite volume increases. More than other cells, pollen tubes, which are among the fastest growing organisms in biology with a speed of up to 30 μm min^{-1}, need high water uptake rates to ensure the fastest possible growth through the stigma tissue. On the other hand, water uptake has to be regulated and well synchronised with other growth-related cellular processes like synthesis and transport of cell wall material, vesicle transport and the cytoskeleton to optimise fast growth to ensure fertilisation.

2.2 Water Potential, Water Transport and Pollen Tube Growth

The physical concept of the water potential is very useful especially on the level of single cells to describe and predict water fluxes between cells and the environment. It has to be noted that the common equations used are an approximation as emphasised by Jack Dainty (1963) more than 50 years ago: 'I am afraid that the only correct theory is that based on the theory of irreversible thermodynamics and this must be faced by all workers in this field'. Nevertheless, the water potential concept for everyday use is practical and allows a comprehensive modelling and prediction of water transport. Basic concepts of water relations in plant cells are described in a number of review article and book chapters (Dainty 1963; Steudle 1989; Zimmermann 1989; Nobel 2009). In analogy to Ohm's law $I = U \bullet R^{-1}$, the water (volume) flow J_V can be described as

$$J_V = L_P \bullet \Delta\Psi \tag{2.1}$$

with L_P as the hydraulic conductivity, and the water potential difference $\Delta\Psi$ acts as the driving force for water flux. The water potential itself derives from the chemical

potential of water and at the cellular level is defined as

$$\Psi = \frac{\mu_W - \mu_W^o}{\overline{V}_W} = P - \pi = \Psi_P + \Psi_\pi \tag{2.2}$$

where μ_W and μ_W^o are the chemical potential of water and the standard chemical potential for water, respectively, and $\overline{V}$ is the molar volume of water ($18.05 \cdot 10^{-6}$ $m^3 mol^{-1}$), P ($= \Psi_P$) is the hydrostatic pressure (or pressure potential) and π ($= -\Psi_\pi$) is the osmotic pressure (or osmotic potential). The water potential is expressed in units of pressure (1 MPa = 10 bar) with 0 MPa as the highest value ($\mu_W = \mu_W^o$), and thus, water is always flowing in the direction to the more negative Ψ_W value.

In the presence of nonpermeable solutes only, the water (volume) flow density (j_V) across the plasma membrane separating the plant cell interior (i) from the medium (o), Eq. (2.1), can be written as

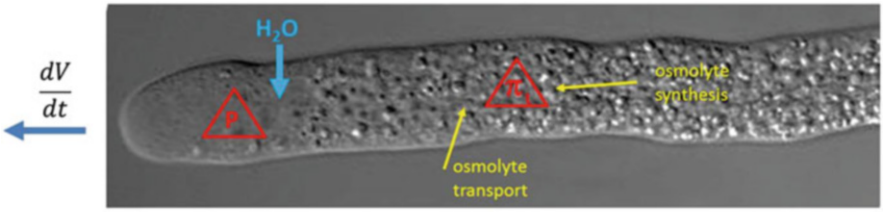
$$j_V = -\frac{1}{A}\frac{dV}{dt} = L_P (P - \Delta\pi) = L_P \left[P - \left(\pi^i - \pi^o\right)\right] \tag{2.3}$$

with A as the surface area of the cell; V, cell volume; L_P, the hydraulic conductivity; P, turgor pressure; R, gas constant; T, absolute temperature; and c^i and c^o, concentration of internal and external nonpermeable solutes, respectively. By considering the van't Hoff relation ($\pi \cong RT \bullet c$), Eq. (2.3) can be written as

$$j_V = -\frac{1}{A}\frac{dV}{dt} = L_P \left[P - RT\left(c^i - c^o\right)\right] \tag{2.4}$$

It has to be noted that only the case for nonpermeable solutes was considered in these equations. For a more general description of water transport including permeable solutes and simultaneous fluxes of water and solutes, please consult the literature (Kedem and Katchalsky 1958; Dainty 1963; Kramer and Boyer 1995; Murphy and Smith 1998).

A simplified relation between water fluxes and pollen tube growth is illustrated in Fig. 2.1. Growth of pollen tubes, namely, the increase in volume (dV/dt), is based on influx of water which according to Eq. (2.1) occurs only if $\Psi_i < \Psi_o$ or if $\Delta\pi > P$ (Eq. 2.3). To reduce the internal water potential, pollen tubes have to decrease their turgor pressure P or increase the internal osmolyte concentration c^i by uptake of solutes from the surrounding medium or by synthesis of osmotic active solutes. The movement of water into or out of a cell is always passive, and water follows the changes in water potential, which can be actively influenced by cells via solute transport or synthesis. The cell, or in this case the pollen tube, may reach an equilibrium when the turgor pressure equals the cytosolic osmotic pressure ($P = \Delta\pi$), and water uptake ceases as well as tube growth.



$$\frac{dV_{tube}}{dt} = J_V = -AL_P[\Delta P - \sigma(\pi_i - \pi_o)]$$

Fig. 2.1 Simplified scheme of water uptake in pollen tubes. Uptake of water changes the pollen tube's volume (V_{tube}), thus leading to tube elongation with a growth rate of $\frac{\mathrm{d}V_{\text{tube}}}{\mathrm{d}t}$. The volume flux ($J_{\text{V}}$) depends on the pollen surface (A), the hydraulic conductivity (L_P), the turgor pressure (ΔP) and the difference in internal and external osmotic pressure, π_{i} and π_{o}, respectively. The σ denotes the reflection coefficient that is 1 for nonpermeable solutes

2.3 Measuring Water Potential Parameters and Water Transport in Pollen

A simple way to estimate the minimal net water uptake in pollen tubes that is the difference between water uptake and water release is to measure the tube growth rate. Because pollen tubes grow with an almost constant diameter and the increase in tube volume is mainly caused by water uptake, the growth rate correlates well with the volume increase per time unit ($\mathrm{d}V/\mathrm{d}t$). For instance, a pollen tube with a diameter of 12 μm that grows with a speed of 10 μm min^{-1} adds a volume of 1.131 pl each minute ($V = r^2 \cdot \pi \cdot h = 1131 \times 10^{-18}$ m^3). This equals a minimal water flux of 1 pmol s^{-1}. This simple estimation already predicts that the growing pollen tube has to be capable to transport at least 1 pl water per minute across its plasma membrane to reach the observed tube growth speed. The parameters, which describe the water permeability of biological membranes, are the hydraulic conductivity (L_P) or the osmotic permeability (P_{os}) that are related as:

$$P_{\text{os}} = RT\frac{L_P}{\overline{V}_w} \tag{2.5}$$

For pollen grains of *Lilium longiflorum*, both parameters have been determined experimentally by using either the pressure probe (Pertl et al. 2010) or a protoplast swell assay (Sommer et al. 2007) under isovolumetric and isobarometric conditions, respectively. It has to be noted that the water permeability differs slightly between intact pollen grains and pollen grain protoplasts due to the contribution of the cell wall to the water permeability. Furthermore, one may also expect different water permeabilities in pollen grains and tubes that are also caused by the differences in cell wall composition between grains and tubes.

2.3.1 Protoplast Swell Assays

Swell assays can be performed with every protoplast originated from plant cells to determine the osmotic permeability of the plasma membrane. There are numerous examples with protoplasts prepared from different plant tissues and plant species with slight variations in methods (Ramahaleo et al. 1999; Moshelion et al. 2004; Murai-Hatano and Kuwagata 2007; Sommer et al. 2007). In principle, the cell wall is digested enzymatically, and the resulting protoplasts are challenged with media of varying osmolyte concentrations. The increase or decrease in volume of the spherical protoplast is monitored with a video-equipped microscope. Although this set-up faces some experimental and theoretical problems, it can be easily applied. One experimental challenge is the fixation of the protoplasts in the measuring chamber without modifying its ball-like geometry. Another major complication results from 'theory-demanded' instantaneous change of the external medium when the transition between two equilibrium states is studied experimentally. This needs fast solution fluxes and can hardly be realised without washing away the observed protoplast. An additional biological problem results from the exocytosis capacity of each cell which brings about a modification of the plasma membrane area during the experiment. However, bearing these limitations in mind, swell assays produce reliable data for the osmotic permeability. Even if the absolute values of P_{os} might not be entirely correct, they can reflect changes in treatments, mutants or growth conditions (Wu et al. 2013). In pollen, protoplast swell assays have only been applied to *Lilium* pollen, giving values for P_{os} of 6.60 $\mu m\ s^{-1}$ and 13.24 $\mu m\ s^{-1}$ for grain and tube protoplasts, respectively (Sommer et al. 2008).

In addition to the direct determination of the osmotic permeability from time-dependent volume changes, swell assays can be used to calculate the water flux densities (Fig. 2.2). In accordance with electrophysiological experiments, the data may be plotted like current–voltage curves, resulting in graphs of the water flux (density) plotted against the osmotic potential difference (driving force) with a slope corresponding to P_{os}. The data in Fig. 2.2c clearly show that water influx into pollen grain protoplasts (swelling, endo-osmosis) is faster than water efflux (shrinking, exo-osmosis). These observations in water transport were also made in other plant cells (Steudle and Tyerman 1983; Murai-Hatano and Kuwagata 2007).

2.3.2 Pressure Probe Technique

The pressure probe was developed by Ulrich Zimmermann and Ernst Steudle to measure the turgor pressure of plant cells (Hüsken et al. 1978), and its use is well described (Boyer 1995; Zimmermann 1989; Steudle 1993; Tomos and Leigh 1999). In comparison with other techniques, the turgor pressure is directly recorded, whereas other techniques like ball tonometry, micro-indentation or cellular force microscopy (Linthilhac et al. 2000; Routier-Kierzkowska et al. 2012; Milani et al.

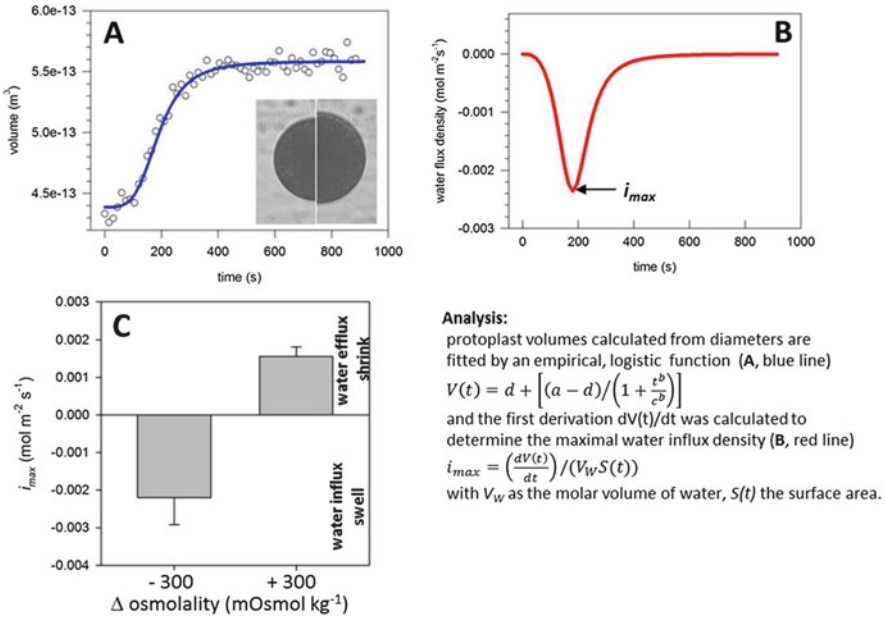


Fig. 2.2 Analysis of pollen grain protoplast swell assays. (**a**) *Lilium* pollen protoplasts are transferred from an iso-osmotic (680 mOsmol kg^{-1}) to a hypoosmotic medium (380 mOsmol kg^{-1}) giving an osmolality difference of ca. 300 mOsmol kg^{-1}. The protoplast's diameter is measured every 10 s and calculated to volume. *Inset* shows a protoplast in iso-osmotic (*left*) and after reaching the equilibrium in hypoosmotic medium (*right*). The volume data were fitted with an empirical, logistic function $V(t)$ similar to Richards (1959). (**b**) The first derivation $dV(t)/dt$ is used to determine the maximal water flux density i_{max}. (**c**) Treatment with hypo- and hyperosmotic conditions shows that swelling of lily pollen grain protoplasts is faster than shrinking despite the same size of the driving force Δ osmolality

2013) measure the cellular micromechanics of a plant cell and extrapolate the turgor pressure value. Although the pressure probe technique needs some experimental skills, it also enables to determine the hydraulic conductivity and the elastic modulus in addition to turgor pressure values in a single experiment (Fig. 2.3). So far, the pressure probe, micro-indentation and cellular force microscopy were used to determine turgor pressure and cellular mechanics of *Lilium* and *Nicotiana* pollen tubes (Benkert et al. 1997; Zerzour et al. 2009; Vogler et al. 2013). The mean turgor pressure of growing lily pollen tubes was about 2.1 bar ranging between 1 and 4 bar (Benkert et al. 1997), while lily pollen grains had a turgor pressure of 3.2 bar (Pertl et al. 2010); both were measured in standard germination medium with an osmolality of ca. 300 mOsmol kg^{-1}. Although the turgor pressure of lily pollen tubes could be deduced by cellular micro-force microscopy after plasmolysis (3 bar, Vogler et al. 2013), the pressure probe might be the only technique, so far, to determine the turgor pressure in the rigid, double-walled pollen grain.

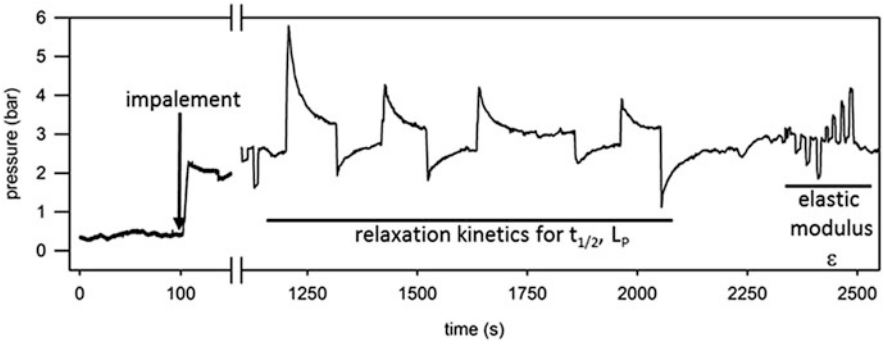


Fig. 2.3 Turgor pressure measurement in *Lilium* pollen grains. An example for a typical pressure experiment is shown. The first 150 s are presented at a higher time resolution. After impalement with the micro-capillary, a sudden increase in pressure is noticed. In addition to turgor pressure, the half-time of the pressure relaxation curves allows determination of the hydraulic conductivity, and known volume changes causing a pressure response can be used to calculate the elastic modulus ε

Hydraulic conductivity, which was also calculated from pressure relaxation curves after application of sudden pressure increases or pressure decreases (Fig. 2.3), gave an average L_P value of 4.9×10^{-8} m $MPa^{-1}s^{-1}$ (Pertl et al. 2010) that corresponds according to Eq. (2.5) to a P_{os} value of approximately 7.4 $\mu m\ s^{-1}$. Although both techniques, the pressure probe and the protoplast swell assay, are sometimes scrutinised due to the experimental manipulation during the measurements, e.g. micro-capillary impalement and cell wall digestion, respectively, the measured values for P_{os} were similar (6.6 $\mu m\ s^{-1}$ for swell assays, see above). Nevertheless, a disadvantage of the pressure probe technique is the need of sophisticated experimental skills due to micromanipulation during impalement and balancing of the oil-cytosol meniscus in the micro-capillary. Single, individual cells or small organisms like pollen grains need to be fixed somehow while forcing the micro-capillary through the cell wall. While pollen grains are fixed with a second holding pipette, the more fragile pollen tubes can be immobilised by agarose or poly-L-lysine-coated cover glasses.

In addition to hydrodynamic parameters like the turgor pressure and the hydraulic conductivity, the pressure probe allows determination of the Young's or elastic modulus (ε) which describes the stiffness of the cell wall. Measurements in lily pollen grains with the pressure probe gave an elastic modulus of 3.9 MPa (Pertl et al. 2010), while cellular force microscopy in combination with a mechanical model based on the finite element method estimated the cell wall elasticity of lily pollen tubes between 20 and 90 MPa (Vogler et al. 2013). Recently, a microfluidic chip technique was tested (Bending Lab-On Chip, Nezhad et al. 2013), and values of 350 MPa were recorded for the longitudinal direction of *Camellia* pollen tubes. These are, so far, the only values from pollen and, thus, do not allow any conclusion before more data are available.

2.4 Pollen Water Transport

In pollen, water transport is an important feature during several stages of flower development. The life of pollen can be classified in five major stages in which water status plays an important role (Firon et al. 2012). In the first phase (microsporogenesis), pollen grains develop inside the anther and are surrounded by the locular fluid which contains sufficient nutrient and water provided by the vegetative tissue. In the dehydration phase, the locular fluid disappears, while the pollen grains mature and dehydrate. The anther opens and the mature, dehydrated pollen is presented for dispersal (presentation phase) followed by the dispersal phase in which the mature pollen grains are exposed to the environment while transported to a stigma. Finally, if pollen grains land on a compatible stigma, they start to rehydrate, germinate and grow pollen tubes towards the egg cells (pollen-stigma interaction phase). This review focusses on water transport during the last phase taking place on the stigma, the rehydration and the pollen tube elongation. The water status during flowering and pollen development is described elsewhere (Firon et al. 2012; Beauzamy et al. 2014).

For both processes, rehydration and tube elongation, a more negative water potential has to be postulated for the pollen interior than in the external medium to allow an influx of water. Although, active water transport against its potential gradient is thermodynamically possible (Onsager 1931; Zeuthen 1995), its role and contribution to water uptake and transport in plants was suggested only recently (Wegner 2015).

2.4.1 Aquaporins in Pollen

Water may enter a cell by three different molecular pathways: (a) diffusion through the lipid bilayer, (b) facilitated transport through aquaporins (AQPs) or (c) cotransport with ions and nutrients via channel or carrier proteins. The latter pathway is very particular since it also enables an 'uphill' transport of water against its potential gradient and might be very interesting in the aspect of osmoregulation and concerted transport of water and ions during tube elongation.

Compared to ions or small solutes with permeability coefficients between 0.1 pm s^{-1} and 0.1 nm s^{-1}, respectively, the permeability of lipid bilayers is quite high for water (1 μm s^{-1}, Hill et al. 2004) which is ten times less than P_{os} values measured for pollen grains. According to Eq. (2.4), the water influx into a 1000 μm long pollen tube with a diameter of 12 μm can be calculated as 0.17 pl min^{-1} when assuming a water permeability of the lipid bilayer alone (1 μm s^{-1}), a turgor pressure of 2 bar and an osmotic potential difference of 380 mOsmol kg^{-1}. This water transport capacity is not sufficient to account for the required water uptake at tube growth rates of 10 μm min^{-1}. However, increasing the osmotic permeability to 20 μm s^{-1}, as measured in lily pollen tubes, allows water uptake of 3.4 pl min^{-1},

which is close to the 11 pl min^{-1} estimated for growth rates of 10 μm min^{-1}. Nevertheless, the question remains: does the pollen need aquaporins for water uptake, and if not which role have the aquaporins known to be expressed in pollen (e.g. Wudick et al. 2014)? If water transport is restricted to a small area and/or is tightly regulated to prevent tube bursting, specific transport proteins for water could play an important role or regulatory function in water homeostasis.

Aquaporins (AQPs) are water-conducting pores formed by four protein subunits of the *m*ajor *i*ntrinsic *p*rotein (MIP) family. One may further distinguish proteins from the plasma membrane intrinsic protein (PIP) and the tonoplast intrinsic protein (TIP) subfamilies which are completed by the SIP and the NIP subfamilies (*s*mall basic and *n*odulin26-like *i*ntrinsic *p*rotein, respectively; for reviews see Chaumont et al. 2005; Ludewig and Dynowski 2009; Maurel et al. 2015). Water channels are mainly built from subunits of the PIP or the TIP subfamilies and, as already implicated by their names, are primarily localised in the plasma membrane and in vacuolar membranes, respectively. One may expect that PIPs are highly expressed in the pollen due to the fast tube growth correlating with high water uptake rates. Evidence for an involvement of aquaporins (PIPs) came from experiments that used mercury ions to inhibit and reducing agents to restore pollen tube growth (Shachar-Hill et al. 2013 and Fig. 2.4), which was taken as an indication for a physiological role of aquaporins because of the reversible effect on a Cys residue close at the permeation pore (Niemietz and Tyerman 2002; Hirano et al. 2010). However, mercury ions have also dramatic effects on all other transport processes as reflected by a large depolarisation of the plasma membrane after addition of mercury compounds (Schütz and Tyerman 1997 and Fig. 2.4) thus questioning the conclusions from such inhibitor experiments.

In opposition to physiological data, no expression of PIP genes could be detected in *Arabidopsis* pollen in various expression and transcriptome studies (Becker et al. 2003; Honys and Twell 2003; Qin et al. 2009). An in silico analysis using *Arabidopsis* microarray data (www.genvestigator.com) revealed almost no detectable PIP-mRNAs in pollen which was confirm by detailed studies (Soto et al. 2008; Wudick et al. 2014). Although PIPs were not detected, 6 out of 35 members of the MIP/aquaporin family were repeatedly reported as expressed in *Arabidopsis* pollen: AtNIP7;1, AtNIP4;1, AtSIP1;1, AtSIP2;1, AtTIP1;3 and AtTIP5;1 (Ishikawa et al. 2005; Bock et al. 2006; Borges et al. 2008; Qin et al. 2009; Loraine et al. 2013). Expression analysis of *Brassica* pollen AQPs was contradictory, showing low expression levels of PIPs in immunoblots, whereas PIP-mRNA could not be detected (Marin-Olivier et al. 2000; Dixit et al. 2001). At least in tobacco pollen, NtPIP1;1 and NtPIP2;1 could be detected at a low level by RT-PCR (Bots et al. 2005), whereas in *Lilium* pollen, partial sequences of PIPs as well as TIP, NIP and SIP sequences were reported in proteome and transcriptome studies (Pertl et al. 2009; Pertl-Obermeyer et al. 2014; Lang et al. 2014).

In summary, a very contradictory image about the presence and possible role of aquaporins in pollen is presented. It might depend on the pollen species, the state of pollen development or the difference in the pollination process itself whether the

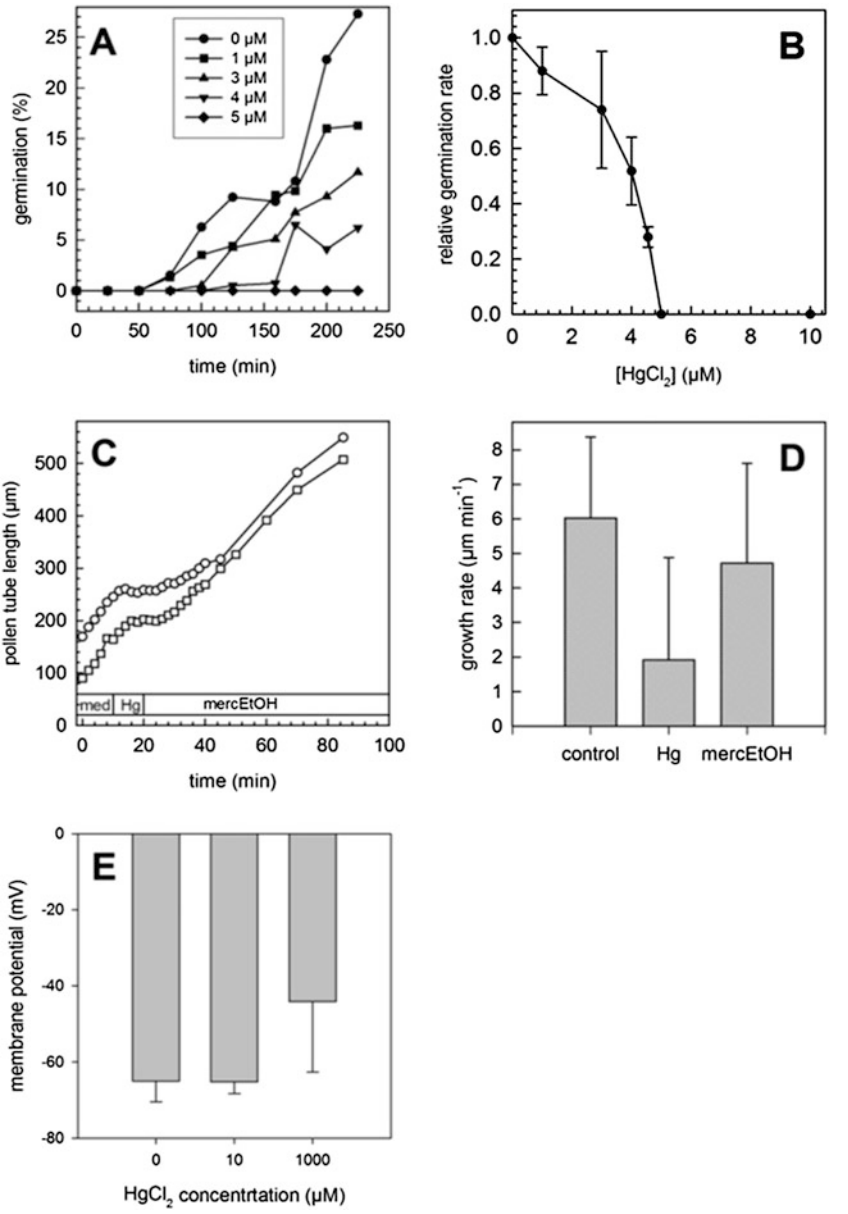


Fig. 2.4 Effect of mercury ions on *Lilium* pollen. (**a**) $HgCl_2$ inhibits pollen germination with a total inhibition at 5 µM. (**b**) Concentration-dependent germination of lily pollen grains. The relative germination rate was calculated from the slopes of germination curves and divided by the control data ($n = 5$) (**c**) Inhibition and recovery of growth of two individual pollen tubes by mercury chloride (5 µM) and mercaptoethanol (10 mM), respectively. Pollen tubes were growing in standard germination medium (med) in a chamber which was perfused with germination medium plus $HgCl_2$ or with mercaptoethanol (mercEtOH). (**d**) Average growth rate of pollen tubes during incubation with 5 µM $HgCl_2$ and recovery in 10 mM mercEtOH ($n = 4$–6). (**e**) Effect on $HgCl_2$ on the membrane potential of pollen grains ($n = 5$)

pollen or the stigma tissue express aquaporins. Pollination involving the so-called 'wet' stigmas might prefer aquaporins in the pollen, while dry stigmas (*Arabidopsis*, *Brassica*) express AQPs in the stigma tissue and, thus, controlling water transport at the stigma site. The lack of large amounts of plasma membrane-localised aquaporins also implies that the water conductance of the lipid bilayer might be sufficient for water uptake to provide the observed tube growth rates, and TIP aquaporins in the tonoplast are adequate to facilitate cell volume enlargements as has been detected during emergence of lateral root primordia in *Arabidopsis* (Reinhard et al. 2016). In pollen, much more research is necessary to obtain a clear and reliable image on the expression and role of aquaporins and PIPs, especially, during pollination.

2.4.2 Pollen Grain Rehydration

Experimental data on the first events during pollen grain rehydration are difficult to monitor, and many observations were collected from studies on different pollen types and species emphasising various aspects during pollen grain water transport. The first synopsis on the hydrodynamics of pollen grains considering the differences in water potential was given by Jack Heslop-Harrison (1979). However, a sound description of water movements seems to be complicated by the state of the plasma membrane bilayer in the dehydrated pollen grain: transition from the lamellar/bilayer structure to a hexagonal (HII) phase occurs under specific conditions, e.g. when the water content of the pollen grains decreases during dehydration, and a lamellar structure becomes thermodynamically instable and turns into a less water-requiring hexagonal or cubic state (Gordon-Kamm and Steponkus 1984; Jouhet 2013). During the first minute, if not the first seconds of the rehydration phase, the cytoplasmic organisation of pollen membranes changes from a micellar (hexagonal) form to a hydrated, continuous bilayer structure (Elleman and Dickinson 1986; Tiwari et al. 1990). A phase transition of phospholipids from the gel to the liquid-crystalline structure was detected during imbibition of dry pollen grains (Crowe et al. 1989; Hoekstra et al. 1991). The reformation of the plasma membrane bilayer implies that a short time period exists during which pollen grains are leaky and might lose cytosolic contents or take up membrane impermeable compounds. This fact might be utilised experimentally to manipulate pollen grains from plants that cannot easily be transformed, e.g. to introduce fluorescent sensor molecules or antisense RNA (Moutinho et al. 2001; Potocky et al. 2007; Mizuta and Higashijima 2014).

After the formation of a continuous lipid bilayer, additional water is taken up across the new-built plasma membrane to allow adjustment of cytoplasmic ion concentrations (pH, Ca^{2+}, K^+, etc.), turgor pressure and metabolite concentrations. For instance, the cytoplasmic K^+ concentration has to be adjusted to allow protein translation (reviewed by Mascarenhas 1993). This early water uptake was postulated as being an initial step in pollen grain germination (Feijó et al. 1995). Although no data on these early events of water uptake are available, one may postulate that water potential inside the pollen grain is much lower than the water potential

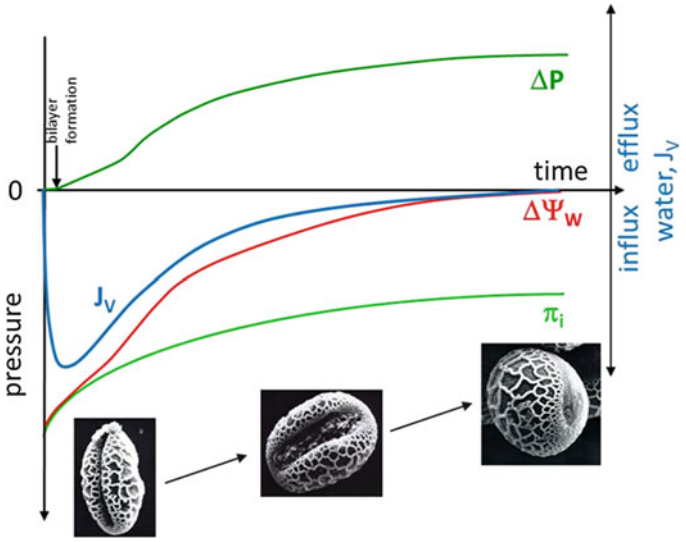


Fig. 2.5 A simple scheme of the water potential relations and parameters during pollen grain rehydration. See text for detailed explanation

in the environment or in the stigma cells which have to supply the dehydrated pollen grain with water. The following scenario might describe the events during pollen rehydration (Fig. 2.5): the mature, dry pollen grain possesses a high osmotic pressure (π_i) caused by ions and metabolites which were concentrated previously during pollen development by the dehydration process. The plasma membrane is not in its bilayer but hexagonal state. Subsequently, no turgor pressure is expected. While water is flowing into the pollen grain following the water potential difference ($\Delta\Psi$), it swells and enlarges its volume; the osmotic pressure decreases due to dilution of the cytosolic and organelle compounds. The plasma membrane transforms into a lipid bilayer, and the turgor pressure ΔP slowly increases as more and more water flows into the grain, enlarging the volume and expands the pollen wall (Katifori et al. 2010). The pollen wall limits the volume of the pollen grain, and the turgor pressure increases faster as more water flows into the grain. Water is taken up until $\Delta P = \Delta\pi$, and any further uptake water needs a decrease in turgor pressure ΔP or an increase in $\Delta\pi$. The osmotic pressure difference ($\Delta\pi$) can either be changed by increasing π_i or decreasing π_o, with both changes leading to water influx. In barley pollen, rapid swelling was observed upon contact with germination medium, and the fast water uptake was correlated with an accumulation of K^+ at the aperture inside mature pollen grains (high π_i) which was suggested to cause the fast swelling in a fraction of a second (Rehman et al. 2004). In addition, volume changes of barley pollen were also observed during pollen development at the stage of anther dehiscence which were probably caused by changes in K^+ concentrations, too (Matsui et al. 2000; Rehman and Yun 2006).

It has to be emphasised that pollen hydration does not mechanically occur when a pollen grain lands on a stigma surface. Of course, the water potential difference between the pollen and its environment is the driving force for water uptake, but whether water is taken up or not is highly regulated. For instance, control of hydration of *Brassica* pollen grains was identified as part of the self-incompatibility response (Sarker et al. 1988). Incompatible pollen grains did not take up water when placed on papillae cells, whereas compatible pollen grains swelled significantly (Dearnaley et al. 1997). On dry stigmas, like in *Brassica* and *Arabidopsis*, pollen coat components of the exine including lipids and proteins form a 'foot' between grain and stigma surface which is necessary for pollen hydration (Ruiter et al. 1997; Mayfield and Preuss 2000; Updegraff et al. 2009). In Arabidopsis, pollen hydration is further favoured by compounds synthesised by the papillar cells (Ma et al. 2012). In species with wet stigmas, e.g. tobacco, lipid compounds of the stigma exudate are involved in the uptake of water and promote pollen hydration (Wolters-Arts et al. 1998) and provide a directional clue in guiding the pollen tube through the stigma tissue (Lush et al. 1998). These studies indicate that water transport is not solely a physical aspect of water potential differences. Instead, many 'biological' factors influence and, much more importantly, regulate the water uptake during the initial phase of pollen germination.

2.4.3 Pollen Tube Elongation

Pollen tubes grow by taking up water to increase their cell volume, consequently, the tube growth rate is strictly connected to the water uptake rate. As estimated before for lily pollen tubes, a growth rate of 10 μm min^{-1} requires uptake of 1 pl water per minute. A study on the hydrodynamics of growing *Agapanthus umbellatus* pollen tubes estimated water uptake rate to be 9.33 μm^3 s^{-1} (ca. 0.56 pl min^{-1}, Malhó and Pais 1992). Again the water transport is driven by the difference between turgor and osmotic pressure ($P - RT(c_i - c_o)$, Eq. 2.4). By changing any of these parameters, the water uptake rate can be regulated, and thus, the tube growth rate may be adapted to new external conditions. Keeping in mind that pollen tube elongation can oscillate in many pollen species or sometimes shows pulses of fast growth rates alternating with slow growth periods, one might expect that at least one of the parameters (P, c_i or c_o) oscillates, too, to modulate water uptake (Messerli and Robinson 2003; Feijó et al. 2001; Zonia et al. 2006; Zerzour et al. 2009). Turgor pressure recordings in lily pollen tubes showed no oscillations, and the pressure values were independent from the tube growth rate (Benkert et al. 1997), but mechanical properties of the cell wall oscillated, e.g. cell wall softening correlated well with phases of faster tube growth (Zerzour et al. 2009). If the turgor pressure stays constant during growth rate changes as well as the external osmolyte concentration, the internal osmolyte concentration c_i (cytosolic osmotic pressure π_i) has to change to cause differential water uptake. Our knowledge on intracellular osmotic events during tube growth and growth rate oscillations are scarce, and the available data permit various

interpretations as reflected by the scientific debate on the specific role of the turgor pressure in driving tube elongation (Zonia and Munnik 2007, 2009; Winship et al. 2010, 2011). This debate illustrated the gaps in the data and in our understanding of osmotically driven tube growth. For instance, physical theory predicts that the hydrostatic pressure is equal at every location in a water-filled system, similar to a pollen tube. However, pollen tubes are filled with organelles and possess cytoplasmic streaming which may cause a bulk flow and circulation of water inside the tube, and the callose plug, even when not entirely closing the tube, may partition the tube and function as valves limiting the internal water flow and solute exchange, thus creating compartments of different osmotic conditions. In addition, it is not known whether water enters across the entire surface of the pollen grain and tube or only at restricted areas, so-called osmotic active surfaces as suggested by Sommer et al. (2008), and which were predicted in a pollen tube growth model (Hill et al. 2012). It has to be mentioned that the plasma membrane of pollen tube protoplasts had a water permeability twice as high than P_{os} of the pollen grain plasma membrane which indicated a favoured water transport across the tube's plasma membrane (Sommer et al. 2008). A locally diverse water influx might also occur if osmolyte concentrations in the cytosol change along the tube and even locally restricted water effluxes might be postulated under particular dissemination of osmolytes causing concentration gradients in the cytosol. However, this implies also a substantial bulk flow of water inside the pollen tube. Irrespectively of such putative distributions of cytosolic compounds, only little is known about quantitative changes of cytosolic osmolytes during pollen tube elongation. Again in *Lilium* pollen, a metabolome study indicated a transition from sucrose synthesis during pollen grain rehydration to sucrose degradation after pollen tube germination and during tube growth, thus increasing the cytosolic osmolyte concentration to probably boost water uptake for tube elongation (Obermeyer et al. 2013). So far, our knowledge on the role of the turgor pressure and the internal osmotic pressure as well as their regulation and their correlation with cell wall rigidity to drive pollen tube growth is still incomplete and needs much more solid data than speculations.

2.5 Osmosensing and Osmoregulation in Pollen

Water transport is essential for pollen tube growth. However, a surplus of water influx increases the turgor pressure and lets the tube burst at its weakest point, the growing tip. In lily pollen, the so-called burst pressure was almost double as high as the turgor pressure of the tube (Benkert et al. 1997). Bursting usually ends the race to the ovule for this particular pollen tube. Therefore, it seems plausible that regulatory mechanisms exist which allow the fastest possible growth while preventing the tube from bursting. Such a regulation involves several processes: firstly, the osmotic state, e.g. turgor and osmotic pressure, of the tube or grain has to be detected; secondly, the pollen needs cellular processes to adjust its osmotic state; and, finally, a kind of

feedback should be possible to adjust the turgor pressure to the current state of tube growth rates and cell wall stability.

In entire plants, water scarceness potentially threatens the survival of the individual. Therefore, several molecular mechanisms have evolved to accurately monitor the environment and very dynamically adapt the metabolism, growth and the development of plants to the new conditions (Claeys and Inzé 2013). Under water-limiting conditions, the uptake of water can be distorted if the external water potential becomes lower than the internal water potential of root cells. The entire plant responds to this drought stress by primarily arresting growth and adapting by several molecular and cellular processes, e.g. ABA biosynthesis, stomatal closure, reduction of photosynthesis, altered source–sink relations and carbon partitioning, etc. (reviewed in Bartels and Sunkar 2005; Bhargava and Sawant 2013; Krasensky and Jonak 2012). These specific adaptions may result in different degrees of drought tolerance. In general, the osmotic stress is possibly sensed by cell surface receptors. For instance, receptor-like kinases, ligand-gated ion transporters or two-component histidine kinase receptors and/or intracellular ABA receptors start a signalling cascade which probably involves phosphorylation and mobile second messengers (e.g. Ca^{2+}, pH or ROS) and, finally, regulates gene expression (Bhargava and Sawant 2013). For comparison with pollen osmoregulation, the plant's response at the cellular level is very interesting and involves osmotic adjustment by synthesis of osmotically active solutes (sugars, cyclic polyols, proline, glycine betaine) as reviewed by Krasensky and Jonak (2012) and enhanced net uptake of ions (Shabala and Lew 2002) as well as a decreased mitochondrial respiration and a modified TCA cycle to prevent the generation of excess reductants (GABA shunt, Fait et al. 2008). These stress-related cellular responses might also occur in the pollen, but it has to be kept in mind that pollen grains are quasi-unicellular organisms (one vegetative cell which surrounds one generative or two sperm cells) and, therefore, may assemble processes for osmoregulation and osmosensing like other unicellular organisms, e.g. yeast cells (Muzzey et al. 2009; Krasensky and Jonak 2012).

In a recent study, it was shown that the plasma membrane H^+ ATPase (PM H^+ ATPase) is involved in osmoregulation of pollen (Pertl et al. 2010). A hyperosmotic treatment of lily pollen grains caused an immediate decrease in turgor pressure simultaneously with a hyperpolarisation of the plasma membrane and acidification of the external medium. These responses were accompanied by an increased PM H^+ ATPase activity and an increase in binding of 14-3-3 proteins to the plasma membrane upon hyperosmotic shock allowing the uptake of external ions or osmolytes to adjust the cytosolic water potential. It has to be noted that the process of 14-3-3 binding to the C-terminus of the PM H^+ ATPase involves a phosphorylation of the penultimate threonine of the C-terminus by a still unknown protein kinase. This step is very likely part of the signal transduction pathway from the osmosensor to the PM H^+ ATPase. Consequently a number of protein kinases, especially receptor protein kinases, as well as Ca^{2+} signalling proteins directly interact with the PM H^+ ATPase in lily pollen as revealed by in vivo cross-linking (Pertl-Obermeyer et al. 2014). In yeast, the high-osmolarity glycerol (HOG) MAP kinase pathway is involved in the adaption to hyperosmotic stress

(Miermont et al. 2011) and ensures water homeostasis and volume recovery by glycerol production. Membrane sensors are activated by osmotic stress and activate a MAP kinase cascade which amplifies and transmits the osmotic signal to further cellular processes. Similarly, hydration of mature, dry tobacco pollen activated a MAP kinase which showed the highest activity at 5 min after rehydration (Wilson et al. 1997). In addition, hypoosmotic treatments affected the cytosolic Ca^{2+} concentration in lily pollen tubes (Messerli and Robinson 2003) and increased phosphatidic acid concentrations in tobacco pollen tubes (Zonia and Munnik 2004). These studies, although not primarily intended to investigate pollen osmoregulation, showed that signalling pathway and components are present in pollen to assemble an osmoregulation signalling network. Furthermore, the osmolyte metabolism may be connected via specific signal transduction pathway allowing a concerted regulation of the cellular response of pollen to changing osmotic conditions.

2.6 Challenges for the Future: Reproduction and Drought Stress

Recent prospective scenarios for agriculture forecast an increase in water consumption and at the same time, postulate a severe shortage in water for agriculture (Gilbert 2012; Okono et al. 2013; FAO webpage: http://faostat3.fao.org/home/E, http://www.fao.org/nr/water/aquastat/main/index.stm). The supply of water during the plant's life is one of the most essential parameters that control crop yield (Boyer 1982; Foley et al. 2011). Generally, drought-stressed plants are retarded in vegetative growth and development. But when drought stress occurs during the reproductive phase, the plant's fertility is affected which not only reduces the crop yield, but can also affect the next plant generation by low seed quality. Drought stress not only reduces plant fertility by disturbing gametogenesis and later on embryo development (Manjarrez-Sandoval et al. 1989; Jäger et al. 2008; Alqudah et al. 2011; Saragih et al. 2013), but, in addition, pollen germination and tube growth through the pistil tissue of drought-stressed plants are also directly inhibited (Alqudah et al. 2011). As described above, water transport plays an essential role during pollination and fertilisation. Under drought stress conditions, the plant's water potential balance may be suboptimal for high water transport rates into the pollen caused by a decrease in the water potential of stigma cells or the stigma fluid. For instance, pollen of sorghum varieties which were grown under different climatic conditions (dry post-rain season or wet rain season), differed in their ability to adapt to osmotic conditions in the style (Patil and Ravikumar 2011). Another study reported lower pollen viability and decreased yield in chickpea when drought stress was applied during the reproductive phase (Fang et al. 2010). So far, the effects of drought stress during the fertilisation phase, namely, the reduction in offspring and crop yield, have been recognised, but almost no molecular mechanisms responsible for low pollen germination frequency and distorted tube growth during drought

conditions have been identified and, more importantly, mechanisms on how the pollen responds to and circumvents the drought stress-dependent water limitation and how it adapts to different osmotic conditions to ensure water uptake have not been investigated. First steps to understand the molecular base of pollen stress response have been made (Tunc-Ozdemir et al. 2013), but a major challenge for basic plant research will be to understand how plants use water in their organs and how they adapt to water shortages to allow targeted improvements for crop plants in the future. Particularly understanding the important molecular processes of osmoregulation in pollen may help to identify 'markers' for breeding new varieties with a less drought-sensitive fertilisation process in the future.

2.7 Conclusion and Perspective

Despite the importance of successful fertilisation for maintaining crop yield under drought stress conditions, the mechanisms of water transport, osmosensing and osmoregulation as processes vital for survival of pollen grains and tube during fertilisation are not well understood. Under all circumstances, pollen grains and tubes need to take up water for the fast growth towards the ovules. On the other hand, a surplus of water uptake causes pollen tubes to burst, and, therefore, water transport has to be regulated. Although studies on pollen osmoregulation are still scarce, the growing pollen tube may present an ideal research subject to study osmotic effects, osmoregulation and also osmosensing in plants due to its very sensitive response to osmotic conditions. Understanding pollen osmoregulation might enable a targeted breeding of crop varieties with a less drought-sensitive fertilisation process to maintain high crop yields for human nutrition in a changing climate environment.

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Chapter 3
The Cytoskeleton of Pollen Tubes and How It Determines the Physico-mechanical Properties of Cell Wall

Giampiero Cai, Luigi Parrotta, and Mauro Cresti

Abstract The growth of pollen tubes is a complex process that requires the synchronized activity of many different factors. Pollen tubes grow by penetrating through relatively solid tissues of the pistil. In doing so, pollen tubes need a specialized shape consisting of a tubular axis culminating with a hemispherical dome. In order to maintain such a shape, pollen tubes must build a dynamic cell wall which is highly adapted to the cell's penetrating activity. Therefore, the molecular mechanism controlling the pollen tube architecture is critical. In growing pollen tubes, the cytoskeleton controls the intracellular transport of organelles and vesicles. Movement of membrane-bounded structures is necessary for the apex-constrained growth of pollen tubes and for proper assembly of the cell wall. This process is strictly related to the fine-tuned deposition of specific proteins and polysaccharides, which contribute to local differentiation of cell wall texture and thus to the growth pattern of pollen tubes. This chapter will focus on the molecular relationships between cytoskeleton and cell wall deposition in pollen tubes in order to highlight how the cytoskeleton controls the shaping of pollen tubes.

Keywords Pollen tube • Cytoskeleton • Cell wall • Directional growth • Membrane trafficking

Abbreviations

ADF	Actin-depolymerizing factors
AGP	Arabinogalactan proteins

G. Cai (✉) • M. Cresti
Dipartimento di Scienze della Vita, University of Siena, via Mattioli 4, 53100 Siena, Italy
e-mail: giampiero.cai@unisi.it; mauro.cresti@unisi.it

L. Parrotta
Dipartimento di Scienze Biologiche, Geologiche e Ambientali, via Irnerio 42, 40126 Bologna, Italy
e-mail: luigi.parrotta@unibo.it

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CDPK	Calcium-dependent protein kinase
GFP	Green fluorescence protein
IP_3	Inositol 1,4,5-trisphosphate
MAP	Microtubule-associated protein
MASC	Microtubule-associated cellulose synthase compartment
PME	Pectin methyl esterase
ROP	Rho of plants
ROS	Reactive oxygen species
SmaCC	Small CESA compartment

3.1 Introduction

The growth of pollen tubes through stigma and styles of receptive flowers is a complex biological process by which pollen tubes accomplish their function (i.e., sexual plant reproduction). The mechanism by which pollen tubes penetrate the pistil also depends on the nature and cytomolecular structure of pistil cells. Consequently, to promote the growth process, pollen tubes must be equipped with a high mechanical strength allowing cells to actively and rapidly grow. The growth of pollen tubes is not "blindly," but it must follow a very specific set of extracellular signals by which pollen tubes grow precisely to the ovary, thereby depositing male gametes in the immediate vicinity of egg cells. The ability of pollen tubes to invade the tissues of pistils may depend on two major factors. On one hand, pollen tubes might hypothetically secrete enzymes capable of weakening the cell wall of pistil cells, thereby allowing pollen tubes to grow within a softer environment. On the other hand, an internal driving force can allow pollen tubes to penetrate the extracellular matrix. This force may depend on two key features: the turgor pressure and the mechanical resistance of cell wall. The two features (turgor and cell wall) are not totally independent, but a precise balance between the two determines the rate of pollen tube growth as well as the ability to invade extracellular substrates. Current models concerning pollen tube growth claim that turgor pressure is relatively constant and unchangeable during the growth process (Winship et al. 2010, 2011). However, we must acknowledge that alternative models of growth claim that turgor pressure can vary in pollen tubes and that oscillation of hydrostatic values would contribute to the growth process (Zonia and Munnik 2011, Chap. 2). The rate of cellular turgor is connected to an external flow of water into pollen tubes, which takes place essentially at the pollen tube apex and that is finely controlled by ion fluxes occurring both at the apex and in the shank of pollen tubes. As pollen tubes grow, the hypothesized steadiness of turgor pressure is maintained through the synthesis of presumably "watertight doors" (callose plugs, Cresti and van Went 1976) that seal progressively older regions of pollen tubes, thereby pushing active cytoplasm closer to the tube apex. In any case, it must be recalled that growth of pollen tubes (and consequently deposition of the cell wall) depends on the structure of the extracellular environment. For example, plant species

whose style is solid (such as *Solanum lycopersicum*) are characterized by pollen tubes exhibiting a discontinuous pectinaceous cell wall (i.e., the presence of regular pectin deposition, the so-called ring). In contrast, plant species with hollow style (such as *Lilium longiflorum*) have pollen tubes with more uniform cell wall and thus with more regular growth (Li et al. 1994, 1995a, 1996).

The pollen tube cell wall is not homogenous along the growth axis and is also highly dynamic. Its composition can vary significantly from one species to another. In addition, the precise composition of cell wall at any given point of the cell may change due to programmed growth events or after various stresses. The relative composition of the cell wall (briefly discussed below) is closely connected to a dual assembly process. A first event, secretion, brings noncrystalline polysaccharides (mainly pectins and xyloglucans) in the cell wall. A second event, synthesis, leads to deposition of either crystalline components of the cell wall (cellulose, β-1,4-glucan) or noncrystalline but highly resistant components (callose, β-1,3-glucan, Mollet et al. 2013). Both processes of secretion and synthesis are supported, directed, and controlled by the activity of cytoskeleton, a network of highly dynamic three-dimensional polymeric as well as monomeric proteins that permeates the entire pollen tube cytoplasm (Fu 2015). Polymeric cytoskeletal proteins (actin filaments and microtubules), supported by a heterogeneous set of regulatory and functional proteins, determine both the fusion rate of pectin-containing secretory vesicles and the rate and localization of cellulose/callose synthesis.

In this chapter, we will discuss the dynamics of pollen tube cytoskeleton in relation to the different composition and strength/softness of cell wall. A proper assembly and strength of cell wall are vital for pollen tubes to grow appropriately through the style so to allow the reproductive success of plants.

3.2 The Cytoskeleton of Pollen Tubes

Like any other plant cell, pollen tubes possess an extensive cytoskeletal apparatus. It consists of actin filaments and microtubules. Both actin filaments and microtubules extend throughout the length of pollen tubes although they show some differences. Unfortunately, quantity of the data currently available is much abundant for actin filaments than for microtubules. This is mainly due to the fact that actin filaments have been also studied in vivo using selective probes or fluorescently labelled proteins. In fact, the data available for microtubules arise solely from the use of antibodies tested on fixed cells. Despite the level of knowledge, it seems clear that actin filaments and microtubules show peculiar configurations in pollen tubes. For example, microtubules are characterized by a longitudinal distribution along the growth axis unlike other plant cells. Actin filaments are characterized by specific arrays, such as the actin fringe, localized between the apex and subapex of pollen tubes. It is assumed that the above patterns are strongly linked to the growth mechanism of pollen tubes, which imposes limits on the distribution of cytoskeletal filaments.

3.2.1 Actin Filaments

The mechanism of tip growth involves the actin filament-based trafficking and accumulation of secretory vesicles at the pollen tube apex. Therefore, actin filaments are arranged in such a way as to facilitate the transport of vesicles toward the apex. Distal areas (the shank) of pollen tubes contain extensive actin bundles in which individual actin filaments are arranged next to each other to create structures capable of supporting organelle and vesicle trafficking (Fig. 3.1c, actin panel). However, it is at the apex that we note key differences with other plant cell systems. The actin fringe is a protein structure that can be relatively extended depending on the species analyzed and on the growth rate. Nevertheless, it is supposed to be essential because it most likely serves to focus secretory vesicles in the apical area. Another peculiarity is represented by a set of short, highly dynamic, actin filaments immediately below the pollen tube tip. It is believed that this pool is in between monomeric actin and newly structured actin filaments that culminate in the actin fringe (Qu et al. 2015).

Therefore, it can be roughly assumed the presence of three distinct subgroups of actin (longitudinal cables, short apical actin filaments, and actin fringe) and that they are balanced to each other. The transition from one state to another is finely regulated by a range of factors (including ions, ROS, proteins). This molecular network allows the cell to detect external and internal information and to modulate the construction of actin filaments in order to transport organelles and vesicles in coordination with speed and growth direction. For example, the overall extension of actin fringe seems to be directly proportional to the growth rate (Dong et al. 2012), while the greater or lesser lateral extension of actin fringe seems to be correlated to the growth direction (Kroeger et al. 2009). Consequently, ultimate transport of pectin-containing vesicles and delivery of pectins via exocytosis are likely controlled by the actin fringe. Using different growth inhibitors, it was possible to degrade the actin fringe inducing the formation of actin aggregates that slow down growth but allow pectin deposition. However, deposition of pectin was observed in a wide arc in the pollen tube tip suggesting that actin fringe contributes to the targeted secretion of pectins in the cell wall and, therefore, to the polarized growth of pollen tubes (Rounds et al. 2014).

3.2.2 Actin-Binding Proteins and Actin-Based Motors: How Do They Affect Cell Wall Synthesis?

Several studies proved that actin filaments are fundamental in the process leading to cell wall deposition in pollen tubes. Almost all of these studies indicate that actin filaments have a critical role in the process of pollen tube growth; even the partial depolymerization of these cytoskeletal elements inevitably leads to termination of growth. This is not surprising because actin filaments have a fundamental role in all plant cells as they are the tracks along which the various classes of organelles

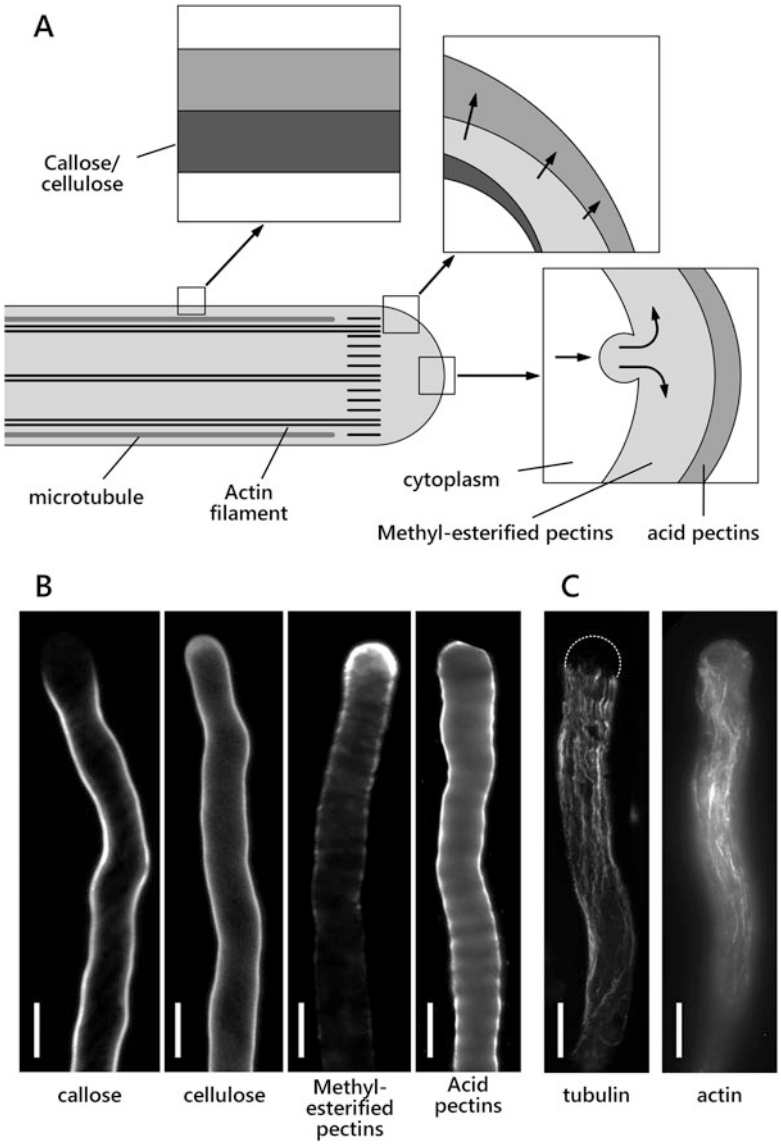

Fig. 3.1 Dynamics of cell wall in pollen tubes and distribution of cytoskeletal components. (**a**) Cartoon showing how the cell wall changes during pollen tube growth. In the tip, the cell wall mainly contains newly secreted methyl-esterified pectins and acid pectins, whose ratio varies according to the growth rate. In the immediate vicinity of the apex, PME converts methyl-esterified pectins into acid pectins, thereby stiffening the cell wall. In the pollen tube shanks, an additional layer of callose and cellulose reinforces the cell wall texture, thus maintaining the tubular shape of pollen tubes. (**b**) Distribution of main cell wall components as revealed by fluorescence microscopy: pectins, callose, and cellulose are shown. (**c**) Distribution of microtubules and actin filaments in pollen tubes as revealed by specific fluorochrome-tagged probes. In the case of tubulin, the apex is indicated by dotted line. *Bars*, 10 μm. Photographs courtesy of Mrs. Claudia Faleri (Department of Life Sciences, University of Siena)

and vesicles move, including the membranous structures responsible for cell wall deposition. Assembly of actin filaments is an elaborate process that is dependent on the presence and activity of several accessory proteins, generally referred to as "actin-binding proteins" (Staiger et al. 2010). These proteins regulate the building of very different actin structures; a particular group of actin-interacting proteins, the so-called motor proteins (Peremyslov et al. 2011), of which myosin is the only representative, are directly involved in the transport of proteins and lipids within the cell. It is not the aim of this review to provide details on the variety of actin-binding proteins in pollen tubes; nevertheless, it is important to give some information on how actin-interacting proteins modulate cell wall deposition.

The cell wall at the pollen tube apex is very dynamic and contains variable levels of methyl-esterified pectins and acid pectins. It is assumed that the dynamics of the apical region is strongly dependent on the presence of a poorly defined actin structure immediately below the apex. Dynamics involves a delicate balance between actin monomers and polymerized actin. Therefore, this region is characterized by the presence of proteins that prevent the assembly of long actin filaments but favor short actin structures. At this level, a critical role is initially played by proteins that interface external signals with intracellular events. Among these, Rho-of-plant (Rop) proteins are undoubtedly an important regulator; in turn, Rop proteins can interface directly with RIC3 proteins, which determine a local increase of calcium ions (Zhao and Ren 2006). The latter are recognized as one of the main factors capable of modulating the assembly of actin filaments; in fact, a local increase of calcium ions leads to depolymerization of actin filaments (Cardenas et al. 2008). However, the picture is not as simple as that because other proteins participate in the regulation of calcium levels, including phospholipase C by releasing IP_3 (Dowd et al. 2006) and NAD(P)H oxidase, which regulates intracellular levels of ROS (Lassig et al. 2014).

Calcium ions are a central controller of actin filaments because they can trigger a variety of proteins involved in regulating actin filament dynamics. Among these, we can mention calmodulin, CDPK, profilin, and the villin/gelsolin group (which also includes ABP29/41/80). In particular, profilin and the villin/gelsolin group are activated by calcium ions, and, as such, they have a depolymerizing effect on actin filaments. In this way, they determine the formation of short randomly arranged filaments immediately below the tip. This local condition is most likely favored by low pH, which usually characterizes the pollen tube apex (Ren and Xiang 2007).

Immediately after the apex (between apex and subapex), local conditions change rapidly, and the most drastic change is the appearance of the alkaline band, which is a rapid increase in intracellular pH (Feijò et al. 1999). The alkaline band could positively regulate a group of proteins known as ADF (actin-depolymerizing factors) that determine the formation of several polymerizing actin ends (Chen et al. 2002). These new local conditions are probably responsible for the production and regulation of the actin fringe, which could be additionally stabilized by proteins such as fimbrins (Su et al. 2012). It is likely that, more or less at this level, new actin filaments are also generated by plasma membrane proteins, the so-called formins (Cheung et al. 2010).

On the opposite side of the actin fringe, different proteins start to build long actin filaments. Among these, villin is a protein that cross-bridges parallel actin filaments (Qu et al. 2013). This protein is negatively regulated by calcium ions; therefore, its activity is low in the apical region. Another important protein is LILIM1, which shows an optimum of activity at low pH (6.25) and is inhibited by high pH, e.g., alkaline band, and very acidic pH as well as by high concentrations of calcium ions (Wang et al. 2008). In addition, it is also worth mentioning SB401 (Huang et al. 2007). Therefore, different and localized concentrations of ions and specific proteins contribute to building different actin structures, each with a specific role. Consequently, trafficking of secretory vesicles carrying enzymes and polysaccharides for cell wall assembly depends strongly on the dynamics of actin filaments. In this context, myosins have a well-defined role because they are the motor by which the vesicle-enclosed material moves along actin filaments. The activity of myosin is most likely regulated by calcium ions (Yokota et al. 1999). Unfortunately, knowledge of these regulatory mechanisms is still very poor, and it is therefore difficult to suggest a reliable model.

3.2.3 Microtubules

As mentioned above, the organization of microtubules in pollen tubes is much less known. Generally, microtubules are described as longitudinal bundles arranged along the growth axis (Fig. 3.1c, tubulin panel). Microtubules appear to be absent in apical and subapical regions of pollen tubes (Onelli et al. 2015). However, this depends on the techniques used to visualize them. In fact, chemical fixation techniques required for immunolocalization could affect the organization and dynamics of microtubules, especially in regions where they are more delicate. A better description by in vivo techniques is therefore required. In addition, we do not have the necessary information concerning the dynamics of microtubules and the proteins that participate in the regulation of microtubule dynamics. The scarcity of available information does not facilitate understanding the role of microtubules in pollen tubes. The situation is complicated by evidence that microtubule inhibitors have no particular effects on pollen tube growth (Lovy-Wheeler et al. 2007).

When visualized by means of immunolocalization techniques, microtubules are easily observed as longitudinal bundles. They seem to be more present in the cortical region of pollen tubes where, according to models accepted in somatic cells, they may participate in the control of cell wall deposition. Nevertheless, it must be considered that microtubules are poorly present, if not completely absent, in the apical region. Consequently, the role of microtubules in cell wall deposition must be carefully evaluated (Cai 2011). Recent data using specific inhibitors suggest a role for microtubules in the process of endocytosis. This possibility seems effective for the internalization of S-RNase during self-incompatibility (Meng et al. 2014). In addition, data of plasma membrane invagination suggested that microtubules play a part (although not defined) in the commencement of endocytosis (Idilli et al. 2013).

How this function is connected to the regulation of cell wall deposition is unclear. As described later, endocytotic events might be required to dose accurately the content of specific enzymes that synthesize cell wall components. Another example of indirect relationship between microtubules and cell wall is the deregulation of deposition of callose plugs (see below) as induced by microtubule inhibitors (Laitiainen et al. 2002).

3.2.4 Microtubule-Associated Proteins that May Take Part in Cell Wall Deposition

Because the exact function of microtubules in pollen tubes is still unclear, the portrait of proteins that interact with microtubules is quite limited. Consequently, the scarcity of information on the function of microtubules prompted researchers to characterize proteins interacting with microtubules, with the hope that such identification sheds light on the role of microtubules.

Most of the available information is related to microtubule-associated proteins with motor capacities. At least two to three types of kinesins have been biochemically identified in pollen tubes. Two of these kinesins of 90 and 100 kDa in molecular weight were localized in association with Golgi vesicles, suggesting that they are involved in the transport of secretory vesicles (Cai and Cresti 2010). Because the speed of transport produced by kinesins as deduced by in vitro motility assays is considerably lower than the actual velocity observed in pollen tubes, it follows that these molecular motors are involved in slower but perhaps more accurate processes. A recognized but not yet sufficiently established model proposes that, in pollen tubes as well as in other plant cells, microtubule-based motors transport vesicles and organelles for short distances, slowly but precisely. Some of these motors may be involved in the transport or localization of the enzyme complexes that synthesize cellulose. Alternatively, they could carry molecules necessary for the correct assembly of cellulose microfibrils. As an example, we can mention a kinesin belonging to subfamily 4. This kinesin (called FRA1) is not likely involved in direct transport of cellulose synthase; on the contrary, it could carry pectic material required for proper cell wall structure (Zhu et al. 2015). In support of this hypothesis, proper secretion of pectins is essential for the assembly of cellulose microfibrils (Yoneda et al. 2010). In cotton as well as in Arabidopsis, the kinesin FRA1 or its homologs are hypothesized to transport pectin material required to control the rate of elasticity of cell walls; this allows an appropriate ratio between cellulose and pectin in order to build a cell wall suited to be deformed during cell growth (Kong et al. 2015). The current state of knowledge does not yet support this hypothesis in pollen tubes; nevertheless, this is a fascinating hypothesis.

Although a number of microtubule-associated proteins have been identified in plant cells, their characterization in pollen tubes is very limited. If we exclude the biochemical and immunological identification of proteins connecting cortical

microtubules to the plasma membrane (Cai et al. 2005), proteins functionally related to well-known CLASP, MOR1, EB1, AtMAP70, MAP65, and MAP18 (Hamada 2007) have not yet been identified. However, microtubules in the cortical region of pollen tubes are arranged in bundles oriented along the pollen tube growth axis. This organization implies at least the presence of proteins capable of bundling microtubules. How this organization is necessary for the proper growth of pollen tubes is questionable since every chemical substance capable of depolymerizing microtubules has no significant effects on growth. For all we know, the relationship between longitudinal arrangement of microtubules and cell wall deposition in pollen tubes is unknown and therefore represents an interesting research area.

3.3 Synthesis and Deposition of the Cell Wall in Pollen Tubes

The cell wall of pollen tubes is a complex structure comprising polysaccharides, proteins, glycoproteins, and other relatively small molecules that define not only the structure but also the function of the cell wall. Being a polarized growing cell, it follows that the tube's cell wall is organized to support dynamically the growth process. Maintaining a cylindrical shape is essential for pollen tubes so that they can grow through the stigma and style of receptive flowers. Therefore, the cell wall of pollen tubes has a polarized structure, more elastic at the apex and progressively stiffer toward the pollen grain. Diversification of cell wall structure is maintained by sequential deposition of different polysaccharides that provide progressively higher strength levels (Fig. 3.1). Like in other plant cells, cell wall deposition in pollen tubes requires the secretion of specific polysaccharides and glycoproteins and the in situ synthesis of other polysaccharides at the plasma membrane level. Both processes are controlled by the cytoskeleton albeit partially different.

3.3.1 Secretion of Cell Wall Components: Pectins, Arabinogalactan Proteins, and the Cytoskeleton

In pollen tubes, pectins are mainly deposited at the apex as methyl-esterified pectins (Bosch and Hepler 2005; Li et al. 1994, 1995b). Shortly after, pectins are converted in their acidic form allowing tight cross-linking with calcium ions. This process leads to the progressive strengthening of cell wall and maintains the cylindrical shape of pollen tubes (Zerzour et al. 2009). Because pectins are secreted at the apex, it is supposed that this process takes place through the transport of pectin-containing vesicles along actin filaments. That distribution of pectins and thus the degree of cell wall stiffness are closely related to the cytoskeleton organization was already suggested (Geitmann and Parre 2004). This close relationship was later proven by experiments with proteasome inhibitors, such as MG132 and epoxomicin.

These substances showed that damages to cytoskeleton have a counterpart in reduction of cytoplasmic streaming and consequently in deposition of pectin and cellulose (Sheng et al. 2006). As already stated, the relationship between pectin deposition and cytoskeleton could be more complex and might involve cellulose. Inhibition of cellulose synthesis in Arabidopsis roots by 2.6-dichlorobenzonitrile caused alterations in the organization of microtubule but not of actin filaments. At the same time, the distribution of pectin is also altered suggesting that cellulose can act as a template for the correct deposition of pectin (Peng et al. 2013). Similar effects have also been observed and described in *Pinus bungeana* Zucc. pollen tubes (Hao et al. 2013).

Arabinogalactan proteins (AGPs) are a heterogeneous class of glycoproteins with a high degree of glycosylation. They have numerous functions, from reproduction to morphogenesis. Therefore, it is not surprising that they are also important in the mechanism of pollen tube growth. In this context, it is supposed that AGPs are involved in controlling the direction of pollen tube growth, probably by acting as biochemical signals through which pollen tubes communicate with pistils (Nguema-Ona et al. 2012). Being highly diversified molecules, distribution of AGP was most often analyzed by specific antibodies directed against often known (but sometimes unknown) epitopes. Using antibodies, different kinds of AGPs were localized in pollen tubes, in pollen grains, and also in generative cells. Collectively, these data suggest that AGPs of pollen tubes are important in mediating the growth process, probably by acting as recognition molecules between male and female parts (Showalter 2001; Tan et al. 2012).

AGPs are most likely secreted by conventional secretory mechanisms although the lack of information does not allow excluding additional scenarios. Because their deposition pattern is critical, it is likely that secretion or insertion in the plasma membrane is precisely driven by the cytoskeleton. In addition, available data suggest further levels of interaction between the cytoskeleton and AGPs. Data on mutant Arabidopsis roots indicated the existence of strong correlations between AGP distribution and microtubule organization, thereby suggesting that the organization of microtubules may also depend on the pattern of AGP (Andeme-Onzighi et al. 2002). Dependency of microtubules on AGP is also attested by the use of chemicals such as Yariv reagent or antibodies against AGP (Nguema-Ona et al. 2007). Comparable analysis with cytoskeleton-depolymerizing agents and Yariv reagent (which binds and perturbs AGP) allowed determining the role of AGP as linker between plasma membrane and cytoskeleton; in fact, Yariv treatment caused depolymerization or disorganization of microtubules. In parallel, organization of actin filaments was compromised. At the same time, treatment with amiprophosmethyl and cytochalasin D, inhibitors of the tubulin and actin cytoskeleton, respectively, induced reallocation of AGP, indicating that microtubules and actin filaments have a role in organizing AGP on the cell surface (Sardar et al. 2006). The effect of microtubules on the distribution of AGP may, however, be indirect and mediated by proper deposition of cellulose fibrils (Domozych et al. 2014). In pollen tubes, it is known that Yariv reagent reduces the growth rate and that this effect is reversible (Mollet et al. 2002; Qin et al. 2007). However, the impact on the cytoskeleton is not known.

3.3.2 *Plasma Membrane-Localized Synthesis of Cell Wall Polysaccharides*

In all plant cells, two mechanisms are basically used to build the cell wall. Along with a process of secretion, which is used to secrete pectins and xyloglucans in cell wall, a critical step is the local synthesis of polysaccharides. In situ synthesis of polysaccharides generally takes place when their synthesis within intracellular membranes is incompatible. This may be related to the necessity of coexisting small secretory vesicles and molecules capable of assembling into crystalline or rigid patterns. Either polysaccharides have the propensity to form crystalline structures or they are otherwise inconsistent with shape and size of secretory vesicles, it follows that synthesis and assembly of polysaccharides must necessarily take place outside the cellular space, that is, at the plasma membrane or directly in cell wall.

Another reason why secretion of specific polysaccharides should take place in situ is the control of the process. If synthesis takes place within intracellular membranes, the length and shape of polysaccharides are predetermined before secretion. Possibly, the polysaccharide chain can be extended subsequently, just when polysaccharides are secreted in the cell wall. Therefore, the secretion process can only regulate the amount of material deposited outside. It follows that the site of secretion depends on the transport of secretory vesicles and thereby on the organization and activity of the cytoskeleton. Any attempt to change the structure of secreted polysaccharides depends on concomitant activity and secretion of enzymes capable of altering the structure of polysaccharides or on the presence of additional components. For example, post-secretory modification of pectins depends on the activity of enzymes, such as pectin methyl esterase (PME) (Pelloux et al. 2007), while the assembly level depends on the relative concentration of calcium ions (Gu and Nielsen 2013).

In situ synthesis of polysaccharides (cellulose and callose) has a number of undoubted advantages. First of all, polysaccharides can be synthesized in an extremely localized manner. This effect is achieved by insertion of synthesizing enzymes at specific sites. In pollen tubes, the insertion site is the apical plasma membrane, according to data currently available in the literature (Brownfield et al. 2008; Cai et al. 2011; Wang et al. 2011). A second advantage is represented by the fact that enzymes, once inserted into the plasma membrane, could be subjected to regulation by both internal factors (e.g., availability of substrates) and external factors (e.g., signals from other cells) that can affect the way polysaccharides are deposited (Lei et al. 2012).

In the pollen tube, cellulose and callose are present along the growth axis but with some significant differences. The main difference is definitely the relative amount. Generally, callose is much more abundant than cellulose. Although the amount depends on the species, it is believed that callose is at least three to four times more abundant than cellulose (Geitmann and Steer 2006) as it accounts for 80% of the secondary cell wall. Generally, callose is a marginal component of plant cell walls being synthesized only under special conditions, such as biotic or

abiotic stress, or during the separation of microspores (Ellinger and Voigt 2014; Shi et al. 2015). The high amount of callose in pollen tubes of angiosperms is most likely related to the high growth rate of these cells compared, for example, to that of gymnosperm pollen tubes (Abercrombie et al. 2011). Theoretically, a higher turgor pressure inside pollen tubes could be related to an increased growth rate, but the available data are not always supporting this hypothesis (Benkert et al. 1997). Nevertheless, turgor pressure should be counterbalanced by the presence of stiff cell walls. The increase in stiffness can be achieved through deposition of callose, which confers a considerable mechanical resistance to cell walls and helps maintaining the cylindrical shape of pollen tubes, which is necessary to allow pollen tubes to grow through the pistil tissues. Callose also supposedly makes the cell walls of pollen tubes more impermeable to water flow, thereby forcing water to enter the pollen tube at the apical growing region only (Parre and Geitmann 2005).

Callose is a polymer whose function does not depend on a crystalline nature. Therefore, it exerts its function as soon as it is produced. For this reason, it is absent from the apex. Indeed, in cases where it is present, pollen tube growth is greatly reduced or even blocked. The presence of callose in cell wall of pollen tubes can be defined as a kind of band surrounding the post-apical region of pollen tubes. This callose sheath has most likely the function to strengthen the cell wall and then to counteract the turgor pressure, which in turn can deform only the apical cell wall (Parre and Geitmann 2005).

Although cellulose is relatively less abundant than callose, it does not have marginal roles. In fact, the few experimental data available indicate that the absence or removal of cellulose from pollen tube cell walls may impact profoundly on geometry and then on growth of pollen tubes (Aouar et al. 2010). Therefore, the two polysaccharides do not play overlapping roles; this is also indicated by their different deposition pattern, especially in the apical region. In many cases, cellulose is present in the apical region with an intensity comparable to that observed in distal regions of pollen tubes. On the contrary, callose is absent in the apical region and can be observed only 10–20 μm from the apex (Chebli et al. 2012; Meikle et al. 1991).

Because the apex of pollen tubes is the growth site, it follows that the presence of cellulose does not impact negatively on growth. The available data do not allow to understand if levels of cellulose at the apex are either constant or, like other molecules, oscillate according to the growth rate of pollen tubes. In any case, apical cellulose must be structured in such a way as to promote pollen tube growth. There may be a form of disorderly organized cellulose where individual strands have not yet been assembled into more consistent cellulose fibrils. This may depend on the lack of appropriate organizational elements or on incorrect organization of proteins (cellulose synthase) responsible for cellulose synthesis. A disorganized arrangement of cellulose synthase could be explained by a poorly arranged cytoskeleton below the apical plasma membrane (Qu et al. 2013).

3.3.3 Cytoskeleton-Based Delivery of Glucan Synthases

The insertion route of callose synthase in the plasma membrane of pollen tubes is not yet clear. Subcellular fractionation experiments showed that the enzyme is conveyed through Golgi membranes, presumably secretory vesicles, which carry the enzyme to the cell surface (Brownfield et al. 2008). Because Golgi-derived secretory vesicles are transported along actin filaments, we assume that actin filaments play a key role in transporting callose synthase to the plasma membrane. Here, the insertion site is not yet clearly identified. Immunolocalization analysis with an antibody to callose synthase suggested that the enzyme is inserted in the apical plasma membrane. This pattern is definitely linked to actin filaments because both actin and myosin inhibitors are capable of significantly altering the apical distribution scheme (Cai et al. 2011). If we accept that callose synthase is inserted at the pollen tube apex, then we must postulate that the enzyme is delivered as non-active. In fact, callose is scarcely present at the pollen tube tip because the presence of callose at the apex would lead to strengthening of the cell wall, thereby preventing distension of pollen tubes and leading to growth arrest. Evidence that callose synthase is inserted into the plasma membrane as a latent form derive from enzymatic analysis in which callose synthase is activated by proteolytic treatments (Li et al. 1999). These observations suggest that a possible way of activating callose synthase is by removing a protein fragment once the enzyme is in the plasma membrane. The hypothetical activation of callose synthase in situ (i.e., in plasma membranes) is also suggested by observation of GFP-fused CalS-5 (which is most probably the only active callose synthase during pollen tube growth) while trafficking in BY-2 cells. In that case, CalS-5 was detected both in Golgi membranes and plasma membranes, but callose was observed only outside the plasma membrane (Xie et al. 2012).

It is unclear how the activity of callose synthase is regulated once it is inserted in the plasma membrane. Assuming that a proteolytic event removes an inhibitory peptide at the boundary between apex and subapex, it is not clear whether the enzyme works exclusively at that site or if its activity is also required in distal regions. Fluorescence images with specific callose probes suggest that this polymer is relatively homogenous in thickness along the axis of pollen tube growth (Chebli et al. 2012; Parre and Geitmann 2005). Therefore, we can assume that callose synthase is activated immediately after the apex, it starts synthesizing callose, and it is subsequently removed or deactivated so as not to produce excessive callose. Data on a possible endocytotic removal of callose synthase are missing. Immunolocalization images suggest that callose synthase accumulates at the apex and subapex of pollen tubes but it is absent or scarcely present immediately after this region (Cai et al. 2011). Therefore, the hypothesis of removal by endocytosis is suggestive but not proven. Data after the use of cytoskeleton or membrane trafficking inhibitors showed no accumulation of callose synthase beyond the subapex, which suggests that, at present, we have no knowledge of possible endocytosis of callose synthase. In addition, depolymerization of actin filaments or microtubules by specific inhibitors does not appear to significantly alter the thickness of callose (Cai et al. 2011;

Laitiainen et al. 2002), indicating that cytoskeletal filaments do not have a regulatory role on callose after it is deposited. Thickness control of callose should therefore takes place at the level of subapex.

Although the callose layer appears relatively uniform along pollen tubes, images obtained with the technique of cryo-field emission scanning electron microscopy showed that callose is deposited in a pattern reminiscent of the ringlike deposition of pectins (Derksen et al. 2011). It is possible that this pattern is the result of the secretion process of callose synthase and of its removal/deactivation. It is also conceivable that the oscillating profile of callose is dependent on the deposition process of pectins, which could act as a "primer" on which callose is deposited.

Distribution of callose is not uniform for the whole pollen tube. After a few hours of growth (depending on the species analyzed), one can observe the formation of so-called callose plugs, centripetal invaginations of the plasma membrane produced by abnormal growth of callose (Cresti and van Went 1976). The process culminates with physical isolation of different portions of pollen tubes. Thus, older regions are progressively separated from still active portions. This allows the cell to save energy and probably to maintain a constant turgor pressure. The invention of callose plugs represents an important evolutionary step, most likely correlated with the increase in growth speed of pollen tubes as observed in angiosperms (Qin et al. 2012; Williams 2008). The deposition of callose plugs is probably under the control of the cytoskeletal apparatus. Depolymerization of microtubules by inhibitors, such as oryzalin, leads to a significant alteration of the deposition pattern, which results in a temporally incorrect deposition but not in the absence of callose plugs. This suggests that microtubules could control either the timing or the site of callose plug deposition rather than activation of callose synthase. The correct deposition of callose plugs also seems to rely on the activity of H^+-ATPase in plasma membrane (Certal et al. 2008). However, it is not clear how the flow of protons may affect the enzymatic activity of callose synthase. Immunolocalization data indicate that callose synthase is also deposited in distal regions of pollen tubes (Cai et al. 2011); the insertion area is relatively large and seems to depend on the correct organization of microtubules. Therefore, it can be assumed that microtubules participate in the insertion of callose synthase in larger areas within which callose plugs will form. A more focused proton flux might trigger synthesis and deposition of callose plugs according to two hypotheses: either the H^+-ATPase locally increases the insertion rate of callose synthase or it locally activates callose synthase.

Unlike callose synthase, the amount of information about cellulose synthase in pollen tubes is relatively scarce. This is likely due to the fact that callose is a polysaccharide more abundant in the pollen tube cell wall and its function has been subjected to deeper studies. Consequently, callose and callose synthase have been investigated by many laboratories. On the other hand, articles related to cellulose synthase are very few. Nevertheless, information on cellulose synthase in other plant cells are conspicuous, and this allows for a number of hypotheses. In somatic cells, cellulose synthase is delivered to the plasma membrane by vesicular trafficking along actin filaments. This allows for the long movement of cellulose synthase, while a short but precise delivery of the enzyme is likely due to microtubules

(Crowell et al. 2010). It is proposed that microtubules take part in two distinct but critical steps. The first is delivery, while the second is removal from the plasma membrane by endocytosis. In both cases, microtubules likely cooperate with specialized membrane compartments known as SmaCC or MASC (Crowell et al. 2009; Gutierrez et al. 2009). This compartment is likely a kind of reservoir of cellulose synthase by which the enzyme is rapidly inserted into the plasma membrane when and where needed. The availability of cellulose synthase gene sequences allowed for direct visualization of the enzyme in living cells, thus establishing the real relationship with microtubules (Paredez et al. 2006).

In pollen tubes, available information has been obtained by immunocytochemistry (Cai et al. 2011) and by using GFP-tagged proteins belonging to the cellulose synthase-like subfamily (Wang et al. 2011). In both cases, enzymes were mainly found in the apex of pollen tubes, likely in association with secretory vesicles. This finding is in agreement with the hypothesis that cellulose synthase (or cellulose synthase-like proteins) is delivered by Golgi-derived membranes that move actively along actin filaments. In support of this, treatment with actin inhibitors revealed that actin is critical for the precise positioning of the enzyme (Cai et al. 2011). On the other hand, the relationship with microtubules is unclear because microtubules do not appear organized at the pollen tube apex. In addition, treatment with microtubule inhibitor did not alter the distribution of cellulose synthase (Cai et al. 2011). Therefore, the relationship between microtubules and cellulose synthase in pollen tubes must be still clarified. Disorganization of cellulose can alter the shape of pollen tubes, thus suggesting that cellulose might be critical for pollen tube shaping (Aouar et al. 2010). By contrast, drug-induced alteration of microtubules does not affect pollen tube shape (Cai et al. 2011). This evidence might indicate that a strong relationship between microtubules and cellulose does not exist at least under the experimental conditions used to test this hypothesis. For example, in vivo analysis, or more precisely *in planta* analysis, might provide additional information. Evidence of the presence of different cellulose synthase-like proteins in pollen tubes (Doblin et al. 2001; Goubet et al. 2003; Wang et al. 2011) suggests that a "real" cellulose synthase might not be required and that a crystalline array of cellulose could not be necessary. This finding also indicates that the pattern of cellulose in the pollen tube cell wall might not be comparable to that of other plant cells.

3.4 Regulation of Cell Wall Synthesis Via Sucrose-Metabolizing Enzymes

Sucrose synthase is a fundamental enzyme in the metabolism of plant cells. In fact, together with invertase, it participates in the degradation of sucrose; this process provides monosaccharides used for energy production (Kleczkowski et al. 2010). It is very well known that invertase and sucrose synthase do not perform exactly the same function. While invertase breaks down sucrose into glucose

and fructose thereby producing molecules that can be directed toward respiration, sucrose synthase breaks down sucrose into fructose and UDP-glucose. The chemical bond between glucose and UDP maintains much of the energy present in the original sucrose molecule. Thus, plant cells manage to save energy. Sucrose synthase is also used with a different timeframe than invertase and also in different physiological conditions. For example, sucrose synthase is predominantly used during anaerobic stress because it slows the processing of monosaccharides to respiration (which is inhibited by the lack of oxygen); in addition, the sucrose synthase pathway requires less ATP consumption (Winter and Huber 2000).

Apart from the regulation of cell metabolism, sucrose synthase is also known because it could participate in cell wall synthesis. The mechanism by which sucrose synthase takes part in this process is anything but clear and maybe very complex. First of all, it should be remembered that sucrose synthase is associated with actin filaments (Winter et al. 1998). Nevertheless, the relationship between actin-dependent localization and cell wall synthesis was still quite unclear at least until sucrose synthase was detected at secondary wall thickenings of tracheary elements in *Zinnia elegans*. In this case, actin filaments and sucrose synthase were found very abundant at the plasma membrane level, suggesting that actin filaments could establish a framework for the positioning of sucrose synthase (Salnikov et al. 2001).

Subsequent studies have shown that sucrose synthase exhibits a large number of interactions. Distribution analysis in cotton showed that the enzyme is localized in correspondence of cortical microtubules as well as in the exoplasmic space (i.e., the cell wall area just outside the plasma membrane, which might be slightly different from the bulk of the mature cell wall, Salnikov et al. 2003). Besides, accumulation of exoplasmic sucrose synthase corresponded to deposits of callose in the cell wall (Salnikov et al. 2003). These findings suggested that sucrose synthase could interact with microtubules and participate in callose synthesis. The complexity of sucrose synthase's function is also attested by the presence of several sucrose synthase isoforms, each with different enzyme capacity and capable of binding differently to actin filaments (Matic et al. 2004).

Involvement of sucrose synthase in cell wall synthesis is probably a complex mechanism and is regulated by several factors. For example, high concentrations of sucrose can promote the binding of sucrose synthase to the plasma membrane, an event that could trigger the synthesis of cell wall polysaccharides. In addition, phosphorylation of specific amino acids promotes the binding of sucrose synthase to plasma membrane. At the same time, adequate levels of sucrose and phosphorylation events can cause the release of sucrose synthase from actin filaments (Duncan and Huber 2007). These data suggest that the start of cell wall synthesis, i.e., binding of sucrose synthase to plasma membrane, is triggered by availability of sucrose, which is quite logical given that synthesis of new cell wall can be started only when energy and material are available.

Evidence of relatively similar behavior were obtained in pollen tubes as well; here, sucrose synthase can bind to the plasma membrane in the presence of sucrose,

and the membrane-bounded form of sucrose synthase is differently phosphorylated than the soluble form (Persia et al. 2008). The final positioning of sucrose synthase seems dependent on actin filaments but not on microtubules (Cai et al. 2011). It is therefore evident that the "metabolic health" of pollen tubes regulates the position of sucrose synthase and its association with the plasma membrane: availability of sucrose and phosphorylation events induces the release of sucrose synthase from actin filaments while inducing its binding to the plasma membrane. Once there, sucrose synthase could associate with enzymes that catalyze synthesis of cell wall polysaccharides. The importance of the phosphorylated form of sucrose synthase in promoting synthesis of cellulose by cellulose synthase was demonstrated by past studies (Nakai et al. 1999). This role was supported by several experimental data; for example, expression of sucrose synthase in poplar stems is capable of increasing the incorporation of carbon from sucrose into cellulose (Konishi et al. 2004). The most recognized hypothesis is that sucrose synthase provides key metabolites (UDP-glucose) directly to cellulose synthase allowing rapid synthesis of cellulose (Brill et al. 2011). Cellulose synthase is carried to the plasma membrane by a process involving transport of membranes along microtubules and actin filaments, while positioning of sucrose synthase is probably under the control of actin filaments. In any case, a complex system of interactions must allow both enzymes to interact in the plasma membrane and, specifically, at sites of cellulose synthesis. Unfortunately, facts may not be so simple. On the one hand, down-expression of sucrose synthase genes in Arabidopsis is reported to have conflicting effects on cellulose production (Baroja-Fernandez et al. 2012; Barratt et al. 2009). On the other hand, overexpression of sucrose synthase in poplar tremendously enhances cellulose deposition in tracheary elements (Wei et al. 2015).

The involvement of sucrose synthase in callose synthesis is supported by a smaller number of data. Beyond the various models indicating that sucrose synthase can be part of the callose synthase complex (Hong et al. 2001), some evidence suggests the presence of sucrose synthase at sites of active callose synthesis (Salnikov et al. 2003) or, more generally, in combination with the production of cell wall carbohydrates (Brill et al. 2011). In specific cases, such as phloem elements in Arabidopsis, specific forms of callose synthase are hypothetically associated with sucrose synthase (Barratt et al. 2011). Although current data do not fully support the above model, it is clear that sucrose synthase can be a regulator of the availability of carbon necessary for cell wall synthesis and that its role is controlled by the cytoskeleton. In this way, a connection is established between cytoskeleton, enzymes that synthesize the cell wall, and the cell wall itself. The role of this molecular network would be to synthesize the cell wall in relation to the energy needs of cells. In the case of pollen tubes, this relationship will be discussed further in the next chapter.

3.5 How the Cytoskeleton Affects the Physicochemical Properties of the Cell Wall

As reported above, it is clear that building of the pollen tube cell wall is an extremely elaborate process. The main components taking part in this process are most likely similar (if not identical) to those found in other plant cells. However, the peculiarity of the pollen tube, coupled with its absolutely specific function, generated a series of changes in the mechanism of cell wall construction. To analyze the details of this process, it is suggestive to split the whole mechanism in separate sections although individual phases cannot be completely independent of each other. In addition, we attempt to describe the role of the cytoskeleton in cell wall assembly when pollen tubes undergo a local modification of cell wall composition, i.e., when it changes direction (Fig. 3.2).

The contribution of the cytoskeleton to the process of cell wall assembly in pollen tubes most likely starts in the cytoplasm by correctly distributing the enzyme sucrose synthase (Fig. 3.2, Box 1). Assuming that sucrose synthase is a critical enzyme in cell wall assembly because it provides metabolites for cellulose and callose synthases, its relative position serves to regulate the entire process of synthesis. If availability of intracellular sucrose is adequate, it is assumed that sucrose synthase is conveyed to the plasma membrane. Since sucrose synthase, when bound to the plasma membrane, is phosphorylated, the involvement of specific kinases is conceivable. Although activation of sucrose synthase-targeting kinases is currently hypothetical, it might be related to the reception of extracellular cues that globally trigger the process of synthesis (Zou et al. 2011). Association of sucrose synthase with the plasma membrane could be facilitated by cortical actin filaments, whereas the direct involvement of microtubules can be excluded (Cai et al. 2011; Persia et al. 2008).

The second step in which the cytoskeleton plays an important role is vesicle and organelle transport. This process is critical for almost all of the physiological aspects of pollen tubes. From the perspective of cell wall assembly, transport of Golgi bodies can be considered an important event because Golgi bodies produce secretory vesicles that contain enzymes and polysaccharides required for cell wall assembly (Worden et al. 2012). The movement of Golgi bodies along actin filaments is relatively proven in plant cells even though it has not yet been sufficiently demonstrated in pollen tubes. If pollen tubes behave like other plant cells, we can deduce that Golgi bodies move rapidly along actin filaments in order to supply the apical region with secretory vesicles (Fig. 3.2, Box 2). This movement is promoted by the mechano-enzyme activity of myosin(s) (Madison et al. 2015). The partial characterization of myosins in pollen tubes does not allow the identification of myosin isoforms specifically (or most likely) involved in the transport of Golgi bodies as already done in other plant cells (Tominaga 2012). We have yet to clarify the role of microtubules in this transport. Several years of research allow rejecting the hypothesis that microtubules are involved in the fast movement of Golgi bodies even though some experimental evidence indicates that microtubules can function

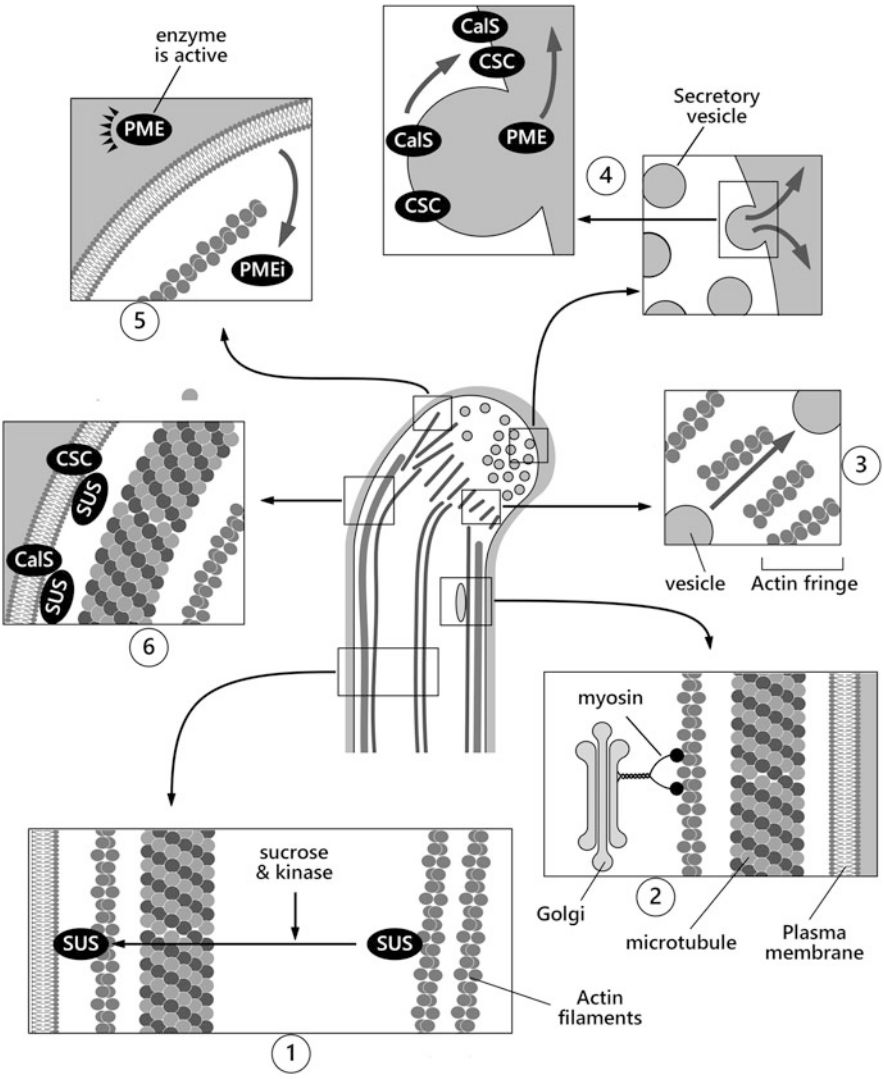

Fig. 3.2 Main events occurring at the cytoskeleton and cell wall level when pollen tubes change growth direction. *Box 1*: displacement of sucrose synthase (SUS) to the plasma membrane and binding to cortical actin filaments. *Box 2*: movement of organelles and vesicles along actin filaments. *Box 3*: accumulation of secretory vesicles as mediated by the actin fringe. The drawing shows the actin fringe as working like a sieve; actually, the actin fringe is more prevalent in the cortical region of the subapex and should function as a regulator of accumulation and/or fusion of secretory vesicles. *Box 4*: fusion of secretory vesicles with the plasma membrane and release of methyl-esterified pectins. Vesicles also release callose synthase (CalS), the cellulose synthase complex (CSC), and pectin methyl esterase (PME). *Box 5*: removal of PME inhibitor allowing activation of PME. *Box 6*: Cytoskeleton-mediated association of sucrose synthase with glucan synthases. See the text for additional details

as regulator of this process (Brandizzi and Wasteneys 2013). Hypothetically, microtubules could "slow down" the transport of Golgi bodies as soon as they arrive at the point of release of secretory vesicles, but this is only a supposition.

Displacement of sucrose synthase and movement of Golgi bodies are unceasing activities in pollen tubes and are independent from the growth direction as well as accumulation of secretory vesicles in the apical area. When pollen tubes need to change direction, e.g., by following specific extracellular signals, it is plausible that accumulation of secretory vesicles is not uniform throughout the apex volume, but they accumulate preferentially toward the new growth direction. Alternatively, we can hypothesize that fusion of secretory vesicles mostly occurs laterally displaced from the tip. Assuming that secretory vesicles do not accumulate evenly at the apex, it is necessary to identify the molecular mechanism responsible for such nonuniformity. As already stated above, the actin fringe could play this role (Rounds et al. 2014). The actin fringe is a highly dynamic structure, and its lesser or greater extension presumably induced by signaling mechanisms could regulate the accumulation of secretory vesicles at the apex. Assuming that a lower extension of the actin fringe causes a greater accumulation of secretory vesicles, it follows that the actin fringe acts as a molecular sieve (Fig. 3.2, Box 3). This is a critical step in generating the asymmetric structure of the cell wall because it leads to the local accumulation and consequently secretion of new cell wall polysaccharides, particularly new methyl-esterified pectins.

Vesicle secretion is undoubtedly a very critical phase in the process of cell wall assembly and then in the shaping of pollen tubes. It is likely that vesicle secretion gives rise to three critical events. The first is deposition of new methyl-esterified pectins. As new secreted methyl-esterified pectins accumulate, they contribute to soften the apical cell wall (Chebli et al. 2012). Thus, the apical cell wall becomes increasingly deformable during secretion. It is supposed that secretion is the slow phase of pulsed growth, while the progressive softening of the apical cell wall and its turgor pressure-induced expansion is the rapid growth phase (McKenna et al. 2009). The second event caused by secretion is the release of enzymes capable of modifying the cell wall structure. This is exemplified by secretion of pectin methyl esterase (PME), the enzyme that converts methyl-esterified pectins into acid pectins, thereby contributing to strengthen progressively the cell wall. The data in the literature suggest that PME is secreted at the tube apex (Bosch and Hepler 2006). Because the enzyme is found only in the very apical region, it is hypothetically removed by a process of cytoskeleton-dependent endocytosis. The third event related to vesicular secretion is insertion of two glucan synthases, cellulose synthase and callose synthase, respectively, in the plasma membrane (Cai et al. 2011; Doblin et al. 2001). As described above, it is likely that the functional state of the two enzymes is different, with cellulose synthase already active when secreted, while callose synthase would be inserted as inactive but activated later (Fig. 3.2, Box 4).

PME is a critical enzyme in the dynamic process of cell wall building at the apex. Its role is to regulate the balance between methyl-esterified and acid pectins, thus making the apical and subapical cell wall more or less elastic. In doing so,

PME contributes fundamentally to the pulsed growth. It is believed that PME is not constitutively active, but its activity is regulated by a specific inhibitor, the so-called PMEi (Zhang et al. 2010). This protein may be secreted in conjunction with PME, but it accumulates exclusively in the apical region, thereby contributing to inactivate PME at the apex. In this way, PME would be inactive at the tip where a majority of methyl-esterified pectins will accumulate. In the shank regions, the absence of PMEi would activate PME that converts methyl-esterified pectins into acid pectin, thereby strengthening the cell wall. Based on the data in the literature, it is plausible that the absence of PMEi in the shanks is mainly due to endocytosis (Rockel et al. 2008, and Fig. 3.2, Box 5). In the event that pollen tubes change growth direction, it is expected that conversion of methyl-esterified pectins into acid pectins is not uniform on both sides of pollen tubes and thus endocytosis is likely tuned differently, perhaps based on different cytoskeletal organizations.

Stabilization of the new growth direction must also include additional reinforcement of the cell wall through deposition of cellulose and callose. As mentioned above, the role of the two polysaccharides is probably different, and their deposition sequence is not likely concomitant. Deposition of cellulose and callose requires the activation of callose synthase and cellulose synthase. This is a complex process that usually requires the simultaneous activity of several accessory proteins. Details of activation are much more known for cellulose synthase (for a comprehensive review, please see Lei et al. (2012) but are largely incomplete for callose synthase. In both cases, it is likely that the cytoskeleton has a key role. In the literature, the molecular relationship between cellulose synthase and microtubules has been highlighted by recent studies showing that actin filaments rapidly convey the enzyme while microtubules are used for precise positioning (Crowell et al. 2010). The relationship between cytoskeleton and callose synthase is unclear, and more data are needed to define the contribution of microtubules and actin filaments in callose deposition. The limited data available for pollen tubes indicate that actin filaments and microtubules are likely to have specific roles in relation to different regions of pollen tubes. However, it is clear that actin filaments contribute to the insertion of both enzymes in the apical plasma membrane (Cai et al. 2011). The regulation of callose synthase and cellulose synthase could also be achieved by fine-tuning the position, relative abundance, and activation of sucrose synthase. As specified above, this enzyme provides substrates to cell wall polysaccharide-synthesizing enzymes (Kleczkowski et al. 2010). Therefore, regulating the activity and position of sucrose synthase can affect the activity of callose synthase and cellulose synthase. In this context, actin filaments will likely have a more critical role than microtubules because they are associated with sucrose synthase and regulate its intracellular positioning based on availability of sucrose (Fig. 3.2, Box 6).

Activation of cellulose synthase at the pollen tube apex and of callose synthase in regions immediately after the subapex might also be achieved via variation of additional factors, such as the local concentration of calcium ions or protons. Currently, there are more evidences concerning callose synthesis. For example, past data indicate that calcium ions could stimulate the synthesis of callose in soybean cell suspension cultures (Fink et al. 1987) or in microsomal preparations from corn

coleoptiles (Paliyath and Poovaiah 1988). In contrast to these findings, calcium ions do not seem to have a role in regulating callose synthesis in carrot cells (Messiaen et al. 1995). In pollen tubes, the apex is usually characterized by a calcium ion concentration gradient, which is essential for proper growth. Because the synthesis of callose starts several micrometers far from the tube tip, this suggests that high concentration of calcium ions inhibits (or does not favor) callose synthesis. The pollen tube apex is also characterized by lower pH values followed by the so-called alkaline band (Feijò et al. 1999). It is not easy to determine the spatial correlation between alkaline band and callose synthesis although the partial overlap between the two systems suggests that changes in pH can regulate callose synthesis. In that regard, it must be mentioned that changes in proton flow along the pollen tube are somehow related to deposition of callose plugs (Certal et al. 2008). This fragmented collection of evidence suggests that many more studies are needed to understand the role of calcium and protons in regulating the synthesis of cell wall polysaccharides.

3.6 Conclusion and Perspective

The relationship between cytoskeleton and cell wall deposition in pollen tubes is far from being clear. Although different experimental data suggest that the cell wall is deposited in a controlled manner, mainly in relation to the way by which pollen tubes grow, control of the deposition process is quite unclear. Nevertheless, exhaustive knowledge of the whole process is essential to understand how pollen tubes grow unidirectionally and how they follow the several extracellular signals encountered during their journey from the stigma surface to the ovaries. The interaction between pollen tubes and style and/or stigma matrix represents an interesting model of study in order to correlate extracellular information, cell wall building, and organization of the cytoskeleton. The simplicity of this cell model should make it possible to outline a relatively established framework of the molecular interactions that modulate pollen tube growth. Hopefully, efforts of the scientific community in the coming years will allow getting a progressively larger number of information spanning from structural chemistry of the cell wall to the cytoskeleton dynamics. A critical point is represented by the communication mechanisms and by our understanding of how extracellular signals are detected and converted into intracellular information, which are then transmitted to the cytoskeleton. The molecular biology of this mechanism is of importance because it is not strictly limited to pollen tubes but almost certainly pertinent to other plant cells in which it might help fully delineating the relationship between plant cell growth, namely, cell wall generation and energy consumption in relation to external "positive" environmental or stress signals.

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Part III
Technical Improvements to Study Tip Growth

Chapter 4
Measuring Cytomechanical Forces on Growing Pollen Tubes

Hannes Vogler, Naveen Shamsudhin, Bradley J. Nelson, and Ueli Grossniklaus

Abstract Cytomechanical measurements are important to unravel the influence of the biochemical composition of the plant cell wall on growth, morphogenesis, and stability. Agronomical research has a great interest in cell wall mechanics because in an ideal situation, crop plants grow as fast and large as possible without loosing the strength to withstand destabilizing environmental influences. Pollen tubes provide a convenient system to study major aspects of cytomechanics. They grow extremely fast but expansion is restricted to the tip region, providing a cellular model where both biochemical and mechanical properties vary spatio-temporally along the cell. The path of the pollen tube from the stigma to the ovary is full of obstacles, which the pollen tube has to overcome to reach the ovule and achieve fertilization. Once an obstruction is sensed, it can be either circumvented or penetrated, which involves mechanosensing, signal transduction, internal physiological changes, and adaptation of the mechanical properties of the pollen tube. As a result, the pollen tube changes its growth direction or increases the pushing force, both of which are controlled by a fine-tuned interplay between turgor pressure and cell wall extensibility. In this chapter, we provide an overview of state-of-the-art methods to measure those two parameters, as well as an outlook on novel technical developments that will allow the precise evaluation of the mechanical properties of the cell wall along the length of the pollen tube.

Keywords AFM • Cell wall • CFM • Cytomechanics • MEMS force sensor • Pollen tube • Turgor

H. Vogler (✉) • U. Grossniklaus
Department of Plant and Microbial Biology and Zurich-Basel Plant Science Center, University of Zurich, Zollikerstrasse 107, 8008 Zurich, Switzerland
e-mail: hannes.vogler@botinst.uzh.ch; grossnik@botinst.uzh.ch

N. Shamsudhin • B.J. Nelson
Multi-Scale Robotics Lab, ETH Zurich, Tannenstrasse 3, 8092 Zurich, Switzerland
e-mail: snaveen@ethz.ch; bnelson@ethz.ch

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Abbreviations

PDMS	polydimethylsiloxane
AFM	atomic force microscopy
SAM	shoot apical meristem
IAA	indole-3-acetic acid
MEMS	microelectromechanical systems
CFM	cellular force microscopy
FEM	finite element method
TEM	transmission electron microscopy
LoC	Lab-on-a-Chip
RT-CFM	real-time cellular force microscopy

4.1 Introduction

On their way to the ovule, pollen tubes face many biophysical interactions (Fig. 4.1). Mechanical forces already play a role in the very first steps of pollination. When the pollen grain lands on the stigma, strong adhesion between the grain and a papilla cell of the stigma is necessary for successful germination of the pollen tube. Adhesion differs between compatible and incompatible pollen grains (Luu et al. 1999). Once germinated, the pollen tube grows through the style towards the ovary. In plants with a solid style, like *Arabidopsis* (*Arabidopsis thaliana*) and the majority of flowering plants, the pollen tube first penetrates a papilla cell and then navigates through a maze of cells within the transmitting tract before leaving this tissue and growing along the funiculus towards the micropylar opening of the ovule. Within the transmitting tract pollen tubes forge their paths between the cells but they can also penetrate cells and grow between the cell wall and the plasma membrane or even within the cell wall (Elleman et al. 1992; Jiang et al. 2005). The situation presents itself totally different in plants with hollow styles, such as lilies (*Lilium longiflorum*) (Jauh and Lord 1996), lemon (*Citrus limon*) (Ciampolini et al. 1981), and bean (*Phaseolus acutifolius*) (Lord and Kohorn 1986). In this case, the pollen tubes adhere to and grow along the transmitting tract epidermis that lines the inner part of the style (Mollet et al. 2000; Park et al. 2000; Chae et al. 2007). While chemoattractants guide the pollen tubes at various steps of their journey (Hülskamp et al. 1995; Shimizu and Okada 2000; Kim et al. 2003; Palanivelu and Preuss 2006; Okuda et al. 2009), it is mechanosensing that makes them aware of obstacles and controls whether they penetrate a cell wall or avoid it by changing growth direction to skirt the obstruction.

Cytomechanical studies have been conducted for more than a century but progress in this field was very slow for many decades (de Vries 1884; Miyoshi 1895; Heilbronn 1914). Over the last 20 years, however, there has been an explosion of publications dealing with biomechanical questions at the tissue as well as at the

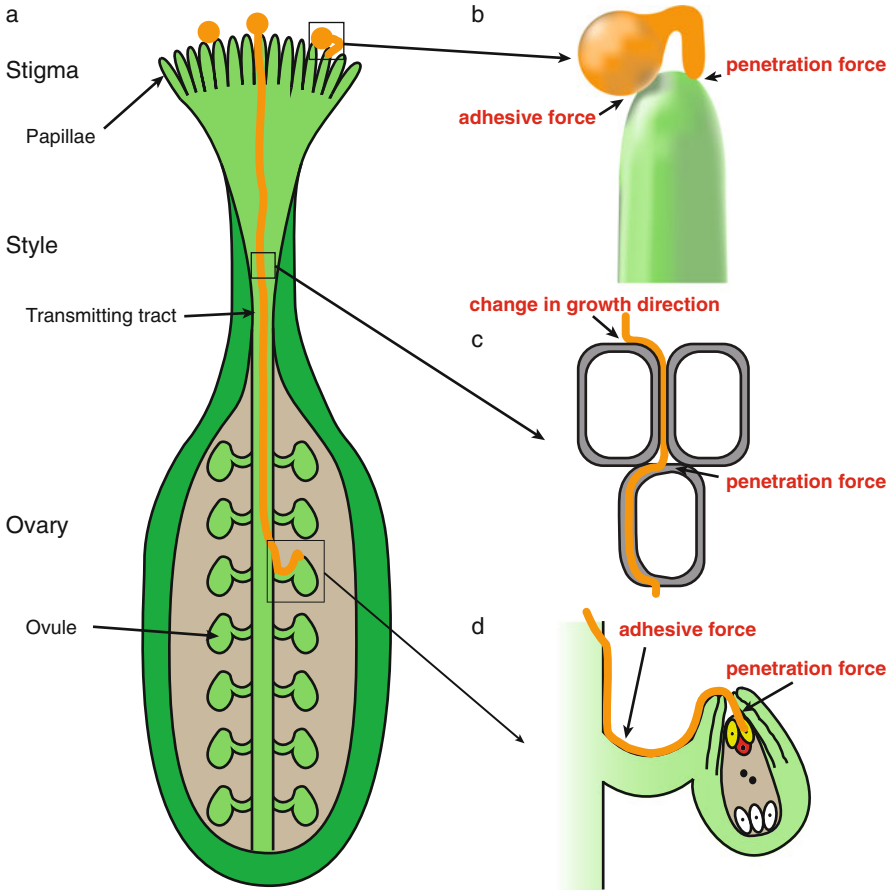

Fig. 4.1 Path of the pollen tube from the stigma to the ovule, illustrating the forces involved in the interactions between pollen tube and female tissues. (**a**) Overview of a pistil with a solid style. Compatible pollen germinates on the stigma and penetrates the papilla cells. A pollen tube grows through the style and transmitting tract into the ovary where it is attracted by an ovule. (**b**) Close-up of a papilla cell with a germinated pollen grain. The grain adheres to the surface of the papilla cell while the pollen tube penetrates the cell to grow within the cell wall or between the cell wall and plasma membrane. (**c**) When a pollen tube encounters a cell in the transmitting tract, it can either change growth direction and grow between the cells or penetrate them and grow within the cell wall or between the cell wall and plasma membrane. (**d**) After exiting the transmitting tract, the pollen tube follows a gradient of chemical attractants, adhering and growing along the funiculus into the micropylar opening of the ovule, where it penetrates a synergid cell to release the sperm cells into the embryo sac. Pollen grains and tubes are shown in *orange*

cellular level. One reason for this increased research activity was the improvement of instrumentation, such as the development of miniaturized, extremely sensitive force sensors and precise positioning systems required to measure the small forces that are exerted by living biological samples.

But why is it interesting at all to study biomechanics? Naively, one would assume that the biomechanical properties of individual cells or tissues are only important to define the shape and size of the resulting organism. Although this is one part of the story, there is much more to it. Apart from the final form, mechanical signals can influence the identity of cells and tissues as well as the stability of an organism. Just to mention a few examples, mechanical disturbances can alter the gene expression patterns and interfere with morphogenesis during embryo development (Shawky and Davidson 2015). Moreover, the magnitude and direction of the forces acting on stem cells influence their differentiation fate (Ho et al. 2016), and tumor cells have been shown to possess different mechanical properties than their normal counterparts (Nagelkerke et al. 2015).

Since plants are mostly unable to escape unfavorable conditions they may be even more prone to respond to mechanical triggers or react to environmental stresses with the modulation of the mechanical properties of their cells. Under insect attack, leaves can become tougher and thus less attractive to herbivores (Dimarco et al. 2012). Agriculture and the dairy industry are struggling with diverse problems that are based on biomechanics. For instance, reduction of the lignin content in maize has a direct implication for the profitability in dairy production. The *brown midrib* mutant has a lower lignin content, which leads to a better digestibility and, therefore, higher production of milk in dairy cattle (Saunders et al. 2015; Vanderwerff et al. 2015). The downside of this is that the mutant *brown midrib* maize plants are more susceptible to lodging. Crop lodging is a general problem and can dramatically reduce yield under unfavorable weather conditions, simply because the plant stems are not stable enough (Berry et al. 2006). Up to this day, the countermeasure to this threat is to produce shorter plants, either by breeding or through the use of growth regulators (Flintham et al. 1997; Crook and Ennos 1995). A better understanding of the mechanical properties of individual cell wall components could help to develop more stable plants that reach their full yield potential.

The mechanical properties of plant tissues or, even more so, entire plants result from a complex interplay between macromolecules that build the cell wall—the exoskeleton of the plant cell—turgor pressure, and mechanical interactions between neighboring cells. To gain a basic understanding of how these individual components affect cytomechanics in growing tissues we need to study simplified cases. Tip growth is an extreme form of polarized cell growth, which occurs in algae, fungi, animals, and plants. Tip-growing cells play pivotal roles in the lifestyle of those organisms employing it. Our brains would not function without the billions of dendrites and axons, formed by tip growth, that connect our neurons. Fungal hyphae can spread over incredible areas and are among the largest organisms on our planet (Smith et al. 1992; Ferguson et al. 2003). They can penetrate host cell walls and form haustoria, specialized feeding structures to acquire nutrients from the host cell (Pérez-de-Luque 2013; Yoshida et al. 2016). Plant root hairs are essential to guarantee sufficient supply with water and nutrients by tremendously increasing the root surface (Nye 1966). Arguably the most amazing cells, though, are pollen tubes. Their sole function is to deliver the male gametes to the ovule to ensure double

fertilization, which they do by growing at incredible speeds to enormous lengths, reaching length-to-diameter ratios far beyond 1000:1.

The pollen tube is an especially well-suited model to study the mechanical aspects of growth, because it is a single cell and no effects of neighboring cells have to be taken into account. The fact that cell expansion is restricted to the apex allows the direct comparison of the mechanical properties of this highly dynamic region with more proximal parts in the shank, where the cell wall does not change anymore. Furthermore, the biochemical composition of the wall changes along the pollen tube axis and can be assessed using cytological methods (Mollet et al. 2013), allowing, in turn, the measurement of different, well-defined composite cell wall materials in a single cell. Cell expansion is a complex process involving the fine-tuned interplay between a driving force that increases the volume of the cell and a containment that limits this expansion. Turgor pressure, the cell's internal hydrostatic pressure, is the driving force of cell expansion. The surrounding cell wall controls expansion by loosening and deformation concomitant with the incorporation of new cell wall material to reinforce the weakened areas. Where exactly pectins and other materials are deposited via exocytosis—in a subapical annular zone behind or at the extreme apex—is still under debate (Bove et al. 2008; Zonia and Munnik 2008; Rounds et al. 2014).

In this chapter, we will provide an overview of existing approaches to quantify the biomechanical properties of growing pollen tubes, with a special focus on indentation methods. Along with the development of measuring methods, there is growing need for automation, increase in throughput, precise calibration, and reproducibility of measurements across research laboratories, as most of the methods depend on statistical evaluation and/or modeling of the measurements. For this reason, we also provide an outlook on potential future developments and alternative approaches to unravel the secrets of pollen tube growth mechanics.

4.2 How Are Cytomechanical Parameters Measured?

4.2.1 Measuring Turgor

Incipient plasmolysis was the very first technique developed to measure internal turgor (de Vries 1884; Beck 1929). Due to lack of alternative methods, for a long time turgor pressure has been estimated using this method. The osmolarity of the extracellular medium is increased until the retraction of the protoplast from the cell wall can be observed, allowing an estimation of the osmotic pressure inside the cell. This method is simple, non-invasive and allows a large number of measurements in a relatively short time. Living cells, however, tend to react to osmotic stress with osmoregulation, which means that the estimated osmolarity may differ from that in the unperturbed cell.

This problem has been overcome with the development of the pressure probe, an oil-filled capillary equipped with a pressure transducer, which is inserted into the cell (Green 1968; Husken et al. 1978; Tomos and Leigh 1999). Turgor is measured directly by adjusting the pressure in the probe in order to keep the cell sap/oil meniscus at the plasma membrane, such that no sap enters the probe and no oil enters the cell. Although the pressure probe is the only direct method to measure turgor until present, it has the disadvantage of being invasive and tedious to use, especially on small cells. This limits the number of measurements that can be taken in a day. Both methods, incipient plasmolysis and the pressure probe have been applied to measure turgor pressure in growing lily pollen tubes, delivering significantly different results (Benkert et al. 1997; Pertl et al. 2010).

Ball tonometry is another method to estimate turgor. A cell is compressed with a large ball with a diameter between 30 and 50 μm. Since the cell wall is only a thin shell compared to the cell diameter, its influence can be neglected if the indentation is considerably larger than the cell wall thickness (Lintilhac et al. 2000; Wei 2001). While this approach is fast and non-invasive, problems arise when the cell to be measured is small. Theoretically, it would be possible to miniaturize the setup but it is not quite clear to which extent the influence of cell wall elasticity would increase and could not be neglected anymore.

4.2.2 Measuring Penetration Forces

Turgor pressure is not only the driving force for cell expansion but also generates the pushing force necessary for plant and fungal tip-growing cells to penetrate (or displace) obstacles. Penetration force measurement in fungal hyphae has a long tradition starting in the late nineteenth century by studying their ability of penetrating gold membranes of varying strength (Miyoshi 1895). Later on, more sophisticated methods, such as strain gauge (Money 2007), optical wave guide (Bechinger et al. 1999), and optical tweezers (Wright et al. 2007), were employed. Work on pollen tubes to measure penetrative forces was taken on only recently. A first attempt using agarose gels with zones of different stiffness as the mechanical obstacle revealed that actin filaments influence the behavior of poppy (*Papaver rhoeas*) pollen tubes when hitting an obstacle. However, this assay did not quantify the penetration force (Gossot and Geitmann 2007). The first quantitative assessment came from lab-on-a-chip experiments, in which *Camellia japonica* pollen tubes were forced to grow into narrow channels carved into polydimethylsiloxane (PDMS) with known mechanical properties. At the narrowest point of the channel, the dilating force was about 15 μN (Sanati Nezhad et al. 2013).

4.2.3 Stiffness and Topography Mapping–Indentation Methods

Plant cell walls are complex composite materials consisting of cellulose, hemicelluloses, pectins, and proteins. It is still under debate how exactly the individual components interact to define the mechanical characteristics of the cell wall (Cosgrove 2016). It is generally accepted, however, that the cellulose microfibrils are the load-bearing component, which are embedded in a more viscous pectin matrix. Hemicelluloses may be directly cross-linked to individual cellulose microfibrils, thus tethering them together (Hayashi 1989; Pauly et al. 1999; Scheller and Ulvskov 2010), or they may be covalently bound to the pectin network and connect individual cellulose microfibrils where they come close together in the so-called biomechanical hotspots (Park and Cosgrove 2012; Zhao et al. 2014; Nili et al. 2015). Although pollen tubes lack a classic, lignified secondary cell wall, they display a bi-layered cell wall with an outer layer dominated by pectins and an inner callose layer. In addition to this radial patterning, the biochemical composition of the cell wall changes along the longitudinal axis with a tip region that consists mainly of methylated pectins. Through the action of pectin methylesterases pectins get demethylated and consequently cross-linked by calcium in the subapical region, where also crystalline cellulose can be detected. Farther behind the tip, deposition of callose is observed in the shank (Mollet et al. 2013). A high callose content and reduced cellulose accumulation, compared with other plant cells (Rae et al. 1985; Schlupmann et al. 1994), nourished the idea that callose may be the main load-bearing component in pollen tube cell walls (Parre and Geitmann 2005a).

To understand the mechanical control of pollen tube tip growth, as well as how the pollen tube keeps its shape during growth, it is necessary to measure the physical properties of the cell wall along the pollen tube axis. Different approaches have been utilized to measure the mechanical properties of tip-growing cells. In order to be able to see local differences in cell wall properties, it is essential that the chosen method provides highly resolved spatial data and must cover the appropriate force range. As the thickness of the cell wall is usually much smaller than the cell diameter, indentation methods have proven valuable to determine the mechanical properties of the cell wall.

The type of information resulting from indentation experiments depends on the indentation depth as well as on the geometry and the size of the indenter. An indentation depth much smaller than the thickness of the cell wall mainly reveals the mechanical properties of the cell wall, while at higher indentation depths, the influence of turgor pressure increases. Equally, the influence of turgor decreases with decreasing indenter diameter. Taken together, a rather big indenter at low indentation depth would reveal in plane stresses of the cell wall, while a pointy probe, as they are frequently used in atomic force microscopy (AFM), would measure forces perpendicular to the plane of the cell wall.

Pioneering work in this field was performed in the Geitmann laboratory. Using a microindentation approach, they could show that poppy pollen tubes have a gradient of cellular stiffness along the tube axis, with a soft tip and a harder

shank. Moreover, the shank was almost completely elastic whereas the apical region displayed considerable viscoelasticity (Geitmann and Parre 2004a). Later, the stiffness gradient from the apex to the shank was confirmed in pollen tubes of lily. Additional experiments revealed that the cellular stiffness in the apical region followed growth oscillations with increased stiffness at high growth rates and vice versa (Zerzour et al. 2009). Taken together, these findings fitted well with the fact that the pollen tube apex, where growth takes place, should be softer than the shank, which does not expand but has to resist tension forces created by turgor pressure. Differences in the biochemical composition of the cell wall in the tip region compared to the shank could explain the observed mechanical difference. Indeed, it has been shown in pollen tubes of *Solanum chacoense* that reduced levels of both pectins and callose lead to a measurable reduction in cellular stiffness and increased viscoelasticity (Parre and Geitmann 2005a,b). However, the above-mentioned studies did not take into account the influence of turgor pressure and the geometry of the pollen tube, which are important parameters as we will discuss below.

More recently, AFM has been utilized to visualize the topography of plant cells (Yarbrough et al. 2009; Zhang et al. 2016). However, the AFM can also be used to evaluate the mechanical properties of cells. After the use of the AFM as a material indenter has been proven useful in bacterial and animal cells, first attempts in plant cells included the study of the nano-mechanical properties of dead wood cells (Clair et al. 2003) and cultured cells of *Vitis vinifera* (Lesniewska et al. 2004). Recently, after a further improvement of the technology, studies have started to assess the mechanical properties of living and growing tissues by probing the shoot apical meristem (SAM) of *Arabidopsis*. Using a sharp, pyramidal indenter and indentation depths significantly smaller than the estimated cell wall thickness, which rendered the measurements insensitive to turgor pressure, Milani and colleagues could show that the cells at the top of the SAM are stiffer than those at the flank (Milani et al. 2011). Glueing large spheres to the AFM probe makes the measurements highly sensitive to turgor (Peaucelle et al. 2011). Other AFM studies investigated the material properties of the cell wall in *Arabidopsis* culture cells (Radotic et al. 2012) or the material plasticity along the *Arabidopsis* root (Fernandes et al. 2012).

Although modern AFM setups provide a larger range of forces (pN–μN) and measuring area (up to a few mm), their strength lies in the measurement of very local nano-mechanical properties along the indentation axis. To quantify the in-plane elasticity, which is influenced by the load-bearing cellulose microfibrils, however, larger indentation depths are necessary. Hence, a lot of effort has been invested to develop microindentation systems that combine the ability to non-invasively map the mechanical properties of living and growing cells with large indentation depths and force ranges.

The first and only reported AFM study on pollen tubes was by Wu and colleagues (Wu et al. 2008). The surface morphology of *Torenia fournieri* pollen tubes treated with the auxin indole-3-acetic acid (IAA) were investigated by intermittent-contact mode imaging in air. The pollen tubes were grown, chemically fixed, and lyophilized

by freeze-drying before imaging. In the presence of IAA, the ultrastructure changed, leading to a modification of cellulose microfibril content and orientation on the outer surface of the pollen tube, which was observed by AFM at the tip and the subapical region. The authors concluded that the cellulose microfibrils were in the form of a random network in both the apical and subapical regions of control pollen tubes, while microfibrils were parallel to each other and predominately oriented 45 degrees to the longitudinal axis in the subapical region of IAA-treated pollen tubes.

4.3 Cellular Force Microscopy

4.3.1 General Considerations

Mechanical measurements of plant cells and tissues impose various technical challenges. The assessment of different parameters, such as turgor pressure, cell wall stiffness, or penetration forces, involve a wide range of forces from nN–mN and require a variety of measurement devices as discussed above. The topography of plant tissues makes it necessary to equip the force sensors with long probes and to have a wide z-positioning range to reach into the depression between adjacent turgid cells. A high degree of automation is important to create high-resolution stiffness maps and to measure a large number of samples. The cellular force microscopy (CFM) combines the high level of automation and spatio-temporal resolution along with the versatility of classic microindentation systems that are able to measure in-plane elasticity, which is important to measure growth (Fig. 4.2a) (Felekis et al. 2015a). The use of microelectromechanical systems (MEMS)-based force sensors allows measuring in locations that are difficult to reach with AFM cantilever probes (Fig. 4.2b and c). The CFM probes demonstrate no hysteresis over their full range of operation and are individually force-calibrated by the supplier (FemtoTools AG,

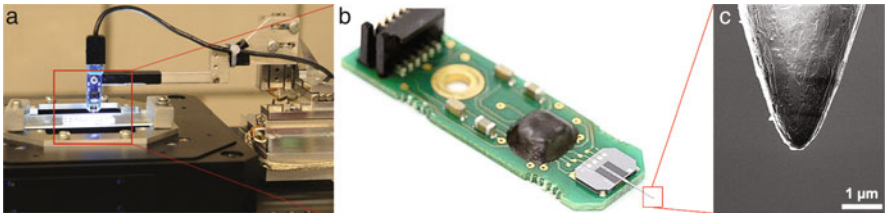

Fig. 4.2 The RT-CFM. (**a**) An overview of the positioning and measuring unit. The force sensor (*blue*) is mounted to an aluminum arm, which is attached to a three-degrees-of-freedom micropositioner that is used to bring the sensor probe close to the sample. To make measurements, the sample is moved against the probe via a piezo-driven fine positioning stage. (**b**) Close-up of the capacitive microelectromechanical systems (MEMS)-based microforce sensor (FT-S100, FemtoTools). A tungsten tip is glued to the actual sensor probe (*red square*). (**c**) Magnification of the tungsten tip

Switzerland). Custom-made software allows for real-time force and position control of the measurements.

First experiments using the CFM demonstrated that the method is suitable for high-resolution stiffness mapping on pollen tubes (Felekis et al. 2011) and onion epidermal cells (Routier-Kierzkowska et al. 2012). In the latter study, a plasmolyzed piece of onion epidermis was measured sequentially over several hours during recovery from plasmolysis and revealed changes in topography and stiffness with increasing turgor pressure. Figure 4.3 shows a typical CFM measurement.

4.3.2 CFM Measurements on Pollen Tubes

Stiffness measurements taken under the conditions used by the CFM and other microindentation systems do not only reflect the mechanical properties of the cell wall alone but also are strongly influenced by turgor pressure, cell and indenter geometry, as well as stresses in the cell wall prior to indentation. Hence, the measured values are referred to as the "apparent stiffness" (Zamir and Taber 2004). First CFM experiments on pollen tubes were performed in the Grossniklaus and Nelson laboratories who developed this technology (Felekis et al. 2011; Vogler et al. 2013). CFM measurements on growing, fully turgid lily pollen tubes confirmed the apparent stiffness results from microindenter experiments showing a soft apical

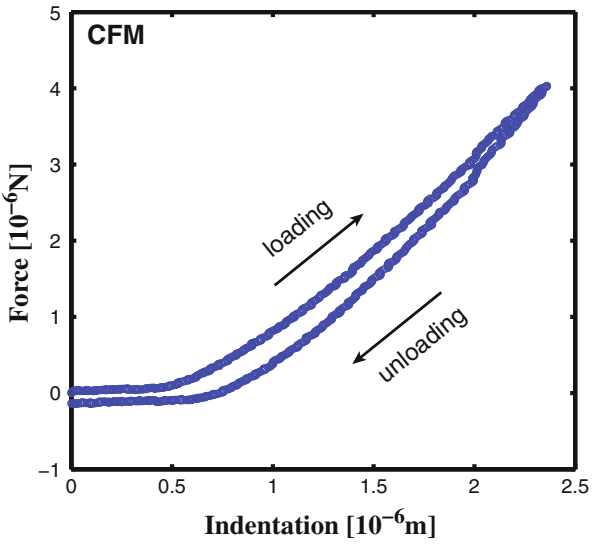

Fig. 4.3 A typical force against indentation plot from a CFM measurement taken in the tip region of a lily pollen tube. The apparent stiffness of the pollen tube, resulting from a combination of turgor pressure, cell wall stiffness, and cell and sensor geometry, is calculated by applying a linear fit to the loading and/or unloading curve. Hysteresis suggests a degree of viscoelasticity in the cell wall

region and a gradual increase in stiffness that reached a plateau 20 μm behind the tip. Finite element method (FEM)-based modeling was applied to disentangle the effects of turgor pressure and cell wall elasticity by adjusting the material properties of a virtual cell wall until the modeled stiffness curve was in agreement with the measured values. Surprisingly, it was unnecessary to modify the elastic properties in the tip region to get an almost exact match of the steep decrease in the measured apparent stiffness towards the very tip of the pollen tube. In other words, the difference in apparent stiffness between the tip and the shank does not reflect the well-documented changes in cell wall composition but can be explained by the geometry of the cell and the contact angle between the probe tip and the cell surface (Bolduc et al. 2006; Vogler et al. 2013; Routier-Kierzkowska and Smith 2013).

Using an FEM-based modeling approach allows the unraveling of the effects of turgor pressure and cell wall stiffness without the necessity to measure turgor by another method, i.e., the pressure probe. However, the model relies on the assessment of several geometrical parameters, such as the initial stretch ratio, which is correlated to the wall stress under pressure prior to indentation. This can easily be measured by comparing the diameter of a pollen tube before and after plasmolysis. Cell wall thickness is another required parameter that is more difficult to measure. To get accurate values it is necessary to analyze and average pictures obtained by transmission electron microscopy (TEM), a difficult and labor-intensive method. However, because the cell wall shows local variation in thickness, it would be ideal to know the thickness at the position of indentation. A method to estimate cell wall thickness from apparent stiffness-indentation curves has been suggested by Forouzesh et al. (2013) but is not yet accurate enough and depends, at least partially, on the pressure state of the cell.

4.3.3 Limitations of the CFM Approach

While the CFM has contributed significantly to the mechanical investigation of pollen tubes and other plant cells (Vogler et al. 2013; Weber et al. 2015), experimental challenges remain with regard to high-throughput measurements at the scale of individual cells. Firstly, ensuring sufficient substrate adhesion of the growing pollen tube is a major challenge for microindentation. While other cells, like yeast cells or oocytes, that exhibit isotropic or near-isotropic growth, can be immobilized by culturing them in solid growth medium (De et al. 2010), by trapping in surface-patterned wells (Ng et al. 2007; Kailas et al. 2009), or by physical fixation through capillary aspiration (Sun et al. 2003), the rapid and three-dimensional nature of pollen tube growth renders such immobilization techniques unusable. Secondly, under conventional *in vitro* growth conditions pollen tubes grow unorderly in all directions, leading to increased operational time to monitor the growth trajectory and to position the microindenter for measurements at the required location on the pollen tube (Felekis et al. 2015b). The random growth direction complicates the

development of automated tools to pre-plan the position, number of measurement points, and trajectory of the microindenter, consequently lowering the measurement throughput.

4.4 Improvements

Several improvements have been proposed to overcome the aforementioned challenges to increase the throughput of microindentation. The resulting higher efficiency in data collection will enable the generation of micromechanical datasets that are amenable to statistical analysis. Such datasets are essential to overcome the inherent biological heterogeneity within a population to decipher the underlying complex biochemical and cytomechanical processes controlling the fast tip growth of pollen tubes.

4.4.1 Microchannel Guidance

Microfluidics has emerged as a powerful technology to isolate and chemically as well as mechanically probe individual cells (Nilsson et al. 2009). Such cytomechanical studies typically involve optical monitoring of the cellular deformation caused by externally applied on-chip forces, e.g., by fluid-driven cell loading onto narrow constrictions (Di Carlo 2012), or by electro-deformation with non-uniform electric fields (Engelhardt and Sackmann 1988), etc. High-throughput micromechanical assays have also been performed using a patterned two-dimensional array of mechanoprobes (Sniadecki et al. 2007; Favre et al. 2011). While a typical μm-sized animal cell, such as an erythrocyte or 3T3 cell, can be mechanically probed using the above-mentioned techniques, it is difficult to apply such immobilization and force measurement methods to pollen tubes because they grow fast and are extremely elongated. Another problem that arises when using single or multiple on-chip force actuators is the difficulty in force calibration at the nN to mN scale typical for cellular forces (Kim et al. 2011). Furthermore, the use of flexible materials, such as PDMS that is commonly used in microfluidics approaches, or the use of magnetic or piezoelectric materials as actuators, can result in actuator hysteresis and nonlinear responses. This behavior can compromise quantitative cytomechanical assays by convoluting them with the actual mechanical response of the cell. The need for well-calibrated mechanical assays incorporating microfluidic technologies is paramount.

Recently, microfluidic phenotyping assays have been developed for cells exhibiting polarized growth such as neuronal axons, filamentous fungal hyphae, and pollen tubes (Taylor et al. 2005; Geng et al. 2015; Yetisen et al. 2011; Agudelo et al. 2013). The reported Lab-on-a-Chip (LoC) devices used microchannels to guide

the tip-growing compartments by isolating them from the rest of the cell body. To increase the measurement efficacy of the CFM-based micromechanical assays, we developed an integrated microfluidic-CFM platform that allows for high-throughput germination, growth, and uni-directional guidance, combined with micromechanical measurements of individual pollen tubes (Shamsudhin et al. 2016a). The LoC has a loading and germination reservoir for the pollen grains, which is surrounded by dozens of collinear microchannels (Fig. 4.4a). These channels guide the growing pollen tubes uni-directionally, with vertical and lateral confinements, enabling long-term observations without the need for active z-focusing on the microscope (Fig. 4.4b). The LoC has an open architecture, which allows it to be integrated with the CFM microindenter probe (Fig. 4.4c). After growing in the microchannels, the pollen tubes exit the LoC and grow onto the open glass slide, being fully immersed in the growth medium. After, emerging from the microchannels, the pollen tubes

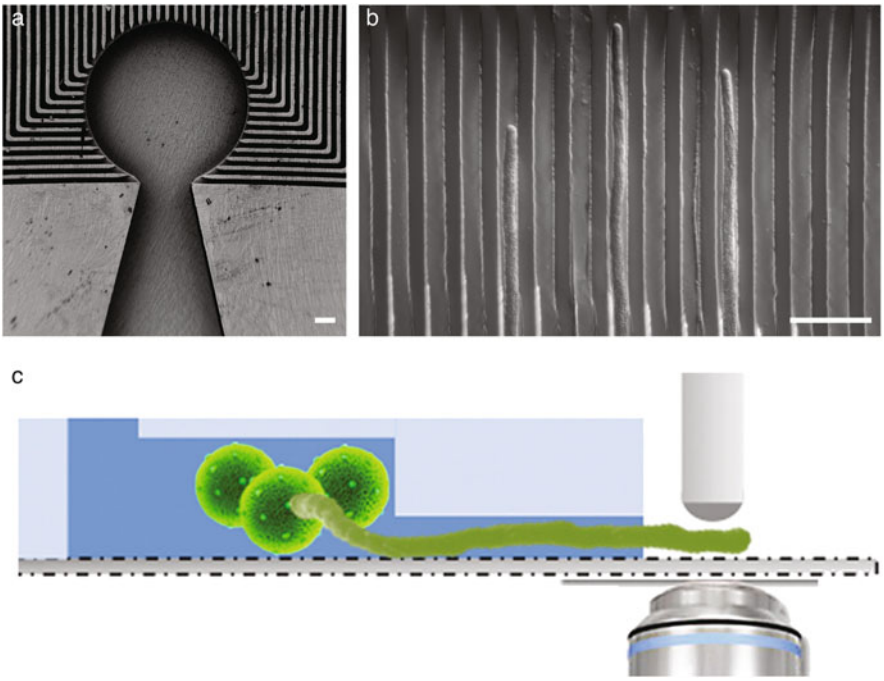

Fig. 4.4 Multichannel Lab-on-a-Chip (LoC) device to parallelize pollen tube growth. (**a**) Part of a multichannel LoC designed for lily pollen. The pollen grains are loaded into the circular reservoir where they will germinate and the pollen tubes will grow into the individual channels. (**b**) Close-up of an array of channels with three growing lily pollen tubes is shown. (**c**) Scheme of the multichannel LoC in a CFM experiment. The sensor probe is placed in front of the channel and measurements are taken when the pollen tube emerges. Scale bars = 100 μm

grow straight for several hundred μm without deviations from their previous growth direction. This allows for a faster positioning of the CFM force sensor on the growing pollen tubes, thus increasing measurement throughput. Additionally, the vertically confined growth in the microchannels increases the adhesion of the pollen tube to the substrate, enhancing the robustness of microindentation.

The microindentation measurements on the integrated microfluidic-CFM system confirmed the previously reported gradient in apparent stiffness along the axis of the pollen tube. The measurements identified two different states of apparent stiffness, corresponding to growing and non-growing (naturally growth-arrested) pollen tubes. While growing tubes exhibited a mean loading stiffness of 2.20 N/m, non-growing tubes exhibited 0.69 N/m. It is well known that the apparent stiffness is affected by the turgor pressure of the cell when forces are in the range of μN and indentation depths applied by the CFM probe in the range of μm (Weber et al. 2015). While it is reasonable to assume that non-growing pollen tubes may have lower turgor pressures (Geitmann et al. 2004), states of modified cell wall biochemistry cannot be ruled out, as pressure-probe measurements on lily pollen tubes have not found a correlation between growth rates and turgor pressure (Benkert et al. 1997).

4.4.2 Real-Time Computer Vision

In optical and mechanical phenotyping assays, the ability to monitor individual pollen tube growth trajectories is crucial. The naturally three-dimensional and disordered growth of pollen tubes increases the time and complexity of optical measurements, as the operator has to constantly refocus in the z-plane to keep the tube in focus for long-term measurements. Additionally, the entangled growth of pollen tubes makes it difficult to distinguish one pollen tube from the other. Currently, pollen tube identification is mostly done manually, but a software algorithm called fluorescent multi-pollen trajectory tracker was implemented by Palanivelu's group as part of the Bioinformatics Toolset of the Pollen Research Coordination Network (Cheung et al. 2013). However, this is primarily a tool for data post-processing and cannot identify the pollen tube's trajectory in real-time. We developed a robotic platform, which utilizes computer vision, for real-time tracking of pollen tubes and for controlling the microindenter probe (Felekis et al. 2015b). In addition, the real-time system allows for image mosaicing, which creates high-resolution images of a large workspace to accommodate pollen tubes that are several mm in length. This mosaic stitching is made possible by a fixed camera-lens system and a movable sample holder mounted on a dual-stage mm range positioner. For more details on the hardware and software algorithm, the reader is referred to (Felekis et al. 2015b).

4.4.3 *Dual-Axis Force Sensors*

The gradient of apparent stiffness measured along the pollen tube's length, with a softer apical region, has been attributed to a biochemical gradient in the cell wall (Geitmann and Parre 2004b; Chebli et al. 2012), an effect of geometry and non-normal surface indentation (Vogler et al. 2013) or a convolution of both the previously mentioned effects (Shamsudhin et al. 2016a,b). The use of multi-axis force sensors may allow the deconvolution of these two effects. The design, fabrication, and calibration of multi-axis μN force sensors have been reported in the literature (Beyeler et al. 2009; Muntwyler et al. 2009). We highlight preliminary results of microindentation performed on lily pollen tubes with a dual-axis force sensor (Burri et al. 2016). The sensor converts the axial and lateral force at the probe tip to an independent change in deflection of orthogonal comb drives (Fig. 4.5). The comb drives convert the displacement change to a differential change in capacitance, which can be read out using on-board electronics. During vertical indentation of the pollen tube, both vertical and lateral forces are simultaneously detected at the very apex of the pollen tube and the subapical region. No measurable lateral forces were observed beyond 4 μm from the apex. The measurement of lateral forces at the pollen tube tip is an indication of a non-normal contact angle. These promising

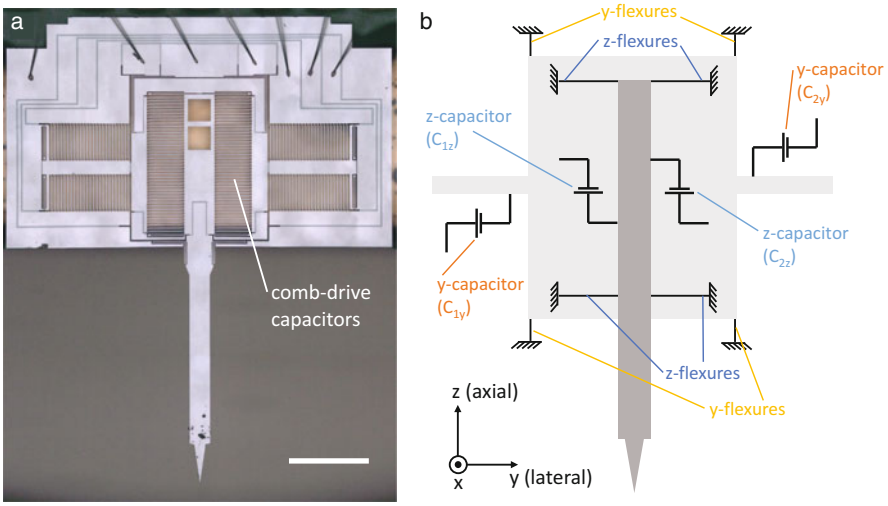

Fig. 4.5 Dual-axis force sensor for simultaneous measurements of vertical and lateral forces. To be able to measure the forces at the curved tip of pollen tubes, values for the vertical and lateral force components need to be known to calculate the force perpendicular to the cell wall. (**a**) Close-up of the dual-axis force sensor, showing the arrangement of the capacitor arrays (comb-drive capacitors). The sensor probe is attached to the capacitor arrays measuring the vertical force component. The lateral force sensing capacitor arrays are arranged on the left and on the right and are connected to the central part via flexures. (**b**) Scheme of the dual-axis MEMS-based force sensor (prototype, FemtoTools). Scale bar = 1.5 mm

results on the use of dual-axis force sensors for microindentation experiments are currently being pursued further.

4.5 Conclusions and Further Developments

On its tortuous journey, which begins at the stigma and goes all the way to the ovule, the pollen tube receives and processes a variety of chemical and mechanical cues from the surrounding environment. While chemoattractants are perceived at a distance, the mechanical environment is only perceived when the pollen tube is in physical contact with the cell exerting the mechanical force. The counteracting mechanical forces generated by the pollen tube in response to the chemical and mechanical signals from the outside is regulated by a complex feedback loop involving the cell's internal machinery. The generation and regulation of internal turgor pressure and the spatio-temporal variation of cell wall stiffness are manifestations of this feedback mechanism. Only in the last decade, with the arrival of micromechanical probes and technologies, have the mechanical forces encountered and generated by the pollen tube been precisely quantified. Quantification of such cytomechanical parameters is essential to formulate better models for pollen tube growth and to gain insights into the interplay between the biochemical and mechanical properties of the cell wall during cellular growth and morphogenesis.

High-throughput measurements are a key requirement for a statistical analysis of the mechanical properties of single cells. In addition to increasing the throughput of measurements and data analysis using novel hardware and software tools, it is important that measurement devices can be precisely calibrated and traced to force standards. This is particularly important for the measurement of forces at nN to mN range, such that cytomechanical data can be compared across laboratories. Another technical challenge is the combination of intracellular physiological monitoring, e.g., by using fluorescent sensors, and mechanical measurements to understand the interplay of biochemical and mechanical factors. Several questions concerning the mechanical basis of pollen tube morphogenesis remain, and there is the need for advanced instrumentation to answer them in the future. For instance, oscillating turgor has been postulated in pollen tubes (Messerli and Robinson 2003; Zonia et al. 2006), which can now be investigated by making precise, quantitative measurements using the micromechanical and LoC devices we described in this chapter. Moreover, the integration of knowledge about the biochemical composition and macromolecular arrangement of cell wall components, determined by immuno-cytochemistry (Mollet et al. 2013), TEM, and AFM, with cytomechanical measurements, promises to yield fundamental insights into the mechanisms underlying the tip growth of pollen tubes, the fastest growing cells on our planet.

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Chapter 5
Microfluidic- and Microelectromechanical System (MEMS)-Based Platforms for Experimental Analysis of Pollen Tube Growth Behavior and Quantification of Cell Mechanical Properties

Anja Geitmann

Abstract Experimentation on pollen tubes has benefited greatly from recent technological developments in the fields of microfluidics and microelectromechanical systems (MEMS). Various design strategies have been developed to expose in vitro growing pollen tubes to a range of experimental assays with the aim to study their behavior and their mechanical properties. The devices allow exposing the cells to chemical gradients, microstructural features, integrated biosensors, or directional triggers, and they are compatible with Nomarski optics and fluorescence microscopy. Microfluidic technology has opened new avenues for both more efficient experimentation and large-scale phenotyping of tip-growing cells under precisely controlled, reproducible conditions. The chapter provides an overview of the different design strategies used and the type of data acquired over the past 5 years since the technique was first adopted by the pollen community.

Keywords Pollen tube growth • Invasive growth • Chemotropism • Directed growth • Tip growth • Microfluidics • MEMS • Lab-on-a-chip

Abbreviations

LoC	Lab-on-a-chip
MEMS	Microelectromechanical systems

A. Geitmann (✉)
Faculty of Agricultural and Environmental Sciences, Department of Plant Science, McGill University, Macdonald Campus, 21111 Lakeshore, Ste-Anne-de-Bellevue, Québec, H9X 3V9 Canada
e-mail: anja.geitmann@mcgill.ca

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5.1 Introduction

The pollen tube is a member of the group of tip-growing cells, which distinguish themselves from other cell types by their extremely polar growth dynamics. All cellular growth occurs at a single point on the cellular surface yielding a cellular protrusion that is cylindrical and that has an extreme aspect ratio of up to tens of thousands. A substantial body of research has been dedicated to understanding how tip-growing cells focus their growth activity onto a single growth region and how they regulate growth dynamics in time and space (Feijó et al. 2001; Hepler et al. 2001; Geitmann and Palanivelu 2007; Palanivelu and Tsukamoto 2011; Malhó 2006; Brand and Gow 2009; Money 2001; Money et al. 2004; Cheung and Wu 2008, 2007; Qin and Yang 2011). In the context of pollen tube growth, this type of research helps us understand how the female and male partners communicate with each other, how a given pollen tube outcompetes others, and how it finds its female partner in the first place. Experimentation on pollen has drawn upon a variety of experimental designs, all of which have their advantages and limitations. Large-scale assessment of pollen tube performance has primarily been based on quantifying seed set (in vivo approach) and on determining the percentage of germination, the average growth rate, and the morphology of the cells (in vitro approach). These parameters can be determined microscopically in bulk samples and require only simple in vitro cell culture techniques, although not all plant species are easy to cultivate in vitro (Bou Daher et al. 2008). Since tip growth is a complex process, multiple efforts have been made to develop more sophisticated methods with the aim to measure cell biological parameters. Such experimentation includes for example monitoring ion fluxes with a vibrating probe (Feijó et al. 1995; Messerli and Robinson 1997, 1998; Messerli and Robinson 2003), using a micro-indenter to measure the biomechanical properties (Geitmann et al. 2004; Parre and Geitmann 2005a, b; Zerzour et al. 2009; Vogler et al. 2013), and experimental setups exposing elongating pollen tubes to mechanical obstacles and directional triggers (Higashiyama and Hamamura 2008; Márton and Dresselhaus 2010; Lord 2003; Gossot and Geitmann 2007; Bou Daher and Geitmann 2011; Palanivelu and Preuss 2000; Palanivelu and Tsukamoto 2011; Malhó et al. 1992). The latter have been designed to understand aspects of pollen tube behavior that pertain to the in vivo growth situation in the pistil. The drawback of most of these experimental designs is the fact that they are time-consuming since only one cell can be tested at any given time. Furthermore, the resulting data are often highly variable and difficult to reproduce. It is therefore not surprising that the pollen tube community rapidly adopted new technology when it became available. Microfluidic and microelectromechanical system (MEMS) technology has been developed and put to use successfully in numerous biological and medical applications to simplify sophisticated functions such as chemical reactions, drug development, and bioassays by integrating them within a single micro-device, called a lab-on-a-chip (LoC, Giouroudi et al. 2008; Nuxoll and Siegel 2009; Cheung and Renaud 2006). The technology offers enormous opportunities for pollen tube research since it allows precise handling of pollen tubes and enables the researcher

to create and control the chemical and physical environment of the elongating cell at micrometer-scale resolution. Using this technology, the microenvironment of the pistil or aspects thereof can be mimicked to understand how the pollen tube navigates this tissue on its way to the ovule. This chapter summarizes the considerations that have to be made when using microfluidic and MEMS technique for the purpose of studying pollen tube biology, and it provides an overview over experimental designs produced over the past 5 years.

5.2 Design Principles and Considerations

5.2.1 Basic Design and Fabrication

Microfluidic devices generally have a planar geometry. The designs developed for pollen tube research typically consist of a microfluidic network that allows capturing either individual pollen grains suspended in growth medium (Sanati Nezhad et al. 2014a; Ghanbari et al. 2014; Agudelo et al. 2012, 2013b), groups of pollen grains (Agudelo et al. 2016), or an entire pollinated stigma (Yetisen et al. 2011; Horade et al. 2014; Sato et al. 2015). In all cases, pollen grains are then left to germinate and form tubes, which once long enough are typically guided into channel structures where they are exposed to some kind of experimental design, either in (small) bulk or individually.

The flat design of the microfluidic network harboring the pollen tubes restrains any interactions between two cells or cell and microstructure to a two-dimensional space. Therefore, all growth activities occur essentially in one focal plane. Planning of the layout has to consider all desired functionalities: pollen grain capture or placement of pollinated pistil, guidance of the tube into a suitable microchannel, and experimental test on the growing tube (Fig. 5.1). In devices that aim at capturing individual pollen grains, the capturing has to be achieved through controlled fluid flow which drags the grains injected into the device to the capturing mechanism, such as V- or U-shaped traps of the TipChip (Fig. 5.1d,e,g), a multifunctional device developed for pollen tube research (Sanati Nezhad et al. 2014a; Ghanbari et al. 2014; Agudelo et al. 2012, 2013b). The design of the microchannel and the experimental test requires detailed understanding of what should be achieved. Depending on the experiment, a simple two-dimensional design feature suffices, whereas others may require multiple structural layers (Agudelo et al. 2013b) or the incorporation of additional features such as metal electrodes (Agudelo et al. 2016). For both pollen grain capture and microchannels, various designs are possible, and modeling of fluid flow allows guiding the optimization of the design (Sanati Nezhad et al. 2014b; Agudelo et al. 2013b; Fig. 5.1c,h). The design pattern is generally drawn in a CAD software, reproduced on a photomask, and transferred to a silicon/SU-8 mold through photolithography (Agudelo et al. 2012, 2013a). This requires coating a silicon wafer with SU-8, an epoxy-based negative photoresist. When exposed to

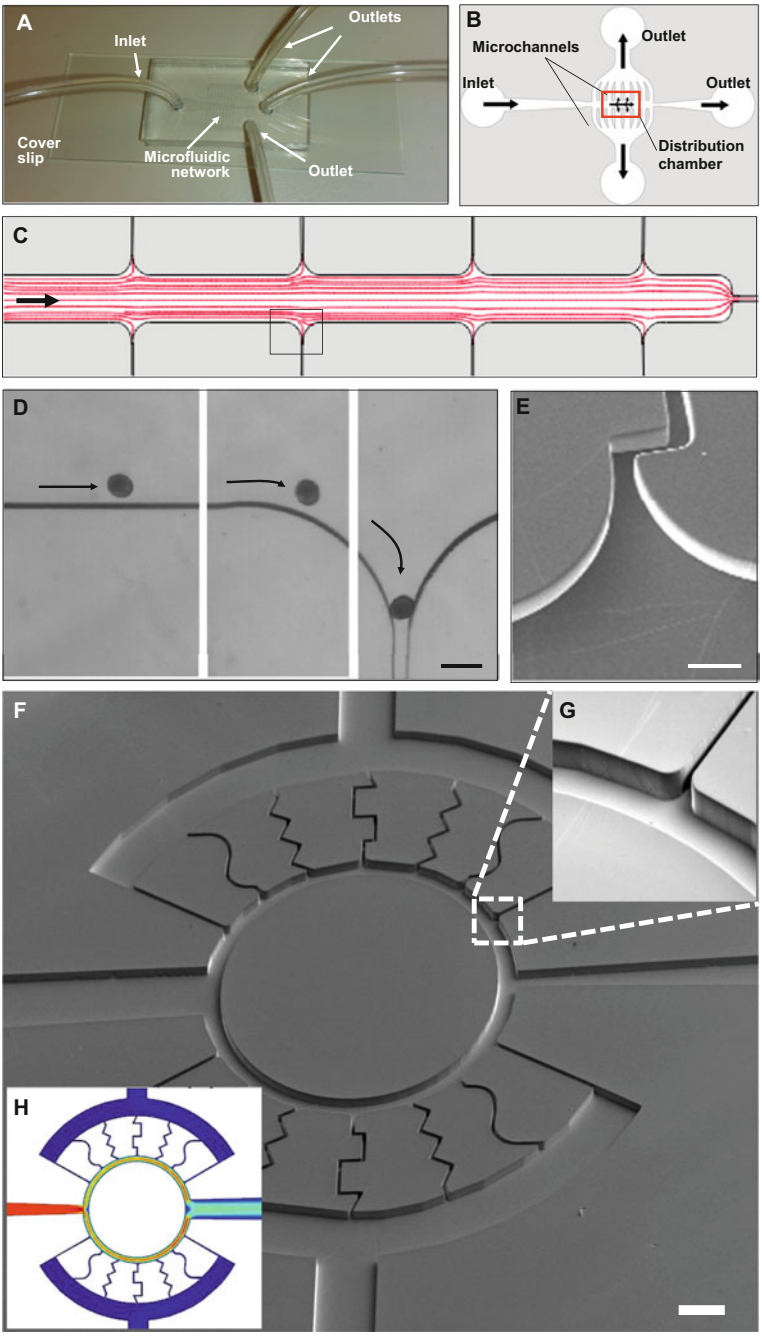

Fig. 5.1 Typical designs of microfluidic platforms used for pollen tube growth. (**a**) Image of the TipChip showing the PDMS layer attached to a cover slip and the attached inlet and outlets.

UV the material becomes cross-linked and polymerizes. This behavior is exploited to irradiate the layer with the CAD-patterned photomask and wash away the portions that remain nonirradiated and soft. The resulting silicon/SU-8 mold serves for the fabrication of the microfluidic network from polydimethylsiloxane (PDMS). The PDMS polymer base is mixed with a curing agent, poured over the mold and baked to cure the material. Subsequently, the PDMS layer is peeled off the mold and the open channel design is sealed to a glass cover slip using oxygen plasma bonding. PVC tubes are inserted through holes punched into the PDMS layer that provide inlets and outlets into and from the microfluidic network. The sealed design of the TipChip enables high-resolution imaging (through the cover slip) and prevents evaporation of the growth medium, a phenomenon which had proved difficult to control in open assays (Yetisen et al. 2011).

5.2.2 *Dimensions and Features of the Microfluidic Network*

In the most straightforward designs, the microfluidic network has the same height everywhere, in the chamber housing the pollen grain traps and in the microchannels harboring the growing pollen tubes. The height must be sufficient to allow the pollen grains to move by fluid flow. To avoid the accumulation of pollen grains into stacks that would represent a mechanical impediment to fluid flow and a visual obtrusion preventing microscopic observation, the height is generally limited to 1.2 and 1.5 times the diameter of the pollen grains. This dimension is species dependent, in *Camellia* the diameter is typically 60 μm, and therefore the height of the microfluidic network is generally set to 80 μm for this species. Pollen grains from *Arabidopsis* are smaller and therefore require a flatter network. Similar to the differently sized pollen grains, the pollen tubes of these two species differ in diameter, and as a consequence, the microchannels have to be scaled accordingly. The diameter of the *Camellia* pollen tube measures between 14 and 20 μm making

Fig. 5.1 (continued) (**b**) General design principle of the microfluidic network in the TipChip. The pollen suspension is injected through the inlet, and the pollen grains move through a central distribution chamber and either get trapped at the entrances of the microchannels or evacuated through the distribution chamber outlet. (**c**) Fluid flow simulation showing the streamlines within the central distribution chamber. (**d**) Micrographs of a pollen grain driven by fluid flow and trapped at the entrance of a microchannel. The stitched images show the same pollen grain at three different times. Bar = 100 μm (**e**) Scanning electron micrograph of trap entrance. Bar = 100 μm (**f**) Scanning electron micrograph of circular design of distribution chamber with various microchannel designs. Bar = 400 μm (**g**) Close-up of microchannel entrance. (**h**) Fluid velocity field simulated by modeling. Heatmap colors represent relative velocity. (**a**–**e**) are reprinted from Agudelo et al. (2013b) and (**f**–**h**) from Ghanbari et al. (2014) with permission of the authors and the respective publishers

it an ideal model system for cytomechanical studies (Bou Daher and Geitmann 2011). Microchannels were therefore generally designed to be 50 μm wide for "comfortable" accommodation, and narrow gaps of 17, 15, or 13 μm were sufficient to challenge the tube mechanically (Sanati Nezhad et al. 2013a). With its diameter of 5–6 μm, the pollen tube of *Arabidopsis* on the other hand requires scaling down the microchannel if it is to be used in a similar way. This is only one of the challenges that this species offers as the soft lithography technique has limits to the spatial resolution it can achieve. The pollen of *Arabidopsis* is generally difficult to germinate in vitro (Bou Daher et al. 2008), and the microfluidic setup does not make the situation easier in the TipChip design. An alternative design improved upon the germination challenges by incorporating a cutoff stigma in front the of microfluidic network. The pollen grains germinate on the stigma, grow through the short section of style, and are thus in much better physiological state when entering the microchannels (Yetisen et al. 2011). The use of pollinated stigmas was also done with *Torenia* (Horade et al. 2014; Sato et al. 2015). Since the stigma is accommodated outside of the microfluidic network, the heights of the microchannels can be reduced to fit the diameter of the pollen tube and thus ensure that pollen tube growth is restrained to one focal plane (Horade et al. 2014).

Physical restraint of the pollen tube in plane has been another important feature for several applications. When the elongating tubes are meant to encounter mechanical obstacles, the grain is not held in place by the U-shaped traps but can be pushed backward by the force generated by the growing tube hitting an obstacle. Similarly, forces applied on the growing tip in perpendicular direction can only be applied in calibrated manner if the base of the tube and/or the grain is immobilized. Additional structural features have helped solving these challenges. In the case of pushback in direction opposite to the growth direction, the incorporation of a simple kink in the microchannel has proven useful to prevent the tube from moving rearward (Agudelo et al. 2013b; Fig. 5.2a,b). Preventing a tube from moving in lateral direction can either be achieved by letting it grow through narrow slits (Sanati Nezhad et al. 2013a; Fig. 5.2c) or by incorporating a second microchannel in perpendicular direction whose fluid flow pushes the tube against the opposite side wall of the growth microchannel (Sanati Nezhad et al. 2013b; Fig. 5.2d).

5.2.3 *Influence of Microchannel Geometry and Fluid Flow on Pollen Tube Performance*

The microchannels are narrow conduits through which the pollen tubes are guided with the intent to have them encounter an experimental test situation. It is therefore important that this environment does not hamper or alter growth, either by limiting the access to nutrients, by causing accumulation of any substances emitted by the pollen tube, or due to any other condition established by the network geometry. This is particularly important since while a narrow conduit may be more similar

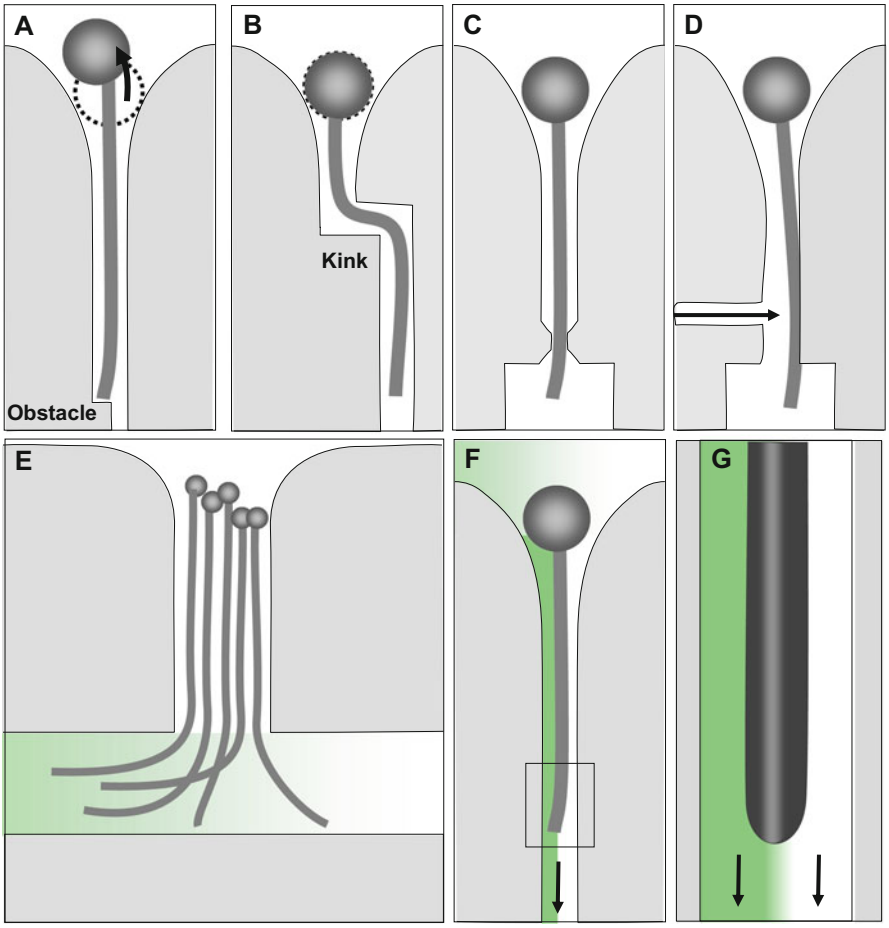

Fig. 5.2 Microfluidic design features enabling analysis of pollen tube behavior (**a**) Pollen tubes growing against an obstacle will experience pushback unless they are mechanically immobilized. (**b**) A kink in the microchannel provides sufficient mechanical immobilization to prevent pushback. (**c**) Lateral movement can be prevented by passage through a narrow slit. (**d**) Alternatively, mechanical stabilization against lateral movement can be achieved through fluid injection through a side channel. (**e**) A static chemical gradient in a T-shaped configuration allows for the investigation of chemotropic growth behavior as shown in Sato et al. (2015) and Yetisen et al. (2011). (**f**) A laminar flow-based gradient can be designed sharp enough to expose the two flanks of the growing pollen tube with different concentrations of an agent of interest. The actual concentrations to which the cell is exposed can therefore be precisely defined. This design can be used to study chemotropic behavior or to apply inhibitors or other agents to selective regions on the cellular surface. The development of the design and experimental data were published by Agudelo et al. (2013b) and Sanati Nezhad et al. (2014b). (**g**) Close-up of the concentration laminar flow-based chemical gradient shown in **f**. The position of the interface between the fluids can be manipulated to align it with the exact position of the pollen tube apex

geometrically to the pollen tube's path through the transmitting tissue compared to a large volume of liquid medium in a petri dish, the PDMS walls of the microchannels are inert and do not provide nutrients (although PDMS does allow for the penetration of oxygen). In early microfluidic devices used for pollen tube growth (Yetisen et al. 2011; Sato et al. 2015), this limitation was not an issue since the channels were comparatively large compared to the dimensions of the cell. The situation is different in more recent devices such as the TipChip (Agudelo et al. 2013b) and the device developed by the group of Tetsuya Higashiyama (Horade et al. 2014). In these the limited volume of medium surrounding the cells could potentially have a negative effect on cellular growth. Experiments performed on parallel microchannels with different dimension showed, however, that the width of the microchannel did not influence the growth rate in microfluidic networks that had a depth of 80 μm versus a pollen tube diameter of 17 μm (Agudelo et al. 2013b). In very flat networks, this might be different, but it has been shown that even microchannel heights close to the dimensions of the pollen tube do not limit pollen tube growth (Horade et al. 2014). Interestingly, the depth of the microchannel did have an influence on pollen tube growth with deeper channels accelerating the growth rate in *Camellia* pollen tubes (Agudelo et al. 2013b) and slowing it in *Torenia* (Horade et al. 2014). Why pollen tube growth is more sensitive to the dimensions in z-direction than to those in-plane is not known, but the finding is consistent with the behavior of other cell types exposed to microfluidic applications (Yu et al. 2005). It has been hypothesized that this difference may be connected to convection occurring primarily in z-direction.

Another factor that influences pollen tube growth is the presence or absence of fluid flow during experimentation. The complete absence of fluid flow in the microfluidic network may cause local concentrations of nutrients around the pollen tube to be depleted due to their consumption by the cell, and it may lead to local accumulation of substances emitted by the pollen tube, which in sufficiently high concentration might affect growth. On the other hand, the application of fluid flow might cause shear forces, a condition that is not normally encountered by in vivo growing pollen tubes. Numerous experiments have shown that in the network geometries used hitherto, the absence of fluid flow has not been correlated with any dramatically negative impact on pollen tube growth over the period of observation. Hence if the experimental design is conducive to the absence of flow, fluid flow can be stopped once the pollen grains are trapped. However, for certain experimental approaches, continuous medium flow through the microchannel is necessary, for example, to establish a laminar flow-based gradient or to move the tube (see below). The effect of shear stress on cellular behavior was therefore assessed experimentally. It is important to note that while the velocity of overall flow can be regulated at the inlet of the microfluidic chip by injecting medium via a motor-controlled syringe, the fluid velocity experienced locally by individual pollen grains and tubes depends on the geometry of the microfluidic network (Sanati Nezhad et al. 2014a; Ghanbari et al. 2014). Modeling of the fluid flow is therefore crucial to predict the velocity of the fluid around the cells when a given inlet velocity is applied. Experiments showed that the presence of slow fluid flow enhanced pollen tube growth, likely because the flow increased the supply of oxygen and nutrients, which were consumed by the

elongating tube. Excessive flow rates on the other hand were found to be damaging (Agudelo et al. 2013b). This effect is likely due to shear forces on the cell surface. At high velocities pollen tubes were observed to burst. Shear stress must therefore be taken into account as possible source of artifacts when designing experimental configurations.

5.2.4 Components for Fabrication of Electrodes and Actuators

The fabrication of microfluidic networks is generally done with PDMS, a material that is nontoxic, oxygen permeable, and optically transparent. These properties make the material highly suitable for pollen tubes which require oxygen to grow, and it is compatible with high-resolution light microscopy and fluorescence microscopy (Agudelo et al. 2013b). Unlike the plastic material from which petri dishes are fabricated, PDMS is not birefringent, and therefore Nomarski optics can be used if the network is sealed with an appropriate cover slip. PDMS has an additional feature that has made it the perfect material—its stiffness or elastic modulus is exactly in a range that makes it suitable to build mechanical obstacles that the pollen tube is able to deform. This feature has been exploited to expose growing *Camellia* pollen tubes to openings smaller than their diameter (Sanati Nezhad et al. 2013b; Sanati Nezhad and Geitmann 2013). Some tubes were able to deform the PDMS material whereas others stalled. This demonstrates that the elastic properties of the material are exactly in a range relevant for experimentation.

In simple microfluidic devices, the only materials used are PDMS and glass. However, incorporation of more sophisticated experimental designs requires features such as actuators, sensors, or electrodes that have to be fabricated from metal. Pollen tubes are highly sensitive to various types of chemical agents, which has motivated their use for toxicity assays (Kristen and Kappler 1995). For this reason, the materials most commonly used in the production of MEMS devices were tested for their effects on pollen tube growth: single crystal silicon wafer, sputtered gold, aluminum film, and laminated copper. Pollen germination in contact with silicon, aluminum, and gold performs very well, but the presence of metallic copper almost completely suppresses pollen germination and tube growth (Agudelo et al. 2013b). Whether this response is related to the electrophysiological properties of the pollen tube (Feijó et al. 1995) or to the toxicity of copper ions released by the metal (Sawidis and Reiss 1995) remains unknown. However, given that under fluid flow conditions the effect is less dramatic than under no-flow conditions, an effect of the copper ions released into the solution is likely. Surprisingly, while gold does not per se inhibit pollen tube growth, the application of a moderate electrical field by means of gold electrodes causes an inhibitory effect in DC fields but promotes growth in AC fields (Agudelo et al. 2016). This field- and material-dependent response is not yet understood.

5.3 Applications

5.3.1 Investigation of Chemotropism and Directional Memory

Pollen tubes are known to respond by changing their growth direction toward chemical attractants or away from repulsive agents (Geitmann and Palanivelu 2007). The increasing number of proteinaceous factors identified to be involved in pollen tube guidance in vivo (Palanivelu and Tsukamoto 2011; Kanaoka and Higashiyama 2015) raises many questions that pertain to the tube's ability to perceive and respond to the signal. For each of these factors, it will be informative to quantify the minimum concentration difference that the tube is able to perceive or to determine whether the features characterizing the turning response are dependent on the nature of signal. These pieces of information will serve to fully understand the roles of chemical and proteinaceous signals in pollen tube guidance.

In vitro, chemical gradients can be applied in various ways, each having their respective advantages and challenges. Diffusion-based, static chemical gradients can be applied by positioning the cells at the interface between two solutions. Through diffusion, the solutions build up a concentration gradient. This can be achieved in a T-shaped or crossroad setup (Fig. 5.2e), and pollen tube orientation can be quantified easily by scoring the percentage of pollen tubes growing toward attractant. The method has been used effectively for *Arabidopsis* and *Torenia* pollen tubes in order to demonstrate that they selectively target ovules that had been placed at one end of the T-intersection (Sato et al. 2015; Yetisen et al. 2011).

The static gradient configuration allows for gradients to be established over extended spatial distances. This configuration can only operate in the absence of fluid flow, and the confined space of the microchannel simulates the *in planta* situation where factors secreted by the pollen tube redistribute only by diffusion. The limitation of this setup is the fact that the resulting chemical gradient is relatively shallow and that the exact concentrations surrounding the pollen tube are not known. The situation is likely complicated through the generation of superimposing gradients of other substances that are released by the tubes themselves.

The accumulation of cell-released agents can be prevented by using a laminar flow-based gradient. This method also allows for the generation of gradients sufficiently sharp to expose the two opposite flanks of the cell with the two source concentrations rather than some intermediate concentration (Fig. 5.2f,g). To establish such a sharp and laminar flow-based gradient, the microchannel is fed from two inlets simultaneously. *Camellia* pollen tubes exposed to growth medium containing 8% and 12% sucrose displayed a clear directional growth response toward the medium with the higher concentration (Agudelo et al. 2013b), and when faced with a choice between 2.5 and 5.1 mM calcium ions, the *Camellia* pollen tubes turned toward the lower Ca^{2+} concentration (Sanati Nezhad et al. 2014b), consistent with the better performance of the cells at this concentration observed in bulk tests (Bou Daher and Geitmann 2011). In addition to assessing the pollen tube's response to a directional trigger, the laminar flow-based configuration can

also be used to administer inhibitors or enzymes specifically to one side of the pollen tube, as was done to test the effect of local application of pectin methyl esterase, an enzyme that modifies the pollen tube cell wall (Sanati Nezhad et al. 2014b). This type of experimental setup will allow for example determining critical concentration differences for proteinaceous attractants or repulsive agents. Furthermore, unlike the relatively shallow static gradient, the concentrations in the laminar flow gradient can be changed in a matter of seconds and the pollen tube response be analyzed in much greater detail with the aim to characterize the cellular machinery that brings about the turning response.

The investigation of directed growth under the effect of exogenous vectorial information provides crucial information on the pollen tube's ability to respond to guidance cues. However, cells also have internal directional mechanisms as is known from root hairs and fungal hyphae, which have a directional memory (Bibikova et al. 1997; Held et al. 2011). This means that they return toward their original growth direction once they bypassed a mechanical obstacle. The TipChip has been used to study this behavior in pollen tubes (Agudelo et al. 2013b). It turns out that the pollen tube does not have directional memory. When the tubes grow through serpentine-like microchannel geometry, the elongation of the tube seems to happen in a "blind" fashion, meaning that a tube grows straight until it encounters a microchannel wall. It then slips along the wall in the direction favored by the collision angle and eventually grows along the wall. If the pollen tube is repeatedly forced to change its growth direction through mechanical obstacles and it is then let to enter a wide chamber, it invariably keeps the last growth direction for extended periods of time (Agudelo et al. 2013b; Fig. 5.3a). The absence of a directional memory in pollen tubes is consistent with the difference in biological purpose of this cell type compared to root hairs and fungal hyphae. To most efficiently accomplish their tasks consisting in water and nutrient uptake, root hairs need to grow away from the root. A directional memory ensures that even after passing an obstacle, the general growth direction of the root hair is maintained to be away from the root. Similarly, the expansion of a fungal mycelium occurs most efficiently, if the hyphae grow in radial direction, even if they are temporarily deviated by an obstacle. Pollen tubes on the other hand must grow toward a target, and the position of the pollen grain is irrelevant for this targeting function. An endogenous directional memory would therefore be an impediment rather than an advantage.

5.3.2 Mechanosensing

It has often been speculated whether pollen tubes are able to perceive and actively respond to the presence of mechanical obstacles and whether they have mechanosensors. No molecular data are available for the latter, but the application of the TipChip has allowed demonstrating the former. When pollen tubes are guided to encounter flat surfaces positioned at various angles (Fig. 5.3b), the growth speed slows down temporarily in dose-dependent manner (Agudelo et al. 2012). However,

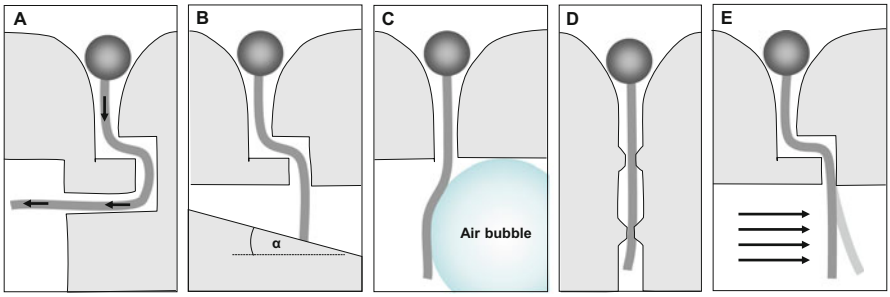

Fig. 5.3 Microfluidic design features enabling analysis of pollen tube behavior and mechanical properties (**a**) Pollen tubes deviated by mechanical obstacles maintain the last direction they were forced into. This means that contrary to other tip-growing cells, they do not have an internal memory as demonstrated by Agudelo et al. (2013b). (**b**) Mechanoperception and response can be assessed using obstacles positioned at different angles as designed by Agudelo et al. (2012). (**c**) Although pollen tubes are able to grow into air bubbles, they typically grow around them as demonstrated by Agudelo et al. (2013b). (**d**) Mechanical assay designed to measure the dilation force exerted by the pollen tube to prevent its collapse under lateral compression. This design forces pollen tubes to grow through slit-shaped gaps made from elastic PDMS material and was developed by Sanati Nezhad et al. (2013a). (**e**) Measurement of the Young's modulus of the pollen tube cell wall was achieved through bending of the pollen tube by fluid flow following the principle of a three-point bending test. This assay was designed by Sanati Nezhad et al. (2013b)

even when an obstacle is placed perpendicular to the growth direction, pollen tubes rarely arrest growth for more than 1 or 2 min before recovering. Similar observations are made when the pollen tubes encounter micro-gap structures. Upon the first contact with these wedge-shaped openings, the growth rate slows (Sanati Nezhad et al. 2013a). Fascinatingly the growth rate does not slow any further despite the fact that the mechanical impedance of the obstacle becomes increasingly difficult to penetrate for the advancing pollen tube. It is hypothesized that this is an active response of the tube that increases its invasive force, and hence the phenomenon suggests the presence of a mechanism that is able to perceive the strength of the obstacle (Sanati Nezhad et al. 2013a).

Mechanoperception is also likely the mechanism behind a different phenomenon: although pollen tubes are able to grow in air (Agudelo et al. 2013b), they typically avoid entering air bubbles trapped in the microfluidic network of the TipChip (Fig. 5.3c). Instead they grow along the fluid side of the fluid/air interface. This suggests that the tube perceives the surface tension at the interface.

5.3.3 Galvano Sensing

Plant tissues exhibit and generate considerable electric fields (Jaffe and Nuccitelli 1977), and it is therefore reasonable to hypothesize that they are involved in pollen tube growth and guidance. However, experimental findings and their interpretations

have been contradictory. Using macroscopic experimental devices, pollen tubes of some species were found to grow toward the cathode and others to the anode, and some do not respond at all (summarized in Agudelo et al. 2016). *Agapanthus umbellatus* pollen tubes behave very differently depending on where in the experimental electric field they are located. Under a constant electric field, they grow toward the nearest electrode when placed in either half of the field but orient randomly in the center (Malhó et al. 1992). One of the biggest challenges is that the details of the experimental setups used for these studies are often not described in detail, and reproducing the data is therefore difficult. Exact dimensions of the electrical field, as well as medium composition, duration of the experiment, and materials used, are crucial for galvano behavior, and therefore MEMS technology offers an excellent opportunity to provide a reproducible experimental setup with precisely defined electrical parameters—the ELoC (electrical lab-on-a-chip, Agudelo et al. 2016). Multiple important pieces of information were gained using such a device in combination with *Camellia japonica* pollen tube, a species that germinates reliably in vitro, tends to grow very straight and rapidly, but is of course not necessarily representative of all species.

The growth medium used for in vitro pollen tube culture typically contains salts and sugars, which makes it an electrolyte. Hence, the electrical behavior of this medium might become considerably complex when an electric field is present. Conductivity in most of the previously published experiments, if it was measured at all, was typically determined with standard devices that operate with AC frequencies, whereas DC current was employed to perform the actual experiment. Due to the complex composition of the growth medium, this distinction is important, and the ELoC study showed that medium conductivity varies significantly with the AC frequency and is very different from the conductivity under DC conditions (Agudelo et al. 2016). Furthermore, when DC conditions are applied over an extended time period, the conductivity of the medium (in the absence of pollen) changes over time. The ELoC allowed for a detailed study to relate applied field strength with the response of pollen in terms of germination rate and pollen tube growth rate. It was also used to show that transient, sublethal electrical fields have a long-term effect on pollen tube growth. The comparison between DC and AC electric fields at different frequencies revealed a dramatic dependence of pollen tube response on the nature of the field applied. Surprisingly, high-frequency AC fields allowed for pollen tube growth to proceed without visible effect compared to the deleterious effect of low-frequency AC (<100 mHz) and DC fields with identical field strength. This pattern correlated rather well with the frequency dependency of the medium conductance, thus suggesting that the effect of the electric field on the cells is mediated by this parameter.

Interestingly, *Camellia* pollen tubes do not show significant turning behavior when exposed to the microfluidic electric field (Agudelo et al. 2016). The pollen tubes do not even avoid a highly localized lethal field when growing toward it. The absence of a turning response in the microfluidic setup is speculated to be due to the specifics of the experimental conditions, one of which being the fact that the tubes are surrounded by liquid medium, whereas the agarose-stiffened medium had been

used in a macroscopic setup that caused turning in a third of the tubes in the same species (Bou Daher and Geitmann 2011). The presence of a stiffening agent likely prevents convection and thus might have a significant influence of the action of the electric field at cellular level. Many more studies on this and other species will be required to gain a comprehensive understanding of whether, how, and why pollen tubes respond in directional manner to electric fields.

5.3.4 Measuring Cell Mechanical Properties

Microfluidics and MEMS technology are uniquely positioned to allow researchers to perform micromechanical measurements on single cells in reproducible manner. The dilating force of the pollen tube was determined letting the tubes grow through obstacles made from PDMS (Fig. 5.3d). Since the elastic modulus of this material is known, it was possible to reverse engineer the force that the tube exerts on the PDMS obstacle and to deduce the exerted pressure from this value. Not surprisingly, the results suggest that the dilating force is generated by the turgor pressure (Sanati Nezhad et al. 2013a).

An important cell mechanical parameter for plant cells is the stiffness of the cell wall or its Young's modulus. Modeling has predicted that pollen tubes grow in polar fashion to form a cylinder because the apical cell wall displays a precisely defined mechanical gradient in deformability (Fayant et al. 2010). Experimental evidence from micro-indentation had confirmed the presence of this mechanical gradient (Geitmann and Parre 2004; Chebli et al. 2012), but this technique does not allow to determine absolute values. The modeling therefore had to rely on educated guesses in terms of the absolute numbers for the mechanical properties of the wall. The micromechanical experiment that eventually allowed determining this parameter was performed using a sophisticated microfluidic design (Fig. 5.3e). The pollen tube was grown in a microchannel and then bent by a perpendicularly oriented fluid flow. From the degree of bending and the speed of the fluid, it was possible to reverse engineer the Young's modulus of the *Camellia* pollen tube to be 350 MPa, similar to Teflon (Sanati Nezhad et al. 2013b).

5.4 Conclusion and Perspective

Microfluidic- and MEMS-based experimental platforms offer huge potential for pollen tube research. The devices allow addressing questions that were impossible to study hitherto, and they enable designing experimental conditions that are highly reproducible. The modular design allows for the incorporation of various features such as sensors, and the data that have been produced since the methods were adopted by the pollen tube community have advanced the field in significant manner.

Future challenges include designing the devices for true high-throughput screening of mutants and to exploit the capacity of the technology to its fullest.

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Part IV
Subcellular Processes

Chapter 6
Polar Protein Exocytosis: Lessons from Plant Pollen Tube

Hao Wang and Liwen Jiang

Abstract Exocytosis is a highly regulated outward secretion process which participates in multiple cellular events including membrane modeling, cell polarization, cell wall formation, and cell signaling. Pollen tube is regarded as an ideal model plant cell system to study the machinery and regulation of exocytosis. Vigorous exocytic vesicle fusion with the apical plasma membrane supports the rapid and polarized pollen tube growth. However, the molecular identities and regulations of these exocytic vesicles still remain largely unexplored. Recent discoveries of various exocytic markers and regulatory proteins of exocytic pathway have advanced this research field rapidly. Here, we review and summarize the recent development and progress of protein exocytic trafficking and regulation in pollen tube, with emphasize on the pertinent questions for future understanding the functional roles of exocytosis in cell polarization and polar cell wall formation.

Keywords Tip growth • Polar exocytosis • Cell polarity • Cell wall • *trans*-Golgi network • Golgi-derived vesicle

Abbreviations

CCP	Clathrin coated pits
EXPO	Exocyst-positive organelle
FRAP	Fluorescence recovery after photobleaching
GDSV	Golgi-derived secretory vesicles

H. Wang (✉)
College of Life Sciences, South China Agricultural University, Guangzhou, 510642 China
e-mail: wanghaohw@gmail.com

L. Jiang (✉)
School of Life Sciences, Centre for Cell & Developmental Biology and State Key Laboratory of Agrobiotechnology, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong, China

CUHK Shenzhen Research Institute, The Chinese University of Hong Kong, Shenzhen, 518057 China
e-mail: ljiang@cuhk.edu.hk

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HPF	High-pressure freezing and substitution
PCV	Pre-vacuolar compartment
PM	Plasma membrane
PME	Pectin methylesterase
RLK	Receptor-like kinase
ROP	GTPase termed Rho of plant
SCAMP	Secretory carrier membrane protein
STORM	Stochastic optical reconstruction microscopy
TEM	Transmission electron microscopy
TGN	*trans*-Golgi network
UPS	Unconventional protein secretion
VAEM	Variable-angle epifluorescence microscopy

6.1 Introduction

The growing pollen tube has become one of the most useful model systems for investigating polarized cell growth in plants. Polarized and rapid pollen tube growth is supported by vigorous tip-focused exocytosis. Large numbers of small exocytic vesicles which carry newly synthesized proteins, cell wall polysaccharides, and plasma membrane (PM) are delivered to the growing pollen tube tip region to support the fast apical membrane expansion and polarized cell growth (Bedinger et al. 1994; Cai and Cresti 2009; Chebli et al. 2013; Fu 2015; Hepler et al. 2001; Hill et al. 2012; Winship et al. 2011; Zonia and Munnik 2009; Qin and Yang 2011). In early electron microscopy studies, the V-shaped subapical clear zone was revealed to be filled with an apparently uniform population of small vesicles. A subsequent morphometric analysis of secretion at the pollen tube tip indicated that more exocytic vesicles fuse with the PM than are required to satisfy the demands of surface expansion (Steer and Steer 1989). This points to an increased removal of membrane via endocytosis, which has been highlighted by FM4-64 dye uptake (Wang et al. 2010a, 2011a; Lancelle and Hepler 1992; Kroeger and Geitmann 2012; Qin and Yang 2011). However, the origin and molecule identities of the vesicles which accumulate in the tip region still remain elusive due to their morphological similarities and the lack of sufficient molecular markers.

The cell wall at the tip of the growing pollen tube has no cellulose and consists almost exclusively of pectins (Dardelle et al. 2010; Ferguson et al. 1998; Wang et al. 2013; Chebli et al. 2012, see also Chap. 3). Pectin, one of the major cell wall components, acts as a gel matrix to provide an amorphous substrate for embedding celluloses, hemicelluloses, and ions to form a complex structural network. Pectin demethylesterification and remodeling catalyzed by pectin methylesterase (PME) have major roles in determining the rigidity and texture of plant cell wall. The major pectin is a homogalacturonan that is cross-linked to two types of rhamnogalacturonans (Bosch et al. 2005; McKenna et al. 2009). The homogalacturonans are synthesized in the Golgi apparatus in a highly methylesterified form and are

released into the apoplast at the pollen tube tip (Bosch et al. 2005; Li et al. 2002). Through the action of PME at the tip, the secreted pectins are de-esterified, a process which also involves the release of protons (Bosch et al. 2005; McKenna et al. 2009; Micheli 2001). The resulting acidification of the tip stimulates the activity of cell wall hydrolases which lead to cell wall loosening and therefore enhances cell expansion. The de-esterified pectins then become cross-linked by Ca^{2+} leading to a stiffening of the wall in the flanks behind the tip (Bosch et al. 2005; McKenna et al. 2009; Parre and Geitmann 2005; Chebli et al. 2012). Thus, the activity of PME is necessary for changes in cell wall rheology and is therefore crucial for pollen tube growth. However, the regulation and molecular details of the post-Golgi exocytic trafficking and targeting of PME to the apoplast remain exclusive.

Tip growth is oscillatory with alternating rapid and slow phases (Feijo et al. 2001; Cai and Cresti 2009; Hill et al. 2012) and is regulated by a highly dynamic actin cytoskeleton (Cai and Cresti 2009; Fu 2010; Gebert et al. 2008), which ensures a regular supply of exocytic vesicles at the tip, and is also responsible for the organized retrieval of excess membrane from the tip (endocytic membrane trafficking). As judged by the existence of Ca^{2+} and H^{+} gradients, the cytoplasm at the tip is highly polarized (Feijo et al. 2001; Hepler et al. 2012). Crucial modulators of Ca^{2+} concentration and actin dynamics at the tip are membrane-located Rho GTPases (Gu et al. 2003; Kost 2008). The localization of RabA4d, a ROP1-type GTPase required for successful exocytosis in pollen tubes, to the V-shaped clear zone, suggests that the clear zone vesicles are exocytic in character (Szumlanski and Nielsen 2009). Thus, ROP1 plays a master role in exocytic vesicle targeting and is present as an apical cap at the tube apex although the underlying mechanisms of the ROP1 signaling network remain to be explored (Gu et al. 2003; Kost 2008; Lee et al. 2008; Szumlanski and Nielsen 2009). For the bulk of this chapter, we will focus on recent progress in exocytic protein trafficking in pollen tubes and the molecular machinery involved. We will particularly discuss the pertinent questions for a future understanding of the functional roles of exocytosis in cell polarization and polar cell wall formation.

6.2 Post-Golgi Exocytosis in the Pollen Tube

Conventional exocytosis is a conserved protein trafficking pathway in eukaryotic cells (Burgess and Kelly 1987). Usually, the newly synthesized proteins are transported from ER to Golgi apparatus for glycosylation, other modifications, and folding. Thereafter, the proteins are further delivered to the *trans*-Golgi network (TGN), packed into exocytic vesicles, and transported to the PM for fusion and protein release. Thus, the ER-Golgi-TGN-PM exocytic pathway is a classical and well-studied protein trafficking process which serves as the major transporting pathway for most of the secretory proteins targeted to the PM or extracellular space (Fig. 6.1). In addition, it is interesting to note that the TGN functions as a transport hub for protein sorting and trafficking by integrating exocytosis, endocytosis, and

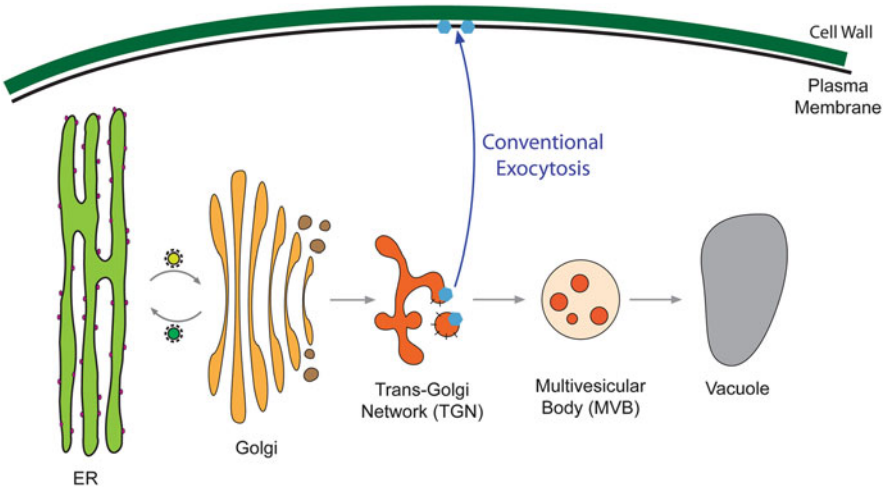

Fig. 6.1 Conventional TGN-mediated exocytosis in plant cells. The conventional exocytosis in plant cells starts from endoplasmic reticulum (ER). The exocytic proteins were first synthesized in the ER. They were then transported to Golgi apparatus for further modifications and proper folding. Thereafter, they were delivered to *trans*-Golgi network (TGN) for sorting and packaging. TGN functions as a critical joint organelle for multiple endomembrane trafficking pathways such as secretory pathway to the vacuole via multivesicular body (MVB). The exocytic proteins were further transported from the TGN to the plasma membrane (PM) or the apoplast

secretory pathways (Jurgens and Geldner 2002; Lam et al. 2007b; Viotti et al. 2010; Tse et al. 2004; Cui et al. 2016; Fig. 6.1). Recent studies have further revealed that different domains of the TGN function respectively in recognizing different cargo proteins prior to sorting and trafficking (Chow et al. 2008; Gleeson et al. 2004; Naramoto et al. 2014). However, in addition to the conventional ER-Golgi-TGN-PM exocytosis, it is of interest to note that unconventional protein secretion (UPS) pathways exist in animal cells and yeast (Malhotra 2013; Zhang and Schekman 2013; Rabouille et al. 2012; Robinson et al. 2016). In plants, UPS usually functions under stress conditions such as pathogen attacks (Ding et al. 2012; Nickel and Rabouille 2009). In addition, recent studies have demonstrated the existence of an exocyst-positive organelle (EXPO)-mediated UPS, indicating that multiple exocytosis pathways are likely to cooperate in plant cells (Ding et al. 2012, 2014; Wang et al. 2010b). In addition to EXPO-mediated UPS, evidence exists for other kinds of UPS, although their underlying molecular mechanisms and biological functions remain largely unexplored (Boutte et al. 2013; Crowell et al. 2009; De Caroli et al. 2011; McFarlane et al. 2013; Poulsen et al. 2014). Although conventional Golgi-TGN-PM exocytic transport mediates a large proportion of the proteins targeted to the PM or extracellular space, UPS appears to perform significant complimentary functions under various environmental conditions or in different plant cell types. This suggests that there are different exocytic routes and mechanisms underlying the regulation of protein secretion in the plant kingdom.

In general plant cells do not move, except for a few highly specialized cells such as pollen tubes which can reach rates of cell elongation up to 1 cm h^{-1} (Bedinger et al. 1994; Hepler et al. 2013). In order to support this rapid and tip-focused pollen tube growth, almost all of the cellular activities including the speed of cytoplasmic streaming, rates of protein synthesis and degradation, efficiency of endomembrane sorting and trafficking, and spatiotemporal reorganization of cytoskeleton are increased to an incredibly high level to support polarized tip expansion when compared to ordinary plant cells (Altartouri and Geitmann 2015; Cai and Cresti 2009; Chebli et al. 2013; Hepler et al. 2001; Keller and Simons 1997; Li et al. 2012; Winship et al. 2011).

In growing pollen tubes, large amounts of the newly synthesized proteins such as transporters, receptors, and cell wall modification enzymes are polarly transported to the tip region via the post-Golgi exocytic pathway. Ultrastructural imaging by transmission electron microscopy (TEM) showed a huge number of tiny single-membrane-bounded vesicles which are 80–150 nm in diameter and fully occupy the pollen tube apical region (Kroeger and Geitmann 2012; Kroeger et al. 2009; Wang et al. 2011a; Weber et al. 2013; Winship et al. 2011). This suggests that the rapid and polarized pollen tube tip expansion is supported by a vigorous polar exocytic vesicle fusion at the apical membrane (Cai and Cresti 2009; Hepler et al. 2001; Kost 2008; Winship et al. 2011; Chebli et al. 2013; Sanati Nezhad et al. 2014).

Recent studies have found that plasma membrane-localized receptor-like kinases (RLKs) at the pollen tube tip control pollen tube growth and mediate male perception during the double fertilization procedure in flowering plants. This solves the problem as to how pollen tubes are attracted by the flower ovules and reveals the molecular basis of this male and female communication process (Takeuchi and Higashiyama 2016; Wang et al. 2016b; see also Chap. 9). However, exactly how RLKs are exocytosed and targeted to the pollen tube apical PM and its underlying regulating mechanism require further exploration.

6.3 Polar Protein Exocytosis in Growing Pollen Tubes

Exocytosis in growing pollen tubes is highly specialized and focused at the tip region to support rapid cell growth. Not surprisingly, cell wall extensibility is one of the key factors in controlling pollen tube growth and morphogenesis (Zonia and Munnik 2011; Hepler et al. 2013; Rojas et al. 2011). The cell wall compositions in the tip and shank regions of the pollen tube are spatiotemporally different and tightly regulated when compared to non-polarized plant cells (Wang et al. 2013; Zonia and Munnik 2011; Dumont et al. 2014; Rounds et al. 2014; Hepler et al. 2013; Rojas et al. 2011; Dardelle et al. 2010b; Rockel et al. 2008). The apical cell wall of the pollen tube is mainly composed of a thin layer of pectin and is mechanically soft enough to allow for fast cell expansion. Whereas the distal pollen tube cell wall, which has both a primary and a secondary cell wall, is hard enough to support the tubular shape. Pollen tube growth oscillation and rate depend largely

on modification and rigidity of the pectic cell wall. However, the detailed molecular machinery controlling polar transportation and exocytosis of pectin and PME to the pollen tube apex remains elusive.

Recent studies have shown that PMEs belong to a large multigene family which share conserved primary and quaternary structures among plant taxa (Weber et al. 2013; Saez-Aguayo et al. 2013; Pelloux et al. 2007; Lionetti et al. 2007; Di Matteo et al. 2005; Jiang et al. 2005). Further research has demonstrated the functional roles of PMEs in plant reproduction, growth, and defense (Weber et al. 2013; Lionetti et al. 2007; Jiang et al. 2005; Wang et al. 2013). A GFP chimeric fusion with *Nicotiana tabacum* pollen pectin methylesterase 1 (NtPPME1) has shown that NtPPME1 is predominantly localized on the apical surface (Fig. 6.2a, b) with some intracellular punctate dots. NtPPME appears to regulate the cell wall dynamics and rheology of the growing pollen tube (Wang et al. 2013, 2016a; Bosch et al. 2005). It is generally believed that PMEs are exocytosed to the apoplast via Golgi-derived vesicles although the detailed post-Golgi route remains unclear. NtPPME1 can therefore be used as a useful tool to study the dynamics and regulation of post-Golgi exocytosis. To better understand the exocytosis of NtPPME1, secretory carrier membrane protein (SCAMP) which is secreted to the PM via the conventional ER-Golgi-TGN-PM route can be included as a control (Cai et al. 2011; Lam et al. 2007a). The expression pattern of a GFP chimeric fusion with *Arabidopsis* SCAMP3 (AtSCAMP3) in the growing pollen tube is given in Fig. 6.2a, c (Wang and Jiang 2011; Wang et al. 2010a, 2011a, b, 2013, 2016a).

Previous attempts at identifying the exact site of exocytosis at the pollen tube tip have been inconclusive (Chebli et al. 2013; Lee et al. 2008; Wang et al. 2013). Recently, however, we have been able to determine accurately exocytic sites for NtPPME1 at the pollen tube tip using fluorescence recovery after photobleaching (FRAP) which has been extensively employed to follow protein dynamics and targeting. We photobleached a median section of the tip region of a growing pollen tube expressing NtPPME1-GFP and traced the signal recovery caused by exocytosis of NtPPME1 into the apoplast at the pollen tube tip (Fig. 6.2d). Before photobleaching, NtPPME1-GFP was concentrated at the apical surface of the growing pollen tube. The bleached area is indicated by the white circle. The exocytosis of NtPPME1 signal is first recovered in the center of pollen tube apical dome and then gradually expanded laterally, as indicated by the numbers 1–3 in Fig. 6.2d. The FRAP analysis of NtPPME1-GFP thus reveals precisely the apical targeting of NtPPME1 and the exocytic process itself in the growing pollen tube. FRAP analysis of the PM-localized GFP-AtSCAMP3 is shown in Fig. 6.2e. The result showed that the polar exocytic targeting of GFP-AtSCAMP3 from TGN to PM is also in the central apex of the pollen tube tip. Taken together, these results demonstrate unequivocally that the center of apical dome is the active exocytic zone for both NtPPME1 and AtSCAMP3 during polar pollen tube growth.

To further characterize the exocytic dynamics and route of NtPPME1 in the pollen tube tip, variable-angle epifluorescence microscopy (VAEM) imaging of a growing pollen tube tip expressing NtPPME1-GFP was performed. The general principle of VEAM imaging of growing pollen tubes is given in Fig. 6.3a. Just as

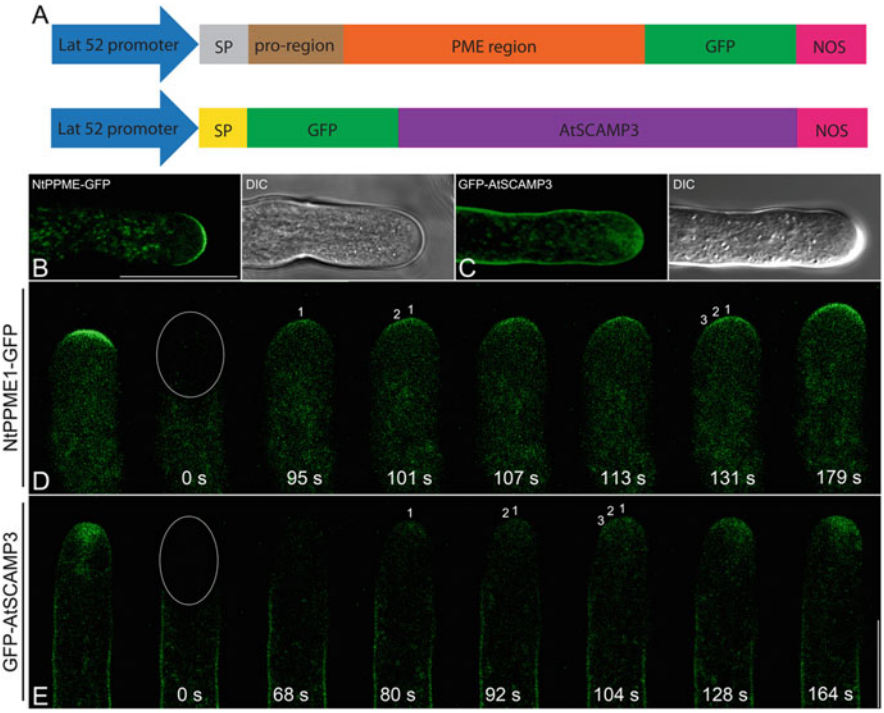

Fig. 6.2 The exocytic process of NtPPME1 and AtSCAMP3 in growing pollen tubes. (**a**) Chimeric fusion of NtPPME1 and AtSCAMP3 with GFP under Lat 52 promoter. (**b**) NtPPME1-GFP predominantly localized in the pollen tube tip apex with some cytosolic punctate dots in the pollen tube. (**c**) GFP-AtSCAMP3 localized to the plasma membrane via the conventional exocytic process and endocytosed into the cell dominantly from the pollen tube tip. Scale bar in (**b**) and (**c**), 25 μm. (**d**) FRAP analysis of NtPPME1 exocytic site in growing pollen tubes. Series of images were shown before bleaching, immediately after bleaching (0 s), and recovery of fluorescence after bleaching at the indicated time points. The bleached area was indicated by the *white circle*. Numbers of 1–3 indicate the order of signal recovery. (**e**) FRAP analysis of AtSCAMP3 exocytic site in growing pollen tubes. Series of images were shown before bleaching, immediately after bleaching (0 s), and recovery of fluorescence after bleaching at the indicated time points. The bleached area was indicated by the *white circle*. Numbers of 1–3 indicate the order of signal recovery. Scale bar in (**d**) and (**e**), 25 μm

in total internal reflection fluorescence microscopy (TIRFM), the illuminating laser in VAEM was twisted, but unlike TIRFM, the incident angle is greater than zero degrees for visualizing exocytic events at or near the PM of the pollen tube tip. The entire pollen tube VAEM time-lapse images were then used for a kymograph analysis to ensure all of the exocytic motions and fusions of NtPPME1-GFP in the pollen tube tip were included and reliably tracked. To make the interpretation of the kymographic analysis easier to understand, three types of representative dynamics of vesicles are demonstrated in three different colors (see Fig. 6.3b). Ongoing vesicle fusion with the pollen tube apical PM is colored in green. The red-colored

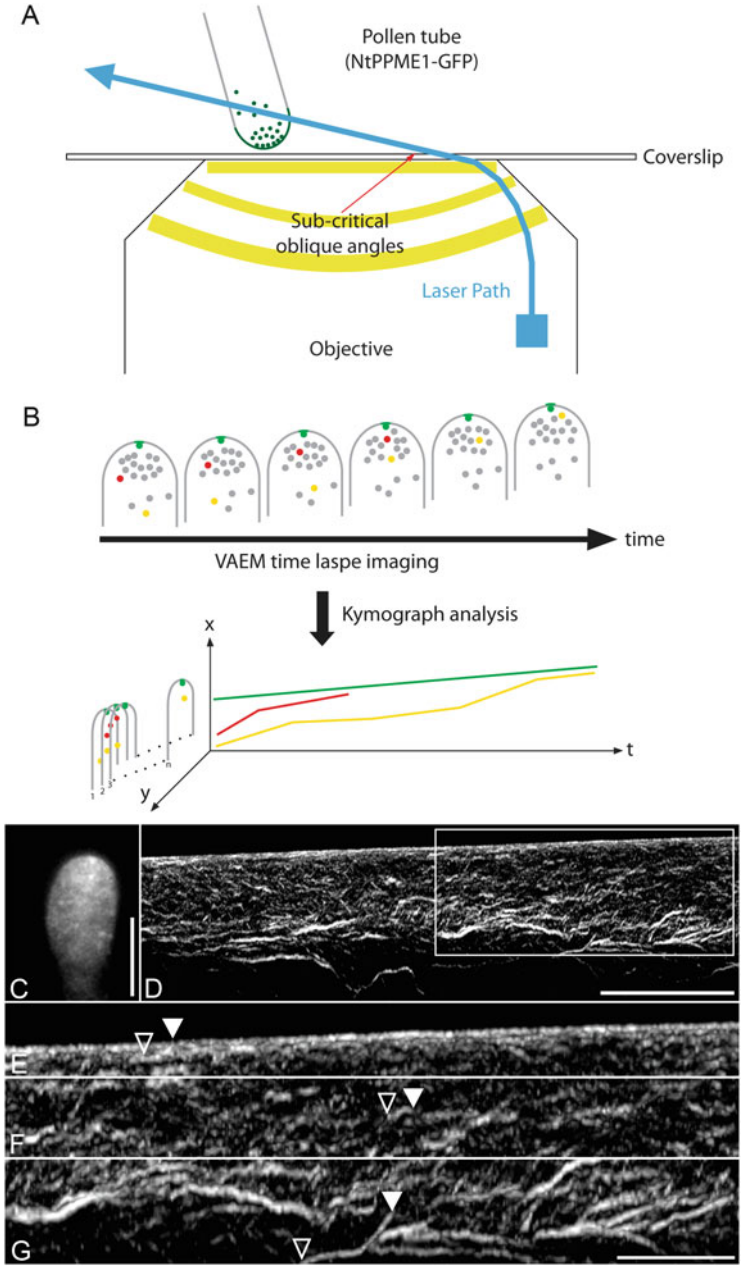

Fig. 6.3 Dynamic analysis of NtPPME1 polar exocytosis in growing pollen tube tips. (**a**) Schematic of VAEM imaging of a growing pollen tube tip. (**b**) Schematic of the kymograph analysis of entire VAEM time-series images of a pollen tube tip. Different types of vesicle dynamics in the tip region were highlighted in different colors. The *green dots* indicate the exocytic vesicle fusion with the apical membrane. The *red dots* represent the come-and-go vesicle

vesicle represents come-and-go vesicles due to moving out of the focal plane during the VAEM imaging. Vesicles continuing to move in the tip region of the pollen tube but without exocytic fusion are highlighted in yellow. A representative scheme for the time-lapse kymograph analysis of using the VAEM time-lapse images from 1 to n is shown in Fig. 6.3b. The dynamic routes of three types of vesicles were revealed along the time axis.

A representative VAEM image of a pollen tube expressing NtPPME1-GFP shows that NtPPME1-GFP-positive polar exocytic vesicles accumulate underneath the apical surface and in the subapical region of the pollen tube (Fig. 6.3c). Kymographic analysis of the whole VAEM time-lapse images including all of the exocytic dynamic tracks of NtPPME1-GFP during imaging as performed are shown in Fig. 6.3d. It reveals the exocytic tracks of polar secretory vesicles containing NtPPME1 to the pollen tube apical surface, and the dynamic differences of NtPPME1-GFP in different regions of the growing pollen tube tip (Fig. 6.3d). The observations demonstrate directly the dynamics of polar exocytosis of NtPPME1-GFP in the growing pollen tube tip. Enlarged data from areas indicated in Fig. 6.3d are shown separately in Fig. 6.3e–g.

Underneath the PM at the pollen tube apex, exocytic vesicles labeled with NtPPME1-GFP were visualized continually fusing with the expanding apical PM as judged by the continuous fluorescence signals (Fig. 6.3e). The acclivitous signal points to a rapid expansion of the pollen tube apex and is supported by the numerous and continued fusion events of exocytic vesicles containing NtPPME1-GFP at the tip. A representative track of exocytic vesicle fusion with the PM was indicated by hollow and solid arrow heads as shown Fig. 6.3e. However, because the apical exocytic vesicles move vigorously in and out of the imaging focal plane as well as the vesicular PM fusion event occurs rapidly, it is a challenging technique to fully track the entire fusion process of exocytic vesicle with the apical PM. In addition, if the exocytic vesicles disappear in the *z*-axis or fail to fuse with the pollen tube apex, the continued fluorescence signal will be disrupted. In the subapical region underneath the pollen tube apex, the tracks of NtPPME1-GFP-positive vesicles were

Fig. 6.3 (continued) which moves out of the focal plane during imaging. The *yellow dots* indicate the dynamics of vesicle moves underneath the apical PM without fusion with the apical PM. After time-lapse VAEM imaging, images from time point 1 to *n* were applied for the whole image kymograph analysis. The dynamic tracks of three different types of vesicles were shown. (**c**) Variable-angle epifluorescence microscopy (VAEM) image of a growing pollen tube expressing NtPPME1-GFP. (**d**) Entire image kymograph analysis of (**d**) shows the polar exocytosis and targeting of NtPPME1 to the pollen tube tip, and the dynamic differences of NtPPME1-GFP in pollen tube apical surface and subapical and shank regions. Scale bar, 70 s. (**e**) Enlarged kymograph of apical surface region from the indicated area in (**e**) showed consistent NtPPME1-GFP exocytic fusion with the PM during pollen tube tip expansion. **(f** and **g)** Enlarged kymograph from the indicated area in (**e**) showed dynamic differences of NtPPME1-GFP in subapical and shank region of growing pollen tube. Punctate appeared, remained static, and disappeared. The *hollow* and *solid arrows* indicate the beginning and end of NtPPME1-GFP dynamic events, respectively. Scale bar, 20 s

short and less continuous (Fig. 6.3f). The hollow and the solid arrows indicate examples at the beginning and end of NtPPME1-GFP dynamics, respectively. In contrast, the movements of NtPPME1-GFP in the more distally located pollen tube subapical region were much more continuous and longer (Fig. 6.3g). Therefore, trafficking tracks of NtPPME1-GFP in different regions of pollen tube tip are distinct, reflecting different exocytic dynamics, which is in agreement with previous working models for vesicle dynamics in growing pollen tubes (Bove et al. 2008; Kroeger et al. 2009).

6.4 A Distinct Polar Exocytic Pathway of NtPPME1 for Plant Cell Wall Formation

Although NtPPME1 matures in the Golgi apparatus and thereafter is exocytosed into the apoplast, details of the post-Golgi exocytic process of NtPPME1 and the underlying regulatory mechanism have remained unclear. By using a combination of conventional laser scanning confocal microscopy and newly developed imaging technology (stochastic optical reconstruction microscopy, STORM), together with pharmaceutical treatments, the exocytosis of NtPPME1 has been shown to be mediated by a novel endomembrane compartment which is distinct from the conventional exocytic trafficking organelles (*cis*- and *trans*-cisternae of the Golgi apparatus, the TGN) as well as the prevacuolar compartment (PCV) and endocytic vesicles (Wang et al. 2016a).

Further ultrastructural studies on the NtPPME1 exocytosis-mediated compartment by using high-pressure freezing and substitution (HPF) for pollen tube fixation (Fig. 6.4a) and immuno-EM with specific antibodies against NtPPME1 have revealed that Golgi-derived secretory vesicles (GDSVs), which are directly released from the *trans*-face of Golgi apparatus, mediate the polar exocytosis of NtPPME1 to the apoplast at the tip (Fig. 6.4b). To further confirm that the subcellular localization of NtPPME1 is distinct from TGN, an OsSCAMP1 antibody which specifically labels the TGN in plant cells was used as a control (Lam et al. 2007a; Wang 2016; Wang et al. 2010a, 2011a, b; Cai et al. 2011). As shown in Fig. 6.4c, the TGN was specifically labeled by the OsSCAMP1 antibodies in the pollen tube. Therefore, GDSVs mediate the distinct exocytic pathway of NtPPME1 from the Golgi apparatus to the apical surface of the pollen tube.

In plant cells, in addition to the highly polarized tip-growing pollen tube, the cell plate is usually regarded as an example for “internal cell polarity” in non-polarized cells (Dhonukshe et al. 2006; Tahara et al. 2005; Nishihama et al. 2002; Jurgens et al. 2015). Plant cells spatiotemporally coordinate the polarized transport to and from the growing cell plate at very high rates via exocytosis and endocytosis (Boruc and Van Damme 2015; Dhonukshe et al. 2006; Reichardt et al. 2007). Expression of NtPPME1-GFP in BY2 cells shows that NtPPME1 is also localized on the forming cell plate during cytokinesis (Fig. 6.5a). Further subcellular localization

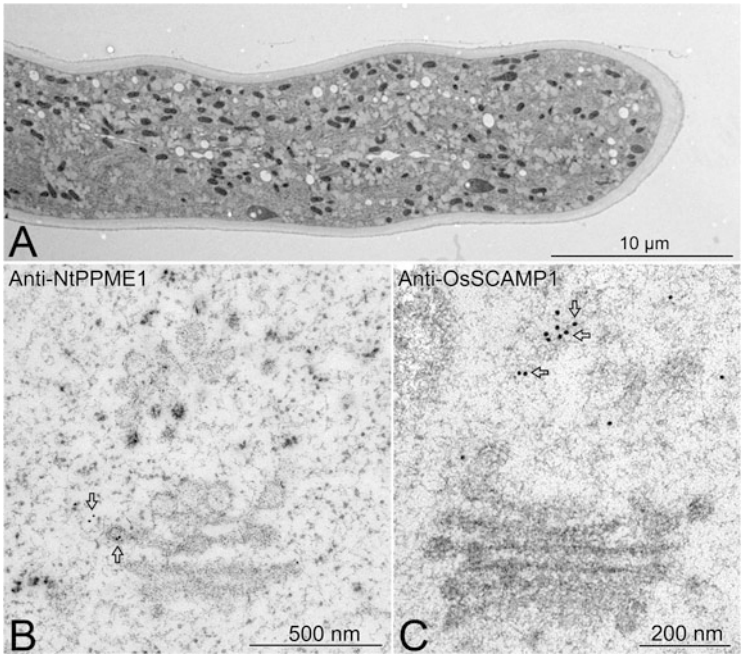

Fig. 6.4 NtPPME1 defined Golgi-derived secretory vesicle as a distinct exocytic compartment for pollen tube tip growth. (**a**) Overview of the ultrastructure of a tobacco pollen tube prepared by high-pressure frozen/freeze substitution. (**b**) NtPPME1 antibodies specifically labeled the Golgi-derived secretory vesicles near the *trans*-face of Golgi apparatus indicated by *arrows*. (**c**) OsSCAMP1 antibodies recognized the *trans*-Golgi network (TGN) as indicated by *arrows*

studies and pharmaceutical treatments during cell plate formation have revealed the novel localization and drug resistance of NtPPME1-GFP-positive intercellular compartments in BY2 cells (Wang et al. 2016a). However, it is of interest to note that NtPPME1-GFP is delivered to the vacuole for degradation in nondividing BY2 cells (Fig. 6.5b). This indicates that the exocytosis of NtPPME1 is functionally activated by the development of a polarization process. It also suggests that polarization in both pollen tube growth and cell plate formation results in the development of an unconventional post-Golgi exocytic pathway.

Previous studies have demonstrated that both the secretory and endocytic pathways participate in transporting membrane to and from the developing cell plate (Dhonukshe et al. 2006; Reichardt et al. 2007; Jurgens et al. 2015; Lam et al. 2008). New cell wall formation occurs during cell plate extension to ensure the success of plant cell division. In this regard, JIM5 and JIM7 are useful antibodies for specifically recognizing demethylesterified versus methylesterified pectins, respectively. However, it is still unclear whether pectins and PMEs are transported within the same exocytic vesicle or not. Immunofluorescent labeling shows that NtPPME1-GFP-positive vesicles are different from JIM7-labeled vesicles (Wang et al. 2016a).

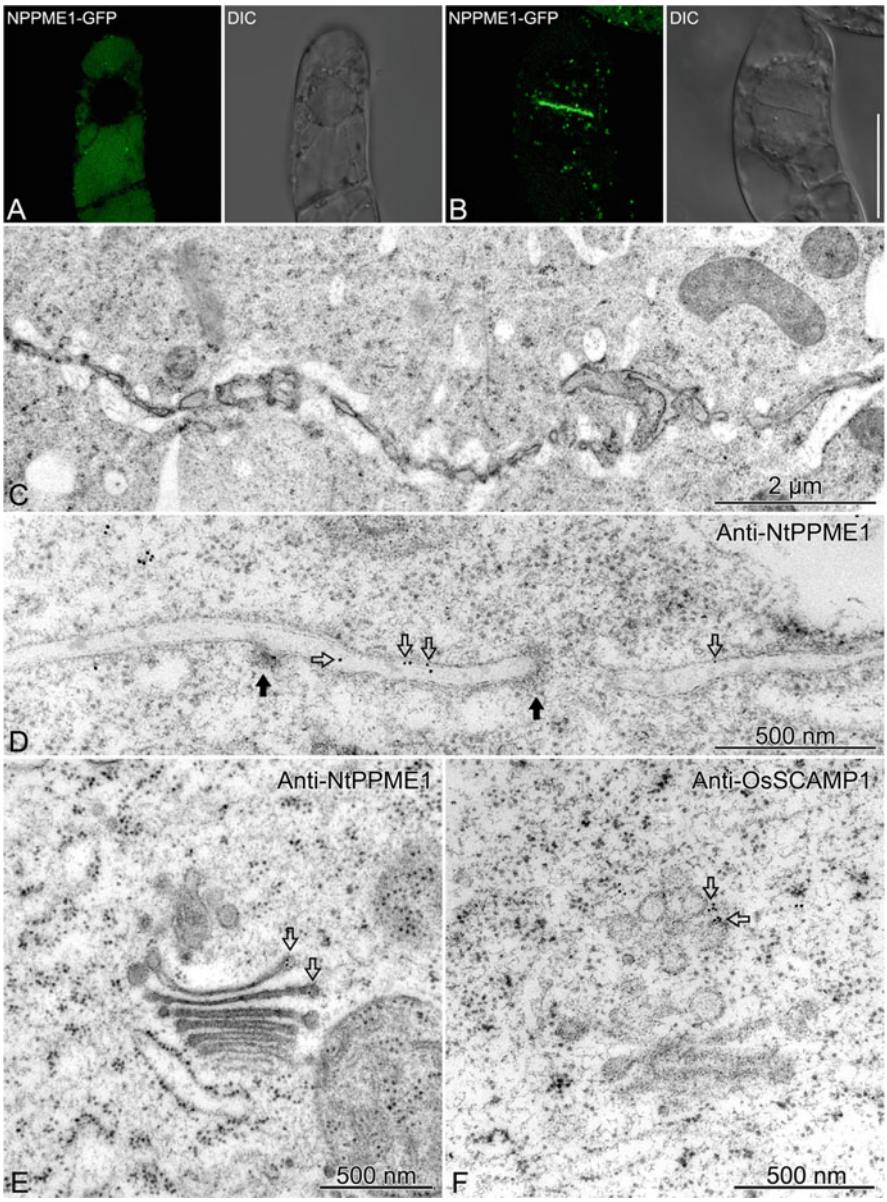

Fig. 6.5 Polar exocytosis of NtPPME1 to the forming cell plate is mediated by GDSV. (**a**) NtPPME1-GFP was polarity associated and highlighted the forming cell plate during cytokinesis in transgenic tobacco BY2 cells but degraded in vacuole of nondividing cell without polarity (**b**). Scale bar in (**a**) and (**b**), 50 μm. (**c**) Overview of the ultrastructure of a cell plate during tobacco BY2 cell division. (**d**) NtPPME1 antibody labeled the cell plate in BY2 cells (*hollow arrows*), whereas the clathrin-coated pits (CCP) were not labeled (*solid arrows*). (**e**) NtPPME1 antibodies specifically labeled the vesicles at the *trans*-face of Golgi apparatus (*arrows*), whereas OsSCAMP1 antibodies recognized TGN indicated by *arrows* in (**f**)

In addition, NtPPME1-GFP-containing vesicles are distinct from JIM5-labeled ones which are possibly derived from endocytic pathway. These results support the hypothesis that highly methylesterified pectins are packed and transported to the cell surface differently to PME probably to avoid their earlier interactions prior to deposition into the apoplast (Pelloux et al. 2007; Bosch et al. 2005). Moreover, KNOLLE, a specialized syntaxin (Q_a-SNARE) which is only expressed in the M-phase of the cell cycle, is required for proper targeting and function in membrane fusion with the cell plate during cytokinesis (Jurgens et al. 2015; Touihri et al. 2011; Teh et al. 2013). NtPPME1-GFP-positive compartments are clearly separate from the endosomes labeled by specific KNOLLE antibodies during cell plate formation (Wang et al. 2016a).

Further detailed ultrastructural characterization of the subcellular localization of NtPPME1 in dividing BY2 cells as prepared by HPF (Fig. 6.5c) and immunogold TEM confirms that NtPPME1 specifically localizes to the forming cell plate as indicated by the hollow arrows in Fig. 6.5d, whereas NtPPME1 antibody labeling was absent from the clathrin-coated pits (CCPs) indicated by the solid arrows on the cell plate. In contrast, GDSVs were specifically labeled by NtPPME1 antibodies, whereas the TGN was labeled by OsSCAMP1 antibodies (Fig. 6.5e and f, Wang et al. 2016a).

Taken together, the exocytosis of NtPPME1 mediated by GDSVs is a highly polarized exocytic process which is specific for cell wall formation in both pollen tube tip growth and cytokinesis. It suggests that plant cells set up unconventional post-Golgi exocytic routes to compliment the conventional exocytic pathway under specific polarizing growth conditions.

6.5 Regulation Machinery of NtPPME1 Exocytosis

To achieve efficient tip growth and maintain cylindrical cell shape, pollen tubes have to tightly coordinate the structural organization with vesicular transport. This implies a requirement for sophisticated signaling networks. The regulation and feedbacks of such signaling networks may directly or indirectly affect the activities of exocytosis and endocytosis in pollen tubes, ultimately leading to alterations in the polarity and/or growth of pollen tubes.

A family of small GTPase termed Rho of plants (ROP), which belong to a highly conserved subfamily of Rho GTPases, act as one of the central regulators of pollen tube tip growth (Gu et al. 2003; Klahre and Kost 2006; Lee et al. 2008; Miyawaki and Yang 2014; Nibau et al. 2006). It functions as a molecular switch by binding, together with GTP or GDP, to biologically active or inactive downstream signaling elements for the regulation of tip growth. In *Arabidopsis* and tobacco, several pollen-specific ROPs have been found to be predominantly localized to the pollen tube apex (Hwang et al. 2005; Klahre and Kost 2006; Kost 2008; Nibau

et al. 2006; Szumlanski and Nielsen 2009; Zhou et al. 2015). ROP1 is one of the best characterized ROPs and specifically functions in determining the polarity of pollen tube at the apical PM. Constitutive expression of an active form of ROP1 (CA-ROP1) dramatically increases the activities of ROP1, causing a balloon-shaped pollen tube tip and cell depolarization, whereas disrupting ROP1 signaling by expression of domain-negative ROP1 (DN-ROP1) strongly inhibits pollen tube tip growth (Lee et al. 2008; Sun et al. 2015; Szumlanski and Nielsen 2009).

Further studies have revealed that the behavior and function of the highly dynamical populations of apical F-actin are regulated by the ROP1 signaling network (Fu 2015; Cheung et al. 2008, 2010; Liu et al. 2015; Qu et al. 2015, 2013; Daher and Geitmann 2011; Wu et al. 2010; Ren and Xiang 2007; Su et al. 2012). ROP1 activates two downstream pathways: the RIC4- and RIC3-dependent pathways. Recent studies have suggested that RIC4-dependent F-actin increases polar accumulation of exocytic vesicles at the tip by promoting actin assembly, whereas RIC3 can cause Ca^{2+} accumulation leading to F-actin disassembly. CA-ROP1 can enhance the accumulation of exocytic vesicles to the apical cortex of pollen tubes, leading to a balloon-shaped tip and resulting in a loss of cell polarity. Expression of DN-ROP1 can promote disassembly of F-actin and prevent polar exocytic vesicle transport and fusion with the apical PM (Fu et al. 2009; Lee et al. 2008). More recent evidence has shown that RIC1, another newly identified ROP effector, regulates the abundance and turnover of apical F-actin by directly interacting with actin in a Ca^{2+}-dependent manner (Fu 2015; Gu et al. 2003; Kost 2008; Lee et al. 2008; Chen et al. 2003). However, the origins and identities of these polar exocytic vesicles regulated by ROP1 remain elusive.

NtPPME1-GFP, which indicates GDSV-mediated polar exocytosis, has been co-expressed either with CA-ROP1 or DN-ROP1 to test the functions of ROP1 in regulating unconventional exocytosis in pollen tube growth (Wang et al. 2016a). GFP-AtSCAMP3, exocytosed to the PM by conventional exocytosis, was also co-expressed with CA-ROP1 and DN-ROP1 as a control. It is interesting to note that ROP1 controls both the polar exocytosis and targeting of NtPPME1 as well as AtSCAMP3 to the pollen tube apex, although NtPPME1 resides on an exocytic pathway completely distinct to that of AtSCAMP3. Besides being a key regulator of the highly polarized pollen tube system, the activities and distributions of ROP1 are also found in the forming cell plate during cytokinesis (Wang et al. 2016a). Expression of the RFP chimeric fusion of ROP1 reveals that it localizes on the cell plate in tobacco BY2 cells. FRAP analysis of CA-ROP1 or DN-ROP1 in NtPPME1-GFP expressions in transgenic BY2 cells showed that ROP1 controls the polar targeting of NtPPME1 to the forming cell plate and affects cell plate extension during cytokinesis. This suggests that besides assuming a master role in controlling pollen tube exocytosis and cell polarity, ROP1 has more general roles in controlling the polarized exocytosis process in plant cells (Wang et al. 2016a).

6.6 Conclusion and Perspective

It is now established that there is a distinct type of polar exocytosis mediated by GDSVs, which is needed for plant pectic cell wall formation and is directly derived from the *trans*-face of Golgi apparatus. This alternative Golgi-to-PM polar exocytic pathway mediates the delivery of NtPPME1 to the apoplast for cell wall rigidity modification during pollen tube tip growth and cell plate formation and is regulated by ROP1 (Wang et al. 2016a). A hypothetical model for this pathway is presented in Fig. 6.6. For both the classical TGN-mediated exocytosis of SCAMP and the unconventional GDSV-mediated NtPPME1 exocytosis, ROP1 acts as a

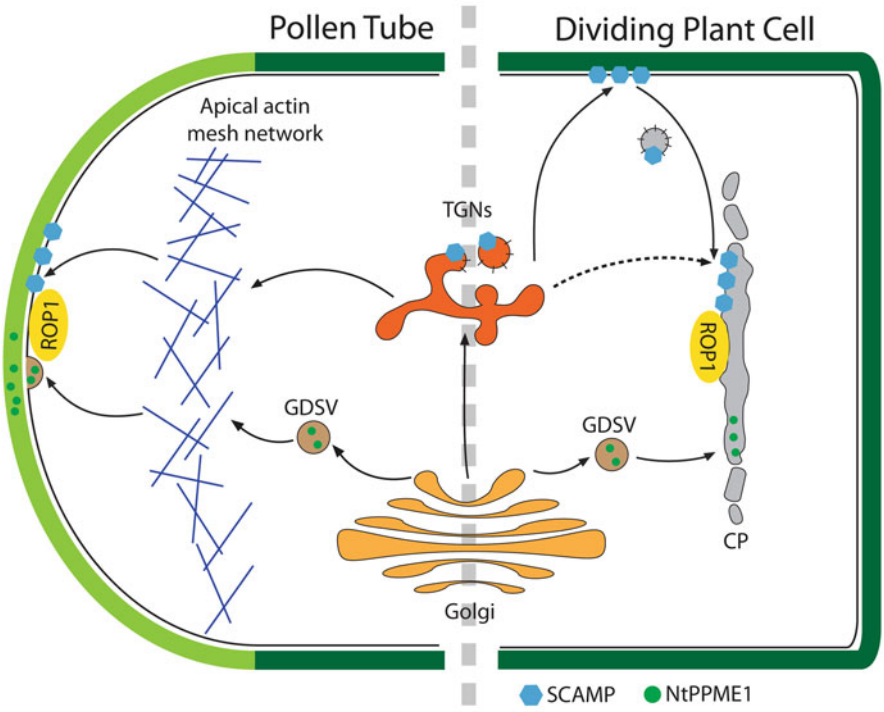

Fig. 6.6 A hypothetical working model of the GDSVs participates in polar protein transport for the cell wall formation. In the growing pollen tube, NtPPME is exocytosed from Golgi by GDSVs to the pollen tube tip and released into the apoplast for the modification of the highly esterified pectin (*light green*) into de-esterified pectin (*dark green*). The GDSV-mediated exocytic of NtPPME1 is distinct from the conventional TGN-mediated exocytosis process in which SCAMP is involved. In addition, during the cell plate formation in non-polarized plant cells, NtPPME1 is polar secreted to the forming cell plate via GDSV which is directly derived from *trans*-side of Golgi apparatus by-passing TGN. For both highly polarized pollen tube growth and cell plate extension, Rop1 functions as a master regulator in controlling the distinct polar exocytosis of NtPPME1 as well as the conventional TGN-mediated exocytosis of SCAMP

master upstream regulator of the growing pollen tube tip and cell plate formation. In addition, the emerging roles of unconventional protein secretion pathways in plant cells generate some thinking about their distinct biological significances and diverse functions under various developmental and environmental conditions or in different cell types in plants. However, many challenging questions about this GDSV-mediated polar exocytic pathway lay ahead of us in future studies. What is the biological significance of GDSV-mediated exocytosis? What other cargos are transported via this polar GDSV-mediated exocytic pathway? What are the specific up-/downstream co-effectors or regulators for the GDSV-mediated exocytosis? What are the underlying molecular regulation networks for this distinct polar exocytosis? To understand these questions, a multidisciplinary approach combining genetics, organelle proteomics, biochemistry, and bio-imaging technologies is required. A more complete knowledge of understanding the molecular working machinery of the unconventional polar exocytosis and its interactions with conventional protein trafficking is needed to gain a better understanding of polarized cell growth and cell polarization.

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Chapter 7
Pollen Tip Growth: Control of Cellular Morphogenesis Through Intracellular Trafficking

Hana Rakusová and Anja Geitmann

Abstract The control of cellular growth in pollen tubes occurs through the fine-tuning of intracellular transport and secretion processes. This does not only apply to the basic genesis of the cylindrical cell through polar expansion but also to the pollen tube's specialized skills including its capacity to respond to directional guidance cues and its ability to perform invasive growth. The control of these specialized activities by intracellular trafficking occurs through the strategic deposition of cell wall material and cell wall modifying agents that soften or stiffen the wall with the aim to regulate the cell wall's mechanical properties both in time and space. Directional and invasive growth of the pollen tube is crucial for successful sperm delivery and fertilization. The mechanisms underlying the regulation and logistics of the endomembrane trafficking in the pollen tube therefore have a direct impact on pollen tube elongation and male fertility. Here, we relate pollen tube morphogenesis and its biological functionality to the intracellular processes that control cellular growth behavior and allow the cell to respond to environmental cues.

Keywords Pollen tube • Tip growth • Cell wall mechanics • Vesicle trafficking • Exocytosis

Abbreviations

CalSs	Callose synthases
CESAs	Cellulose synthases A
FRAP	Fluorescence recovery after photobleaching
HG	Homogalacturonan
PGs	Polygalacturonases
PME	Pectin methyl esterase
PMEIs	Pectin methyl esterase inhibitors

H. Rakusová • A. Geitmann (✉)
Department of Plant Science, McGill University, Montreal, QC, Canada
e-mail: anja.geitmann@mcgill.ca

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RIPs	ROP-interacting partners
ROPs	Rho family of GTPases
SNAREs	Soluble NSF attachment protein receptors
SYP	Syntaxin of plants
TGN	Trans-Golgi network

7.1 Introduction

The fertilization success of the male gametophyte in plants relies on the speed with which it outcompetes its competitors on the way to the finite number of receptive ovules, its efficiency to find these ovules and deliver the sperm cells, and its capacity to drill its way through or around any mechanical obstacles along its path. Pollen tube elongation occurs in highly polar manner and requires the delivery of cell wall and membrane material as well as enzymes and other agents to the growing tip. This applies to the basic growth process that produces the characteristic cylindrical morphology of the cell and becomes even more critically important when the pollen tube has to accomplish specific tasks such as responding to a guidance cue or navigating a mechanical obstacle. These specific tasks require a tight regulation of the morphogenetic process, which in turn is controlled by the cellular machinery delivering and assembling the cellular envelope (Kroeger et al. 2009; Bou Daher and Geitmann 2011; Qin and Yang 2011). The present chapter explains how the pollen tube forms a tube, how it accomplishes specific tasks, and how the intracellular transport machinery regulates all of these processes.

7.2 Mechanics of Tubular Cellular Expansion

Pollen tubes have a cylindrical shape with a diameter of 5–20 μm, depending on the plant species. Their length is indeterminate and can reach several centimeters. In some species, the pollen tube can grow tens of centimeters long, although the living portion of the cytoplasm is confined to the tip region of the cell. The rapid polar cell growth requires structural and functional organelle compartmentalization and high rates of cell wall assembly (Cheung and Wu 2007). An exquisitely orchestrated intracellular transport mechanism ensures the delivery of the material necessary for the construction of the expanding cellular envelope—cell wall polymers and phospholipids. These materials are delivered to the growing tip in small vesicles (Fig. 7.1). Larger organelles such as mitochondria and Golgi bodies are predominantly segregated to the cytoplasm distal to the subapical region. The apex of the rapidly elongating cell is roughly hemisphere shaped, and all growth activity is confined to this region implying that the bulk of new soft cell wall material is added here. The result of this polar mode of growth is a cell wall, the thickness of which does not vary much around the cell (100–200 nm) but which shows a high

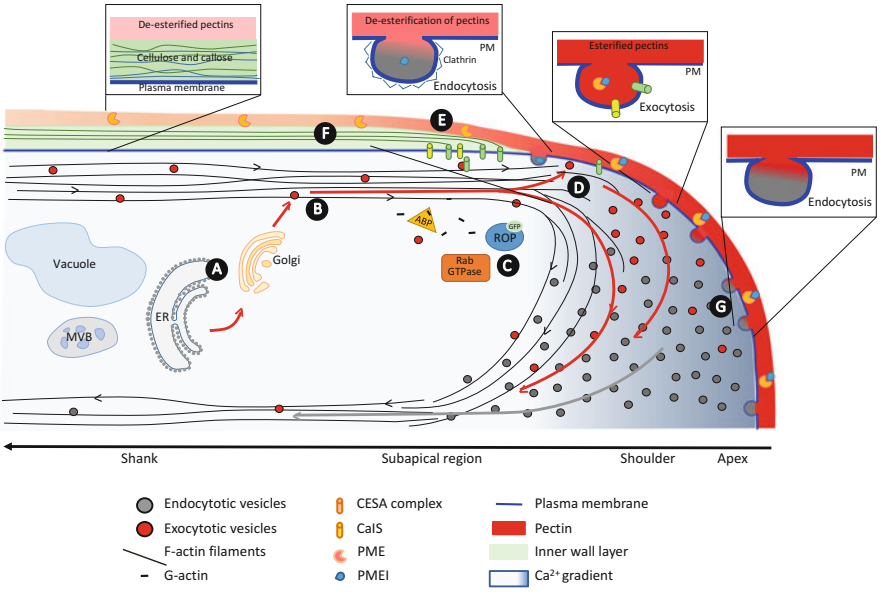

Fig. 7.1 Spatial distribution of pollen tube cell wall components is regulated by vesicular trafficking. Components of pollen tube envelope (plasma membrane and cell wall) are synthetized in the ER and Golgi (**A**) and transported to the cell surface via vesicular trafficking. The vesicles are transported preferentially along the actin filaments (**B**). Exocytosis is regulated by GTPases such as Rab or ROP (**C**) and in some species takes place preferentially in the shoulder region of the apex (**D**). CESA and CalS enzymes are incorporated in the plasma membrane and start to synthetize cellulose and callose. At the same time, endocytosis of PMEI (PME inhibitor) (**E**) allows activation of PME leading to pectin de-esterification and a stiffened cell wall (**F**). The endocytosis process helps to regulate the proportion between the plasma membrane and cell wall component secretion at the pollen tube apex (**G**)

degree of nonuniformity along the longitudinal axis concerning the distribution of its components (Chebli et al. 2012). Mechanical modeling has predicted (Fayant et al. 2010) and micro-indentation has confirmed that the mechanical properties of the cell wall change along the length of the tube, although absolute values for cell wall stiffness are elusive for the tip region (Bolduc et al. 2006; Zerzour et al. 2009; Chebli et al. 2012; Vogler et al. 2013). The cell wall of the shank has been measured to possess a Young's modulus of 350 MPa, a value that resembles that of Teflon (Sanati Nezhad et al. 2013b), and scanning electron microscopy has shown that microfibrils are oriented at a helical shallow angle in this portion of the cell (Aouar et al. 2010). The latter has been speculated to protect the tube from kinking when it is bent, thus ensuring that sperm cell transport is not hampered.

The nonuniform distribution of cell wall components and the resulting polarity of wall mechanical properties are a prerequisite for the successful formation of a cylindrical tube (Fayant et al. 2010). Just as other plant cells, pollen tubes grow by controlled yielding of the wall to the internal turgor pressure (Geitmann and Ortega

2009; Guerriero et al. 2014). The spatial gradient in mechanical properties ensures that the yielding occurs in one direction only. Since turgor is a scalar and pressure is exerted equally in all directions, turgor-driven cell wall expansion at the very apex of the pollen tube must exhibit spatial regulation in biomechanical properties of the cell wall between the apex and the shank of the cell (Geitmann and Steer 2006). Typical pressures in plant cells range between 0.1 and 1 MPa, which corresponds to the pressure in a car or bicycle tire. The only absolute value for turgor in pollen tubes was measured in lily (Benkert et al. 1997). Turgor is a hydraulic pressure maintained by osmosis—the movement of water across a semipermeable membrane along a concentration gradient (Hill et al. 2012). In addition to driving growth, turgor plays a role as a hydroskeleton that maintains plant cell shape against external forces. In the pollen tube, turgor is therefore not only necessary to drive growth; it is the pressure that prevents the cell from collapsing under the lateral compression force of the pistillar tissues (Sanati Nezhad et al. 2013a, Fig. 7.2). Finally, the hydrostatic pressure is also thought to provide the force necessary for the invasive penetration of the pistillar tissues (Sanati Nezhad and Geitmann 2013). The importance of turgor as a driving force for pollen tube elongation is easily illustrated by exposure of these cells to altered osmotic conditions (Liu and Hussey 2014), but this does not mean that the growth rate is actually regulated by the turgor. Rather, it is thought

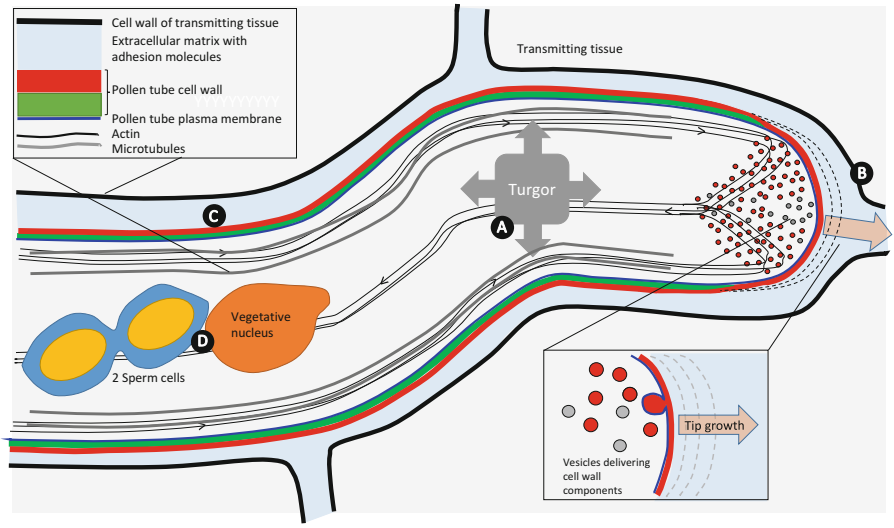

Fig. 7.2 Pollen tube invasive growth within the flower tissue. The pollen tube cytoplasm is compartmentalized—at the very tip, the softer cell wall yields to the turgor pressure (**A**), and simultaneously, cell wall assembly occurs (**B**); in the shank of the pollen tube, the cell envelope is stiffened in order to resist against compressive stress provided by the turgor (**C**). The two sperm cells and the vegetative nucleus are transported along the cytoskeleton toward the tip region. Their transport can occur even in bending tubes since the helical winding of the cellulose microfibrils prevents kinking (**D**)

that the mechanical properties of the cell wall and its assembly rates control the velocity with which pollen tube growth occurs (Winship et al. 2011). Consistent with this notion, it was shown experimentally (Benkert et al. 1997) and explained by modeling (Kroeger et al. 2011) that the average growth rate is uncorrelated from the turgor pressure.

The nonuniform distribution of mechanical properties required for polar growth is generated by a precisely controlled distribution of the individual polymers composing the pollen tube wall (Dardelle et al. 2010; Chebli et al. 2012). It has been shown in lily (Roy et al. 1997), tobacco (Li et al. 1995; Ferguson et al. 1998), and *Arabidopsis thaliana* (Lennon and Lord 2000; Dardelle et al. 2010; Chebli et al. 2012) that the pollen tube cell wall in the shank of the cell is composed of two layers: a fibrillar outer layer and a weakly electron-dense inner wall. The former is rich in pectins and can contain cellulose. Cellulose microfibrils can also be found in the inner layer in certain species, but the main component is callose (Taylor and Hepler 1997). At the pollen tube tip, this inner layer is generally lacking (Roy et al. 1997; Lennon and Lord 2000). The relative abundance of certain cell wall components in the different regions of the cell is therefore one of the features that underlies the spatial gradient in mechanical properties.

The different components of the pollen tube cell wall are synthesized and assembled in different ways. Pectin and hemicellulose are synthesized at the Golgi apparatus and secreted in their final forms. Cellulose microfibrils and callose on the other hand are synthesized directly at the plasma membrane by synthase complexes (Driouich et al. 2012). The deposition and enzymatic modification of all components occurs at precisely defined locations relative to the pollen tube tip. The latter is particularly important for pectins which are complex polymers including homogalacturonan (HG). In most angiosperms, HG is present in highly methyl-esterified form at the tip, whereas the shank of the cell displays acidic or weakly methyl-esterified HG epitopes (Dardelle et al. 2010; Chebli et al. 2012). Given its lower ability to cross-link, the methyl-esterified pectin in the apical cell region is believed to provide sufficient plasticity to provide a strong but malleable surface that permits rapid cell expansion (Cheung and Wu 2008) consistent with predictions made through mechanical modeling (Fayant et al. 2010). Such spatial change in the chemical configuration of a single type of cell wall polymer is therefore the second important feature that contributes to the gradient in mechanical properties.

HG synthesis is carried out in the Golgi apparatus, and the polymers are secreted via Golgi-derived vesicles (Driouich et al. 2012). Their deposition at the apical cell wall occurs in a highly methyl-esterified form explaining the high concentration at this location. As the apical wall matures and becomes part of the shank, the HG is de-esterified, and because the molecules are now negatively charged, they attract Ca^{2+} ions that cross-link the polymers in a process called gelation (Micheli 2001; Geitmann and Steer 2006). The de-esterification is accomplished by the enzyme pectin methyl esterase (PME), which is co-secreted with the esterified pectins in the same secretory vesicles (Bosch and Hepler 2005; Kim and Brandizzi 2014). PME activity has to be tightly regulated in space and time to allow pollen tube growth to proceed. Exogenous application of the enzyme or overexpression inhibits pollen

tube growth, likely by causing the soft apical wall to stiffen prematurely (Bosch et al. 2005).

To prevent PME from acting prematurely (in the secretory vesicles or at the apical wall), its activity is regulated by inhibitors (pectin methyl esterase inhibitors, PMEIs) which are co-secreted (Röckel et al. 2008). The inhibitor is present in the apical wall only, leading to the speculation that it is internalized through endocytosis in the shoulder of the tip coinciding with the location of beginning pectin maturation and PME activity. Treatment of pollen tubes with exogenous PMEI results in an abnormal germination and bursting of pollen tubes in *A. thaliana* confirming the role of HG in the mechanical properties of the cell wall (Lehner et al. 2010; Mollet et al. 2013; Leroux et al. 2015). PMEIs and their endocytosis are therefore thought to play a crucial role enabling polar growth in pollen tubes through confining the "soft spot" in the cell wall to the pollen tube apex (Zhang et al. 2010b; Palin and Geitmann 2012).

The pollen tube also produces other pectin-modifying enzymes such as polygalacturonases (PGs) and pectate lyases. They cleave the HG backbone thus affecting the rigidity of the cell wall (Micheli 2001). However, their main target may not be the tube itself but the substrate through which it has to grow. PGs are detected in the tip region of pollen tubes during papillar cell penetration (Dearnaley and Daggard 2001) suggesting that PGs are probably involved in the loosening of the stigma and transmitting tract cell walls to facilitate the penetration of the pollen tube during pollination.

The stiffening of the cylindrical portion of the pollen tube cell wall is not only accomplished by pectin cross-linking but also by the addition of polymers such as cellulose and callose. Callose is a β-glucan with β-1,3-linkages and is produced, for example, to temporarily block the flow in the phloem during dormancy (Sager and Lee 2014) or as a defense mechanism against invading pathogens (Lee and Lu 2011; Voigt 2014). It is abundant in the pollen tube cell wall but absent from the growing tip (Parre and Geitmann 2005; Derksen et al. 2011; Chebli et al. 2012). Callose clearly has a stabilizing function since treatments with lyticase, a callose-digesting enzyme, cause an increase in the cellular diameter, a reduction in cellular stiffness, and an increase in the cellular viscoelasticity (Parre and Geitmann 2005; Aouar et al. 2010). Callose is also employed to plug the active part of the pollen tube cytoplasm from the distal region, thus enabling the cell to maintain a relative constant cytoplasmic volume despite its continuous expansion (Geitmann and Steer 2006; Abercrombie et al. 2011).

Callose is synthesized at the plasma membrane by callose synthases (CalSs) (Ferguson et al. 1998) and activated in vitro by proteolysis and detergents (Brownfield et al. 2007). Immunogold labeling indicated that CalSs localize in the ER, Golgi bodies, and vesicles located adjacent to the plasma membrane in the distal pollen tube region. CalSs are incorporated into the plasma membrane in the shank and presumably sites of future callose plugs (Ferguson et al. 1998; Brownfield et al.

2008). CalS in *Nicotiana alata* pollen tubes consists of a 220 kDa polypeptide, which is likely to be delivered to the plasma membrane by exocytosis of Golgi membranes (Brownfield et al. 2008). The delivery and accumulation of CalS in the distal region depends on actin, while microtubules seem to be involved in the distribution and maintenance of distal CalS (Cai et al. 2011). Thus, CalSs are involved in cell wall modification in the pollen tube tip and formation of callose plugs in the very distal part of the pollen tube (Cai et al. 2011).

Cellulose, a β-glucan with β-1,4-linkages, is a major component in most plant cell walls. It is present in pollen tubes but in relatively low amounts (Schlüpmann et al. 1994; Cai et al. 2011). Despite its low abundance, cellulose plays an important role in stabilizing the pollen tube tip wall especially in the transition zone between the tip and the shank (Geitmann 2010; Derksen et al. 2011) where it contributes to the maturation of the wall and to its ability to resist the tensile stress generated in the cell wall by the internal turgor pressure (Aouar et al. 2010). This is evident from the fact that cellulase treatments as well as pharmacological or mutational inhibition of cellulose crystal formation result in larger pollen tube diameters, tip swelling, and eventually bursting (Anderson et al. 2002; Lazzaro et al. 2003; Aouar et al. 2010; Derksen et al. 2011). Similar to other plant cells, pollen tubes are able to compensate to a certain degree for a failure in the production of one cell wall component by overproducing another. Partial cellulose digestion results in pollen tubes with increased amounts of pectins, illustrating a certain flexibility in the cellular construction process (Aouar et al. 2010). Most pollen tubes do not possess much cellulose in the apical cell wall, but some do (Chebli et al. 2012; Hao et al. 2013). Whether or not there is a causal relationship between the relatively slow growth rate in these species and the presence of cellulose in the apex has not been investigated (Lazzaro et al. 2003).

Cellulose microfibrils are produced by transmembrane cellulose synthases A (CESAs) deposited into the plasma membrane in a form of rosette terminal complex (Doblin et al. 2002). Different CESA families are implicated in cellulose biosynthesis in plants, depending on tissue and cell type (Doblin et al. 2002; Persson et al. 2007; Chebli et al. 2012). In the pollen tube, cellulose synthases are inserted into the plasma membrane in the apical flanks, and deposition of cellulose begins immediately, if not already prior to insertion. In *A. thaliana* pollen tubes, crystalline cellulose was found in the trans-Golgi network and in cytoplasmic vesicles suggesting that the synthesis of cellulose microfibrils could be initiated before the incorporation to the plasma membrane. This could potentially enable the pollen tubes to optimize cell wall assembly to promote rapid elongation (Chebli et al. 2012). Alternatively, the presence of crystalline cellulose in cytoplasmic vesicles could be indicative of a progressive removal of this polymer from the shank of the cell by endocytosis and a relocalization to the apex (Chebli et al. 2012). Both explanations remain speculative.

7.3 Generation of a Biochemical Gradient in the Pollen Tube Cell Wall by Targeted Intracellular Transport

From the description of the growth mechanism, it becomes obvious that the genesis of a perfectly cylindrical cell with a uniform diameter requires the insertion and removal of polysaccharide building blocks, synthesizing and modifying enzymes, as well as their inhibitors at precisely defined locations at the plasma membrane. This is illustrated even better with a pollen tube that changes its growth direction in order to steer toward or away from an attractant or repulsive agent, respectively. Such a reorientation requires the spatial redefinition of the subcellular region that yields to the turgor pressure and grows. By consequence, the delivery of new cell wall building material and regulatory agents has to be targeted toward newly defined surface areas (Bou Daher and Geitmann 2011). Other substances that need to be synthesized and delivered to the apex include digestive enzymes and signaling molecules that enable the pollen tube to dissolve the apoplast of the pistillar tissue and to communicate with the female partner. All of these delivery processes occur through intracellular trafficking. Exocytosis and endocytosis of cell wall components and their regulated enzymes both contribute to maintaining membrane and cell wall domains over time in the elongating pollen tubes (Onelli and Moscatelli 2013). When this traffic is altered experimentally, the spatial distribution in the mechanical properties of the cell is affected, and as a consequence, cell shape changes from the cylindrical default, either by swelling or tapering of the tube or even by rupture of the wall. Such interference can, for example, result from manipulation of the transporting machinery, the cytoskeleton (Ischebeck et al. 2011; Abenza et al. 2015). Despite the need for a precisely tuned mechanical gradient (Fayant et al. 2010), there is some plasticity built into the system, as is evident when moderately interfering with cell wall assembly (Chebli et al. 2013a, b). Understanding how material is transported and used for morphogenesis is therefore an important endeavor to understand how plant cells are regulated in general.

Depending on the species, endocytosis occurs either in a single apical domain (Guan et al. 2013) or two membrane domains—the extreme apex and the shank region adjacent to the apex (Bove et al. 2008; Zonia and Munnik 2008; Moscatelli and Idilli 2009; Zonia 2010; Onelli and Moscatelli 2013). These two domains seem to be separated by a ring-shaped domain in the shoulder of the apex in which exocytosis dominates (Geitmann and Dumais 2009). It is thought that vesicles endocytosed at the tip region mix with vesicles that are delivered to the apex but fail to exocytose in the inverted cone in which vesicle motion appears to be governed by diffusion (Kroeger et al. 2009) and possibly convection. Secretory vesicles and all other organelles in the pollen tube are moved actively by motor proteins in the shank of the cell. The resulting drag forces move the surrounding cytosol and cause it to move resulting in a fluid flow that transports dissolved substances over significant distances in the "go with the flow" manner (Lord and Russell 2002). The overall cytoplasmic streaming, or movement of cytosol and organelles, shows different streaming patterns in angiosperm and gymnosperm pollen tubes. In angiosperms,

organelles move forward on cortical arrays and backward on a centrally located array resulting in a reverse fountain-like streaming pattern in the tip region (Bove et al. 2008; Geitmann and Nebenführ 2015). In gymnosperm pollen tubes, the direction of movement is reverse, where a fountain-like streaming pattern is governed by forward movement along the central axis and backward movement in the periphery of the cell (Lazzaro 1996). The opposite streaming pattern in angiosperm and gymnosperm pollen tubes was suggested to be generated by an opposite polarity of the actin filaments (Lenartowska and Michalska 2008; Kroeger et al. 2009). Albeit in differently shaped aggregates, both directions of fountain streaming are associated with an accumulation of vesicles in the apical region (Parton et al. 2001; Bove et al. 2008). The bigger organelles such as mitochondria and Golgi compartments generally do not reach the apical cytoplasm but turn around in the subapical region of the cell.

Whether they are delivered through the periphery or the central region of the cytoplasm, the Golgi-derived secretory vesicles containing cell wall components (Toyooka et al. 2009; Kang et al. 2011) have to reach the apical plasma membrane and deliver their cargo (Young et al. 2008; Chebli et al. 2013b; Gendre et al. 2013). It is interesting that the highest rate of cell wall assembly and expansion does not necessarily occur at the extreme apex of the tip but in some species at least at an annular region surrounding the apex (Geitmann and Dumais 2009; Zonia and Munnik 2009). This mechanism could serve to provide a greater resilience against factors that might disturb the steering mechanism. These exocytosis patterns point at the necessity to understand how exactly vesicles are delivered to their target region. Monitoring individual secretory vesicles in living cells is challenging since their size is typically between 60 and 150 nm—below the diffraction limit of the optical microscope. Direct observation and quantitative tracking are difficult, even by TIRF microscopy, since in the pollen tube these vesicles move rapidly and are very densely packed, and even if the pollen tube lies directly on a coverslip, the key processes are located several micrometers away from the glass surface (due to the curvature of the pollen tube apex). To circumvent these challenges, vesicle dynamics in growing pollen tubes have been analyzed quantitatively using fluorescence recovery after photobleaching (FRAP) and spatiotemporal image correlation spectroscopy (STICS) (Bove et al. 2008; Zonia and Munnik 2008). These data have informed theoretical modeling approaches that have been employed to make predictions on the functioning of the transport mechanism (Kroeger et al. 2009). The modeling has been particularly useful to understand how vesicle motion might be driven in cellular regions devoid of cytoskeletal arrays. The simulations have shown that the motor-driven movement of vesicles in the shank of the tube and the flanks of the apex is sufficient to explain the fountain and inverse fountain-shaped motion patterns through the apex even if only diffusion is assumed in apical cytoskeleton-free regions (Kroeger et al. 2009). Another conclusion from the simulations is that the vesicle motion patterns are very stable even when the growth rate changes. This is consistent with microscopic observations made in various species.

7.4 Cytoskeletal Control of Intracellular Trafficking

Motor-driven transport of organelles and vesicles is mediated by a cytoskeletal array that consists of microtubules and actin filaments oriented predominantly parallel to the long axis of the cylindrical cell (Geitmann and Emons 2000; Idilli et al. 2013). Generally, as is typical for plant cells, microtubules play a less prominent role in organelle transport processes (Gossot and Geitmann 2007; Cheung et al. 2008). They are involved in the movement of the male germ unit (Miyake et al. 1995), but drugs such as oryzalin and colchicine do not interfere with cytoplasmic streaming. However, recently nocodazole, another inhibitor of tubulin polymerization, has been demonstrated to impede plasma membrane internalization of the vesicles in the apex of tobacco pollen tubes (Idilli et al. 2013), but more detailed analyses are wanted. No doubt exists that actin plays a crucial role for transport processes in pollen tubes, including the long-distance transport of secretory vesicles (Zhang et al. 2010a, Moscatelli et al. 2012). Actin polymerization and depolymerization therefore need to be tightly regulated to ensure accurate delivery of vesicles to the tip for exocytosis (Cheung et al. 2008; Kroeger et al. 2009). This regulation is performed by numerous actin-binding proteins covered in excellent reviews (Ren and Xiang 2007; Chen et al. 2009; Staiger et al. 2010).

Vesicle trafficking does not only consist of long-distance transport but also comprises processes such as budding, sorting, navigating, and fusing. These processes involve the molecular machineries necessary for specific interactions such as contact of a vesicle with the plasma membrane. The effect of failure or deficiencies in any of these processes are illustrated when analyzing pollen tubes of mutants affected at the various steps in the vesicular trafficking pathway including cytoskeletal functioning (Zhu et al. 2013), endocytosis (Kitakura et al. 2011), or secretion (Cole et al. 2005; Silva et al. 2010; Richter et al. 2012; Doyle et al. 2015). In most of the cases, this affects the assembly of the pollen tube cell wall and as a consequence results in altered morphogenesis of the cell.

For both polar and diffuse plant cell growth, vesicle trafficking involves protein complexes that catalyze vesicle formation (clathrin), transport (RABs/cytoskeleton), tethering (exocysts), and docking and fusion (RABs/SNARE) (Sanderfoot and Raikhel 1999). Vesicle trafficking and fusion in the pollen tube are known to be regulated by small Arf or Rab GTPases (Saito and Ueda 2009; Szumlanski and Nielsen 2009; Kato et al. 2010). RabA4d localizes in the tip-localized membrane compartments in growing pollen tubes, and its function is important for polarized membrane trafficking during tip growth. Mutations *raba4d* and *rab11b* inhibit targeting of exocytotic and recycled vesicles to the pollen tube inverted cone region and compromise the delivery of secretory vesicles; this results in defects in the cell wall composition as well as pollen tube guidance, suggesting that Rab functions in selective delivery of cargo to the pollen tube tip (de Graaf et al. 2005; Szumlanski and Nielsen 2009).

The plant-specific Rho family of GTPases (ROPs) strictly localizes at the tip of the pollen tube thus providing a spatial signal function at the intersection of polarity

and vesicle trafficking (Lee and Yang 2008). ROP GTPases control polar growth by regulating the actin cytoskeleton and Ca^{2+} signaling in pollen tubes and root hairs which in turn regulate vesicle transport and fusion (Molendijk et al. 2001; Chen et al. 2003; Lee and Yang 2008; Jamin and Yang 2011). ROP1 also regulates the temporal control of exocytosis as its concentration varies during oscillatory growth (Hwang et al. 2005; Lee et al. 2008; Lee and Yang 2008). Brefeldin A, a drug inhibiting the secretory pathway, reduces pollen germination and eliminates growth and polar plasma membrane localization of ROPs and ROP-interacting partners (RIPs) (Molendijk et al. 2001) by disrupting the secretory vesicles at the tip. All this leads to significantly decreased content of pectin in the apical region (Wang et al. 2005; Richter et al. 2012). Other polarity markers are RIC3 and RIC4. The spatially regulated presence and activity of signaling agents such as ROP1, RIC3, and RIC4 are believed to be involved in such spatial regulation of the vesicular secretion in the pollen tube through regulation of actin dynamics (Gu et al. 2005; Lee et al. 2008). An increase in Ca^{2+} concentration suppresses ROP1 function and thus balances the actin-RIC4 ROP1 apical activation (Yan et al. 2009), but the ROP1-Ca^{2+}-RIC3 interaction is not clearly characterized.

Tethering and fusion of the vesicles is mediated by the exocyst complex (Hala et al. 2008) and soluble NSF attachment protein receptors (SNAREs) (Sanderfoot and Raikhel 1999). When the vesicle reaches its destination, vesicle (v)-SNAREs interact with target (t)-SNAREs to mediate vesicle fusion (Saito and Ueda 2009). Chemical inhibition of V-ATPase activity at the trans-Golgi network (TGN) by concanamycin A leads to secretory defects and related growth phenotypes (Dettmer et al. 2005, 2006; Brux et al. 2008; Viotti et al. 2010; Guo and McCubbin 2012) suggesting that the vesicular trafficking from TGN plays a central role in polysaccharide secretion to the tip. Also *Arabidopsis* SYP124 and SYP125, pollen-specific SNARE subfamilies of syntaxin-like proteins required for docking and fusion of secretory vesicles, participate in vesicle trafficking between the vacuole and the TGN, mediating exocytotic membrane fusion at the pollen tube apex (Enami et al. 2009; Kato et al. 2010; Silva et al. 2010). Secretory vesicles are exocytosed involving the exocyst complex (a multiprotein tethering factor) important for secreting vesicles to get delivered to specific exocytosis sites (Cole et al. 2005) suggesting that the exocyst is one of the major determinants of pollen tube growth and, hence, of cell morphology (Cole et al. 2005; Hala et al. 2008).

The second messenger Ca^{2+} is believed to be one of the central intracellular modulators of vesicular trafficking and subsequently regulating tip growth in pollen tubes (Qin and Yang 2011; Steinhorst and Kudla 2013). Both the cytoplasmic Ca^{2+} concentration and also the pH change significantly over short distances within the apical and subapical region of the pollen tube cytoplasm (Chebli and Geitmann 2007; Qin and Yang 2011). The concentration of cytosolic Ca^{2+} is high at the growing pollen tube tip and decreases rapidly in the subapical region. The magnitude of this gradient oscillates with the same periodicity as pollen tube growth (Pierson et al. 1996; Holdaway-Clarke et al. 1997; Messerli et al. 2000). Previous observations suggest that the Ca^{2+} gradient is essential for polarity of ROP1 and that it regulates its tip growth polarity (Hwang et al. 2005, 2008). Tip-localized

ROP1 GTPase effectors RIC3 and RIC4 are proposed to promote Ca^{2+} influx through the plasma membrane and thus to establish the Ca^{2+} gradient (Gu et al. 2005) and regulate the actin dynamics at the tip by way of actin-binding protein activity, which in turn might promote exocytosis (Gu et al. 2005; Lee et al. 2008; Bou Daher and Geitmann 2011). Most importantly, when intracellular trafficking is modulated by experimentally blocking the release of intracellular Ca^{2+}, cell wall structure is altered significantly as callose accumulates at the tip and the tip-localized methyl-esterified HG is de-esterified (Chen et al. 2008). The roles of Ca^{2+} in the regulation of pollen tube growth are therefore multiple—through its regulation on actin dynamics and hence secretion and through its action on the mechanical properties of pectin.

Pollen tube assembly requires not only exocytotic but also endocytotic activities. The latter are necessary for the controlled removal of agents (e.g., PMEI) but also to recover excess membrane material. Because of their different geometry, the cellular envelope—cell wall and plasma membrane—and the delivery vesicles have a different ratio of the two materials (polysaccharides/phospholipids). As a consequence, the relative amount of membrane material delivered to the outside is too high and has to be endocytosed (Bove et al. 2008). Therefore, a tight correlation between endocytosis and exocytosis during pollen tube growth is necessary, both in time and in space (Moscatelli and Idilli 2009). The immediate reinternalization of membrane material could potentially also be accomplished by "kiss-and-run" endocytosis (Bove et al. 2008) similar to the endocytosis described in synapses as a clathrin-independent internalization process (Alabi and Tsien 2013), but evidence for this process in plant cells is still lacking.

7.5 Invasive Growth

The regulation of pollen tube growth dynamics through intracellular trafficking becomes a particularly intriguing question if one considers that pollen tubes do not usually grow in the uniform and artificial growth medium provided in a Petri dish but within the complex, maze-like structure existing in the pistil (Lennon et al. 1998; Lord and Russell 2002; Chapman and Goring 2010). This complex environment requires the tube to have the abilities (1) to follow guidance cues by reorienting its growth direction, (2) to navigate or penetrate mechanical obstacles, and, in some species, (3) to adhere to the pistillar tissue. All three mechanisms can be linked to intracellular trafficking. The adhesion mechanism is thought to enable the pollen tubes to attach to the transmitting tissue lining the hollow style in certain species (Zinkl et al. 1999; Park et al. 2000). Arabinogalactan proteins (Cheung and Wu 1999; Nguema-Ona et al. 2012), pectic polysaccharides, and stigma/stylar cysteine-rich adhesin (Lord 2000) have been identified in this context, and all clearly require a secretion mechanism to reach the cellular surface.

Pollen tube reorientation and the calibration of the invasive force exerted by the pollen tube are linked to intracellular trafficking since cell wall mechanics is regulated through controlled secretion either in space (reorientation) or time (invasion). To invade the pistillar tissue in solid styles, an advancing pollen tube has to exert an invasive force that is higher than the resistance posed by the apoplast of the invaded tissue. The invasive behavior of the pollen tube has been studied using the TipChip, a microfluidic platform (Agudelo et al. 2013). This lab-on-a-chip device allows the researcher to expose individual pollen tubes to precisely calibrated mechanical obstacles with simultaneous high-resolution and fluorescence imaging. When pollen tubes were presented with slit-shaped openings, they had to exert a force that could be measured quantitatively (Sanati Nezhad et al. 2013a). Intriguingly, pollen tubes maintained a constant growth speed despite increasing mechanical resistance. The growth speed is likely modulated by all acting forces, the internal turgor pressure and in the opposite direction, the cell wall and the external mechanical obstacle. The stiffer the cell wall, the smaller the available force acting against the obstacle. The fact that growth speed remains constant despite increasing external resistance of the obstacle suggests, therefore, that, when required, the growing tip can soften its wall so that the pressure acts directly against the obstacle instead of being dissipated in the wall (Sanati Nezhad et al. 2013a).

The softening of the apical cell wall can also occur in repeated manner resulting in an oscillatory growth pattern that has been hypothesized to be employed as a sledge hammer (Geitmann 1999). Oscillatory pollen tube growth is generated by periodic changes in the extensibility of the apical cell wall (McKenna et al. 2009; Zerzour et al. 2009; Winship et al. 2010; Kroeger and Geitmann 2013). This in turn is regulated by periodically changing rates of exocytosis which are subject to control by a feedback mechanism that is hypothesized to involve mechanosensors located in the apical plasma membrane (Kroeger et al. 2008).

A change in growth orientation required to either follow a chemical guidance cue (Sprunck et al. 2012) or circumvent an obstacle (Gossot and Geitmann 2007) has been shown to be preceded by a spatial reorientation of the secretory machinery in the pollen tube (Bou Daher and Geitmann 2011). Both the fluorescent signals for the apical vesicle cone and the dynamics of the actin cytoskeleton show asymmetric distribution prior to a visible change in pollen tube shape symmetry hence suggesting a causal relationship between these parameters. This behavior is consistent with that of other walled cell types such as fission yeast (Abenza et al. 2015). The turning response can also be triggered by directly modulating the cell wall mechanical properties. Local application of PME asymmetrically stiffens the apical cell wall and thus causes the tube to turn away from the agent, to the side where the wall remains soft (Sanati Nezhad et al. 2014).

7.6 Conclusion and Perspective

Understanding the intracellular underpinnings of the invasive and directional growth of pollen tubes requires analyzing how the massive amount of cell wall deposition and assembly processes are coordinated to promote fast cell elongation. Tight control of cell wall remodeling during the morphogenetic process ensures that the mechanical properties of the cell wall are regulated in space and time. Cell wall assembly and maturation are directly influenced by spatial control of vesicular delivery of cell wall components and related enzymes. The functioning of the pollen tube including its growth, its targeting, and its force generation is therefore all connected through these intracellular membrane transport processes.

The research on pollen tubes is highly interdisciplinary because it combines cell biology with quantitative and mechanistic modeling and draws upon novel technological developments such as microfluidics and high-resolution live-cell imaging to address questions of global cell biological relevance. The challenge is now to integrate the multitude of different types of molecular, biological, and mechanical data to conceive a meaningful picture of pollen tube biological functioning.

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Chapter 8
Directional Growth for Sperm Delivery

Subramanian Sankaranarayanan and Tetsuya Higashiyama

Abstract Flowering plants use a polarized projection of the pollen grain called a pollen tube, to precisely deliver two sperm cells to the female gametes, which are deeply buried in the female gametophyte of the ovules for fertilization. The pollen tubes navigate a long route from the stigma, through the transmitting tract over a considerable amount of time before targeting the individual ovules. How is this precise cellular targeting achieved by the pollen tube? A lot of progress has been made toward identifying the sources of guidance cues that the pollen tube receives and the molecules that act as such guidance cues during its journey toward the ovule. In this chapter, we discuss these pollen tube guidance cues and also the latest tools in bio-imaging and microfluidics that would enable us to gain a better understanding of this process of directional growth for sperm delivery.

Keywords Bio-imaging • In vivo pollen tube growth • Microfluidics

Abbreviations

AGP	Arabinogalactan protein
Ca^{2+}	Calcium ion
ECM	Extracellular matrix
GFP	Green fluorescent protein

S. Sankaranarayanan (✉)
Institute of Transformative Bio-molecules, Nagoya University, Furo-cho, Chikusa-ku, Nagoya, 464-8601 Japan
e-mail: subbu@itbm.nagoya-u.ac.jp

T. Higashiyama (✉)
Institute of Transformative Bio-molecules, Nagoya University, Furo-cho, Chikusa-ku, Nagoya, 464-8601 Japan

Division of Biological Science, Graduate School of Science, Nagoya University, Nagoya, Japan

JST, ERATO, Higashiyama Live-Holonics Project, Nagoya University, Furo-cho, Chikusa-ku, Nagoya, Aichi, 464-8602 Japan
e-mail: higashi@bio.nagoya-u.ac.jp

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LoC Lab-on-a-chip
TPE Two-photon excitation microscopy

8.1 Introduction

Sexual reproduction in flowering plants begins with the deposition of pollen grains containing the male gametes, onto the receptive surface of the "female" stigmatic tissue. In case the pollen-stigma interaction is compatible, the pollen grain hydrates, germinates, and produces a pollen tube that penetrates the stigma and grows directionally toward the ovule for fertilization (Knox 1984; Nasrallah et al. 1994; Lush et al. 1998; Chapman and Goring 2010). The ovule contains a female gametophyte or embryo sac consisting of seven cells including the egg cell within it (Yadegari and Drews 2004).

The pollen tube interacts with the sporophytic cells during its initial journey toward the ovule. Once it encounters the synergid cells in the embryo sac, the pollen tube stops its growth and releases its content that interacts with the gametophytic cells. Both the sporophytic and gametophytic cells of the pistil contribute toward the directional growth of the pollen tube (Higashiyama et al. 2001). Several key molecules have been identified which aid in the process of pollen tube guidance including the ovular attractant peptides for precise, species-specific attraction (Higashiyama and Takeuchi 2015). Growing evidences suggest that competency control of the pollen tube is essential for making the pollen tube responsive to the molecular guidance cues. Since the process of directional pollen tube growth occurs deep within the female tissues of the flower, it is difficult to observe and to analyze the intercellular communications in real time. The development of improved live-cell imaging systems has enabled the visualization of this process in real time. Advanced micro-engineering in form of microfluidic devices can be used for in vitro testing of pollen tube growth by providing a microenvironment that resembles the in vivo growth conditions. In this book chapter, we will discuss in detail the multistep control of pollen tube guidance: pre-ovular guidance and ovular guidance, including the concept of competency control which is an important aspect of pollen tube guidance. We will also discuss the importance of emerging techniques in live-cell imaging and micro-engineering in understanding the directional growth of pollen tubes during its journey toward the ovule.

8.2 Pre-ovular Guidance During Directional Growth

Upon landing of the pollen on to the stigmatic surface of a flower, adhesion of pollen grain to the stigma is facilitated by the pollen wall surface and extracellular matrix (ECM) of the stigma. Following this, the pollen grain germinates and sets out a pollen tube that grows straight toward the ovary, through the style tissue. The

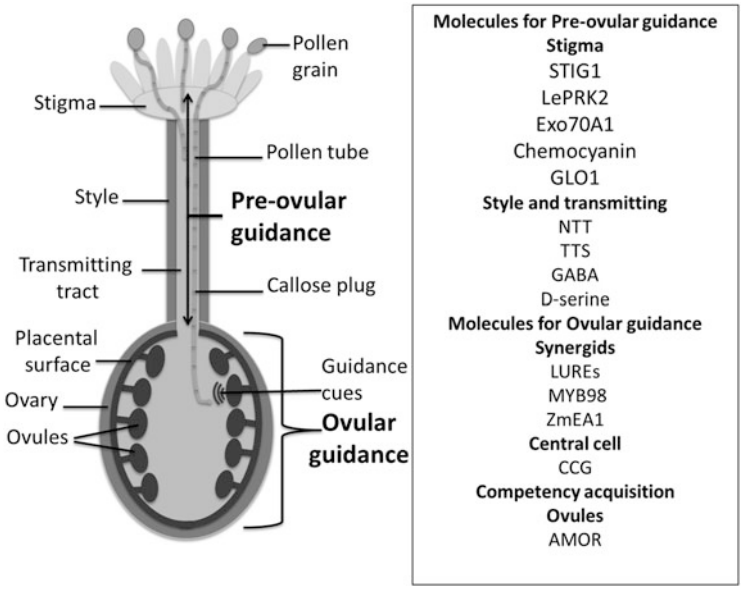

Fig. 8.1 Pollen tube guidance to the ovules. The process of pollen tube guidance can be separated into two phases, pre-ovular guidance and ovular guidance. The key molecules responsible for each of these phases have been listed on *right*

sporophytic cells of the stigma, style, and transmitting tissue control the early stages of pollen tube guidance known as the pre-ovular guidance (Fig. 8.1).

Stigmatic exudates rich in lipids have been shown to be essential for the directional growth of pollen tube, enabling the pollen tube to penetrate the stigma in tobacco (Wolters-Arts et al. 1998). Stigma-specific protein1 (STIG1), a class of cysteine-rich peptides/proteins (CRPs) abundant in the stigmatic exudate of tomato (*Solanum lycopersicum*), stimulates pollen tube growth in vitro and in the pistil, by interacting with the pollen receptor kinase LePRK2 (Tang et al. 2004; Huang et al. 2014). In case of plants that have a dry stigma (e.g., *Brassica*), the pollen coat fulfils the role of exudates seen on wet stigmas. Pollen coat also induces the transport of an important secondary messenger, Ca^{2+}, from the papillae to the tip of growing pollen tubes. Upon receiving the compatible pollen, the activity of ACA13 calcium transporter is induced in stigmatic papillary cells of Brassicaceae (Iwano et al. 2014). Ca^{2+} oscillations was observed in the tip region of the growing pollen tube, and remarkable increases in the concentration of cytosolic-free calcium ions ($[Ca^{2+}]_{cyt}$) is seen in the pollinated papilla cell, following pollen grain attachment and pollen tube penetration into the papilla cell wall (Iwano et al. 2004).

The stigma provides resources including compatibility factors to the growing pollen tube through the process of exocytosis (Samuel et al. 2009). RNAi silencing of the exocyst genes in *Arabidopsis* stigma impaired pollen acceptance and pollen tube growth (Safavian et al. 2015). Compatibility factors like glyoxalase I (GLO1)

promote the germination of pollen and pollen tube growth in stigmas in *Brassica* (Sankaranarayanan et al. 2015). One molecule that mediates the initial guidance of pollen tubes in wet stigmas is chemocyanin. It is a small basic protein of the plantacyanin class isolated from lily stigma that can attract and reorient lily pollen tube growth in vitro (Kim et al. 2003). Overexpression of a chemocyanin homolog in *Arabidopsis* stigma resulted in aberrant growth of wild-type pollen tube on the papillae, and the pollen tubes growing on these stigmas made numerous turns around the stigma before entering the style (Dong et al. 2005).

Specialized cells of the transmitting tracts are indispensible for the growth and guidance of pollen tube in *Arabidopsis* (Crawford et al. 2007). Disruption of the *no transmitting tract* (*NTT*) gene that encodes a C2H2/C2HC zinc finger transcription factor in *Arabidopsis* affects the production or composition of the extra cellular matrix (Crawford et al. 2007). In *Arabidopsis ntt* mutants, seed set occurs only in the upper half of ovary, as the pollen tubes grow slowly and terminate their growth prematurely (Crawford et al. 2007).

Arabinogalactan proteins (AGPs) are among the most abundant proteins in the ECM of the transmitting tract. The transmitting tissue-specific (TTS) arabinogalactan proteins found in the stylar matrix of tobacco get incorporated into the walls of pollen tubes during growth, facilitating their growth toward the ovary (Cheung et al. 1995; Wu et al. 1995). In transgenic tobacco expressing antisense *TTS* gene, pollen tubes were arrested in the style, and a reduction in seed set was observed. Nonprotein amino acids also facilitate the directional growth of pollen tube in the style and transmitting tract (Palanivelu and Tsukamoto 2012). A role for gamma-aminobutyric acid (GABA) in pollen tube guidance was identified in *Arabidopsis* (Palanivelu et al. 2003). In *Arabidopsis pop2* mutant that is defective in the gene encoding a gamma-aminobutyric acid transaminase, GABA accumulates in the pistil, and the self-pollen tube fails to elongate beyond the middle point of the transmitting tract. POP2 activity is essential for GABA degradation and generation of a GABA gradient in the pistil, with highest concentration in the ovule micropyle. Another rare amino acid D-serine aids in normal pollen tube growth in the style, transmitting tract and the ovules. D-serine activates glutamate receptor-like channels (GLRs) in the apical region of the pollen tube, which regulate calcium ion influx and dynamics of pollen tube growth (Michard et al. 2011). However, in vitro pollen tube attraction activity for both these uncommon amino acids has not been demonstrated until now.

8.3 Ovular Guidance During Directional Growth

From the transmitting tract, pollen tubes move to the septum and grow along the surface of the funiculus of an ovule. It then enters the female gametophyte through the ovule micropyle to complete double fertilization. The ovule controls pollen tube guidance after it reaches the ovary (Fig. 8.1). Different plant species have varying numbers of ovules in their ovary, e.g., 50–60 ovules in *Arabidopsis*,

around 500 ovules in *Torenia*, or a single ovule in rice and maize (Higashiyama and Takeuchi 2015). Though many pollen tubes emerge from the transmitting tract and transverse the septum, only one pollen tube associates with each ovule (Palanivelu and Tsukamoto 2012).

In case of densely pollinated pistils, pollen tubes tend to emerge from the transmitting tract onto the surface of the septum more frequently at the upper part (distal end) of the ovary in *Arabidopsis*. In sporophytic mutants defective in ovule and female gametophyte development, the pollen tubes showed little preference for emerging at the upper part of the ovary, suggesting that pollen tube guidance by the ovule is effective over a long range (Hulskamp et al. 1995). Semi-in vitro quantitative assays in *T. fournieri* using microfluidic devices also suggest such long-range pollen tube attraction by the ovule (Horade et al. 2013). The female attractant molecule that mediates this long distance attraction of the pollen tube toward the ovule is yet to be discovered.

Once the pollen tube nears the ovule, the female gametophyte mediates the micropylar guidance of the pollen tube (Ray et al. 1997; Shimizu and Okada 2000). The synergid cells contained within the female gametophyte were found to be the source of micropylar guidance signals because pollen tubes failed to enter the ovules of *Torenia* after the synergid cells were laser ablated (Higashiyama et al. 1998, 2001). In *Arabidopsis*, *MYB 98*, a transcription factor predominantly expressed in synergid cells was found to be essential for pollen tube guidance (Kasahara et al. 2005). A transcriptome analysis of the synergid cells identified two cysteine-rich polypeptides (CRPs) named LURE1 and LURE2 as the pollen tube attractants responsible for guidance to the ovule (Okuda et al. 2009). Recombinant LURE proteins display species-specific chemoattraction activity in vitro toward competent pollen tubes and suppression of LURE expression by antisense repression abolished pollen attraction in vivo (Okuda et al. 2009). A similar orthologous peptide from *Torenia concolor* synergid was also shown to function as a chemoattractant in a concentration-dependent and species-preferential manner (Kanaoka et al. 2011), which is consistent with a report that pollen tube attractant from the synergids displays species preferentiality (Higashiyama et al. 2006).

In *Arabidopsis*, a cluster of six paralogous genes, AtLURE1.1–1.5 and the pseudogene AtLURE1.6 encode the functional pollen tube attractant peptides, collectively named the AtLURE1 peptides (Takeuchi and Higashiyama 2012). RNAi suppression of all the AtLURE1 peptides resulted in reduced pollen tube attraction at the micropyle, whereas *Arabidopsis thaliana* pollen tubes were attracted to enter into a protruding embryo sac of *T. fournieri* expressing AtLURE1.2 in the two synergid cells (Takeuchi and Higashiyama 2012).

In case of the monocot maize, a small-secreted peptide egg apparatus 1 (ZmEA1), which is predominantly expressed in the egg and synergid cells, plays a role in micropylar guidance (Marton et al. 2005). Antisense or RNAi suppression of ZmEA1 prevents the wild-type pollen tubes from entering mutant ovules and also results in significant reduction in seed set. Similar to the LUREs, expression of ZmEA1-GFP in *A. thaliana* synergid cells enabled in vitro-germinated maize pollen tubes to be preferentially guided to the micropylar opening of *A. thaliana*

ovules (Marton et al. 2012). ZmEA1 guidance signal is again species specific, as the mature ZmEA1 peptide specifically binds to *Zea mays* pollen tubes but not to *Tripsacum dactyloides* nor to *Nicotiana benthamiana* pollen tubes (Uebler et al. 2013). Interestingly, another peptide predominantly expressed in synergid cells of maize, ZmES4, causes the arrest of pollen tubes and induces pollen tube rupture via opening of the potassium channel (Amien et al. 2010).

Pollen tube guidance is not restricted to the synergid cell. The central cell also contributes to pollen tube guidance. A *central cell guidance* (*ccg*) mutant was isolated in *Arabidopsis*, which is defective in micropylar pollen tube guidance (Chen et al. 2007). *CCG* encodes a nuclear protein with a conserved N-terminal C2C2-type zinc finger motif that is typical to transcription factor IIB family. CCG is expressed in the central cell of the female gametophyte, and the expression of CCG in the central cell completely rescues pollen tube guidance in the *ccg* mutant (Chen et al. 2007).

Several male factors also serve to guide the pollen tube toward the ovule. A pair of potassium ion transporters, CHX21 and CHX23, is essential for pollen tube guidance to the ovule in *Arabidopsis* (Lu et al. 2011). A role for CHX in shifting the pollen tube axis polarity has been proposed. Polarized tip growth was not affected in *chx21 chx23* mutant pollen, but the failure to target the ovules in vivo and semi-in vivo experiments suggested that the mutant pollen tube had lost competence to shift its directional growth toward the funiculus and the micropyle.

Ca^{2+} gradients in pollen tube tips are known to regulate pollen tube guidance, and Ca^{2+} channels present in the plasma membrane of the pollen tube tips contribute to the regulation of Ca^{2+} gradient and influx. Cyclic nucleotide-gated channel 18 (CNGC18) is a Ca^{2+}-permeable channel in the pollen tube tips of *Arabidopsis* that plays a role in pollen tube guidance (Gao et al. 2016). Point mutations in CNGC18 resulted in abnormal Ca^{2+} gradients and pollen tube guidance defects, which were rescued by complementation.

Pollen defective in guidance 1 (POD1) is an endoplasmic reticulum (ER) luminal protein that is required for normal ovular attraction of pollen tubes (Li et al. 2011). POD1 interacts with calreticulin3 (CRT3), a Ca^{2+}-binding ER chaperone protein, which is essential for folding of membrane receptors. In *pod1* mutant, the pollen tubes fail to target the female gametophyte, implying that protein folding mediated by POD1 may be essential for maintaining the integrity of membrane proteins including the receptors for ovular attraction (Li et al. 2011).

Cobra-like 10 (COBL10), which is a glycosylphosphatidylinositol (GPI)-anchored protein localized at the tip of the pollen tube plasma membrane, also plays an important role in pollen tube growth and guidance (Li et al. 2013). *COBL10* mutants displayed male sterility as a result of reduced pollen tube growth and impaired directional sensing in the female transmitting tract (Li et al. 2013).

Lost in pollen tube guidance1 (LIP1) and LIP2 are membrane-anchored receptor-like cytoplasmic kinases, localized at the tip of the pollen tube (Liu et al. 2013). LIP1 and LIP2 double mutants show defects in pollen tube morphology and impaired micropylar guidance (Liu et al. 2013). The ability to perceive the ovular attraction signal AtLURE1 was also reduced in these mutants, suggesting that they could form

complexes with the receptors of the LURE peptides and aid in signal transduction into the pollen tube cytoplasm (Liu et al. 2013).

Plasma membrane-localized receptor kinases male discoverer 1 (MDIS1) and MDIS2 and MDIS1-interacting RLK1 (MIK1) and MIK2 have been recently identified as the pollen tube receptors of the female attractant LURE1 secreted by the ovules (Wang et al. 2016). The extracellular domains of these plasma membrane receptors showed specific binding to LURE1, and the knockout mutant's *mdis1*, *mik1*, and *mik2* responded less sensitively to LURE1. AtLURE1 binds the receptor-like kinases MIK1 and MDIS1, promoting their dimerization and inducing MIK1 autophosphorylation and phosphorylation of MDIS1. *Capsella rubella* pollen tubes expressing AtMDIS1 were able to target the wild-type *A. thaliana* ovule in semi-in vitro systems but not the wild-type *C. rubella* pollen tubes. Another receptor named pollen-specific receptor kinase 6 (PRK6) with an extracellular leucine-rich repeat domain is also an essential receptor for LURE1 peptides in *Arabidopsis* (Takeuchi and Higashiyama 2016). The tip-localized PRK6 interacts with itself and other PRK family receptors, e.g., PRK3 and receptor-like cytoplasmic kinases, LIP1 and LIP2, which are involved in pollen tube growth and attraction by ovular signals. PRK6 receptor also interacts with the pollen-expressed Rho of plant guanine nucleotide-exchange factors (ROPGEFs) that activate the Rho GTPase proteins of plants (ROPs), leading to targeted growth toward the ovule. Similar to MDIS1, PRK6 also conferred responsiveness to AtLURE1 in pollen tubes of related species *Capsella rubella* (Takeuchi and Higashiyama 2016). Both of these groups of receptors change their localization in the presence of AtLURE1 asserting that these are bona fide AtLURE1 receptors. It yet remains unclear whether these groups of receptors interact and influence the functioning of each other. The functional redundancy between the receptors complicates the understanding of the process of AtLURE1 reception capable of mediating ovule targeting. Biochemical studies aiming to access the interactions among these receptors and AtLUREs would be beneficial to address these open questions.

8.4 Competency Control During Directional Growth and Cessation of Pollen Tube Growth

Not all the pollen tubes growing through the pistil in a semi-in vivo system are competent to be attracted to the ovular attractant molecules. In *Torenia* semi-in vivo pollen tube guidance assay (pollen tubes growing through a cut, pollinated style), attraction of pollen tubes by the ovule and double fertilization happened only when the growth medium was pre-cultivated with ovules. Pollen tubes germinated in the medium without growth through a style rarely get attracted to the ovules placed in front of them and also are not attracted to the ovular LURE peptides (Okuda et al. 2013; Mizukami et al. 2016). *Torenia* pollen tubes that grew through a 15 mm long style were able to bind LURE peptides and were attracted by

them, whereas pollen tubes growing through shorter styles could bind LURE peptides at their tips but were not attracted (Okuda et al. 2013). These experiments suggested that pollen tubes undergo maturation in the pistil tissue to enable them to respond to the guidance cues. This can be compared to the capacitation of sperms in animals that are activated on their way to the egg (Ickowicz et al. 2012). Recent research has shed light into this process of ovular competency control. An ovular factor, methyl-glucuronosyl arabinogalactan, induces competency for the pollen tube to respond to LURE peptides in *Torenia fournieri* (Mizukami et al. 2016). This factor named activation molecule for response capability (AMOR) was purified by a biochemical approach from the ovule extracts of *Torenia*, and it was deduced to be an arabinogalactan by using enzymes that degrade specific structure of arabinogalactan sugar chain (Mizukami et al. 2016). Further, it was found that chemically synthesized β isomer of the disaccharide methyl-glucuronosyl galactose (Me-GlcA-Gal) showed high activity of AMOR (Mizukami et al. 2016). The micropylar pollen tube attraction was reconstituted by LUREs and AMOR in the absence of other ovular attractors, suggesting that AMOR activates LURE signaling pathway in the pollen tube. Growth of the pollen tubes through the style was still an essential step that could not be replaced by the presence of synthetic AMOR in the growth media, suggesting that another unknown factor in the style also contributed to competency control (Mizukami et al. 2016).

Once the pollen tube enters the ovule and encounters the synergid cell, the source of pollen tube attractants, it terminates its growth and will rupture and finally release sperm cells for fertilization. Several genes that contribute to the termination of pollen tube growth and release of sperms have been identified. A serine/threonine receptor-like kinase, *FERONIA/SIRENE* (*FER/SRN*), is one of the key factors that is essential for the termination of pollen tube growth by synergid cells (Escobar-Restrepo et al. 2007; Rotman et al. 2003; Huck et al. 2003). In *fer/srn* mutants, the pollen tube does not arrest its growth, shows excessive growth around the synergids, and fails to discharge the sperm cells (Huck et al. 2003; Rotman et al. 2003; Escobar-Restrepo et al. 2007). It has been proposed that FER localizes to the synergid cell plasma membrane and the extracellular domain of FER binds to a ligand from the pollen tube resulting in autophosphorylation of the kinase domain and initiation of an unknown signal transduction cascade ultimately leading to pollen growth arrest (Escobar-Restrepo et al. 2007). Recently, two new members of the pollen tube reception pathway that is mediated by *FERONIA* were reported (Lindner et al. 2015). These two members *TURAN* (*TUN*) and *EVAN* (*EVN*) mediate pollen tube reception through protein N-glycosylation in the endoplasmic reticulum and encode a putative uridine diphosphate (UDP)-glycosyltransferase superfamily protein and a dolichol kinase, respectively. Apart from these three mutants, other mutants like *lorelei* (*Ire*), *scylla* (*syl*), and nortia (*nta*) also display pollen tube growth arrest (Tsukamoto et al. 2010; Capron et al. 2008; Kessler et al. 2010; Rotman et al. 2008). *LRE* encodes a putative glycosylphosphatidylinositol (GPI)-anchored membrane protein, *NTA* encodes a seven-membrane receptor protein of the powdery mildew

resistance locus O (MLO) family, and the molecular identity of *SYL* is yet to be identified (Tsukamoto et al. 2010; Capron et al. 2008; Kessler et al. 2010; Rotman et al. 2008). Recent studies have identified that the female gametophyte-expressed *LORELEI* and the seedling-expressed LRE-like GPI-AP1 (*LLG1*) act as a chaperone and co-receptor for FERONIA (Li et al. 2015). Both LRE and LLG1 bind to the extracellular juxtamembrane region of FER, and this binding is necessary for FER function. LLG1 was shown to interact with FER in the endoplasmic reticulum and on the cell surface, and loss of LLG1 function induces cytoplasmic retention of FER. It was further demonstrated that LLG1 is component of FER-regulated RHO GTPase signaling complex.

Pollen tube discharge completes the process of synergid degeneration that is initiated upon arrival of the pollen tube (Leydon et al. 2015). The sperm cells then get access to the female gamete, the egg cell, and the central cell for fertilization (Drews and Yadegari 2002; Sandaklie-Nikolova et al. 2007; Hamamura et al. 2011, 2014). There are several molecular events that occur after pollen tube discharge that are essential for successful fusion of sperms with the egg and central cell. There is a chance that multiple pollen tubes are attracted by a single ovule (polytubey in analogy to polyspermy). Polytubey block occurs after gamete fusion that prevents multiple pollen tubes from entering the same ovule to ensure that only two sperms are delivered to the female gametes (Beale et al. 2012). Polytubey block results from the rapid dilution of pre-secreted pollen tube attractant in the persistent synergid cell. This is accomplished by ethylene signaling induced programmed cell death of the persistent synergid cell following egg cell fertilization and synergid endosperm fusion induced by fertilization of the central cell (Volz et al. 2013; Maruyama et al. 2015). Reviewing the detailed molecular events that lead to successful fertilization and polytubey block is beyond the scope of this chapter.

8.5 Techniques to Study Directional Pollen Tube Growth

8.5.1 Live-Cell Imaging

Direct visualization in real time of the dynamic process of pollen tube growth during their journey toward the ovule and subsequent events leading to fertilization can greatly advance our understanding of the whole process of sexual plant reproduction. However, there are several hindrances that limit our ability to observe these events in vivo, including the confined nature of plant reproduction process that occurs deeply hidden within several cell layers of the pistil. Recent advances in the development of in vitro (or semi-in vivo) systems and the use of advanced imaging techniques have enabled real-time visualization of events in plant reproduction (Kurihara et al. 2013).

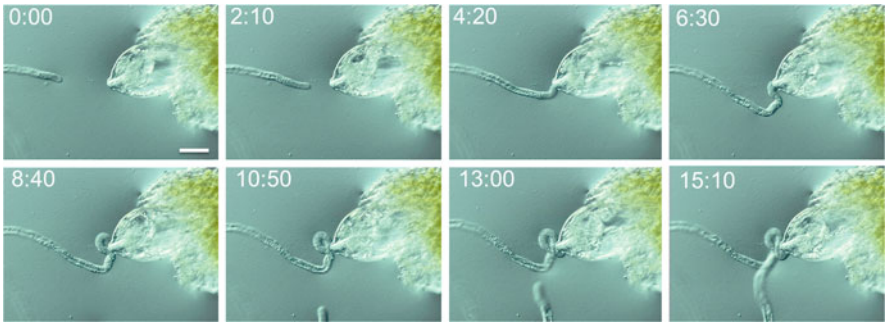

Fig. 8.2 Pollen tube attraction by the *Torenia* embryo sac. Two pollen tubes are attracted to the micropylar end of a protruding *Torenia* embryo sac in the in vitro guidance assay system. Images taken from Higashiyama and Hamamura (2008). Bar, 30 μm

The embryo sac in most of the flowering plants is found inside the ovule and is deeply embedded within thick layers of sporophytic tissues, which makes it difficult for imaging. However, in some plant species including *Torenia*, the embryo sac protrudes from the ovule enabling us to visualize the process of pollen tube guidance in vitro (Higashiyama et al. 1998). It is now possible to culture both the ovule and pollen tube in vitro and semi-in vivo to study the growth of pollen tube toward the ovule. In vitro systems in *Torenia* and *Arabidopsis* have broadened our knowledge of the requirement of the female sporophytic tissues in the generation of guidance cues for the growing pollen tube (Higashiyama et al. 1998; Palanivelu and Preuss 2006). A time-lapse observation with light microscopy is used for live imaging of pollen tube guidance in *T. fournieri*. As shown in Fig. 8.2, the pollen tube grows precisely toward the entrance site of the protruding *Torenia* embryo sac. Micromanipulation of the ovule with a glass needle established the fact that the entrance site of the embryo sac releases some diffusible attractant, as the pollen tube moved toward the entrance site even when the attracting ovule was moved away from the pollen tube (Higashiyama and Hamamura 2008). Later, it was established that these diffusible attraction signals were cysteine-rich peptides derived from the synergid cells (Okuda et al. 2009).

Advanced imaging technology like the use of two-photon excitation microscopy (TPE) enables live imaging of the pollen tubes journey inside the pistil tissue (Cheung et al. 2010). Using this technique, it is now possible to penetrate deep into the plant tissues and obtain higher resolution of thin optical sections that is not possible with wide-field fluorescence or confocal microscopy (Hepler and Gunning 1998; Fricker and Meyer 2001; Feijo and Moreno 2004; Cheung et al. 2010). A noninvasive procedure to monitor pollen germination and tube growth has been developed in the *Arabidopsis* using TPE in combination with a transgenic line carrying a pollen-specific promoter fused with GFP (LAT52::eGFP) (Twell et al. 1989; Cheung and Wu 2001; Cheung et al. 2010). Using this method, the growth of a pollen tube can be followed until the final stages of growth along the stigmatic

papillae and prior to its entry into the transmitting tract tissue. Thick and optically dense tissues surrounding the transmitting tissue preclude the detection of GFP from the penetrating pollen tubes, but monitoring of pollen tube growth can resume after they emerge from the initial septum wall of the ovary locules. Two-photon imaging using longer excitation wavelengths is useful for deep imaging of intact *Arabidopsis* tissues (Mizuta et al. 2015). The results are promising as it is possible to decrease the chloroplast autofluorescence as well as the autofluorescence of leaves, roots, pistils, and pollen grains. Two-photon imaging at 980 nm enabled multicolor imaging by simultaneous excitation and allowed deep imaging of intact cells in root tips and pistils (Mizuta et al. 2015). In future, it should be possible to visualize the entire process of pollen tube growth, guidance, and fertilization using a combination of near-infrared TPE and appropriate or improved fluorophores which are resistant to photo bleaching.

Traditional method of visualizing pollen tubes growing though a pistil in fixed samples relies on aniline blue staining. The limitation of this method is that different genotypes of pollen tubes are indistinguishable. Multicolor imaging with two-photon imaging and the use of a recently developed clearing method using a specific chemical solution, ClearSee, might overcome these limitations (Mizuta et al. 2015; Kurihara et al. 2015). ClearSee can significantly reduce chlorophyll autofluorescence while maintaining fluorescent protein stability and is suitable for both confocal and two-photon excitation microscopy as well as for multicolor imaging of fluorescent proteins (Kurihara et al. 2015).

Live-cell imaging of double fertilization was achieved using a semi-in vivo system and spinning disk confocal microscopy in *Arabidopsis* (Hamamura et al. 2011). All the stages from pollen tube discharge to the fusion of the sperm nuclei with the target female nuclei (karyogamy) were visualized continuously by photolabeling of the isomorphic sperm cells before pollen tube discharge. The study revealed a three-step process for sperm delivery. The sperms were released together to the boundary between the two female gametes, and after a long period of immobility, each sperm cell fused with either female gametes in no preferential order (Hamamura et al. 2011). Live-cell imaging can now be used to visualize embryogenesis in real time. The process of cell division and cell fate specification following zygote formation was successfully visualized in *Arabidopsis* using an in vitro ovule culture system (Gooh et al. 2015). The day is not far when we could observe all the process starting from pollen tube germination and growth to fertilization and embryogenesis in vivo and in real time.

8.5.2 Microfluidic Devices Aid in Understanding Directional Growth of Pollen Tube

Pollen tubes are the perfect model system to study polarized cell growth as they elongate rapidly and can be cultured in vitro. Development of an in vitro growth environment that physically resembles the in vivo growth conditions can greatly

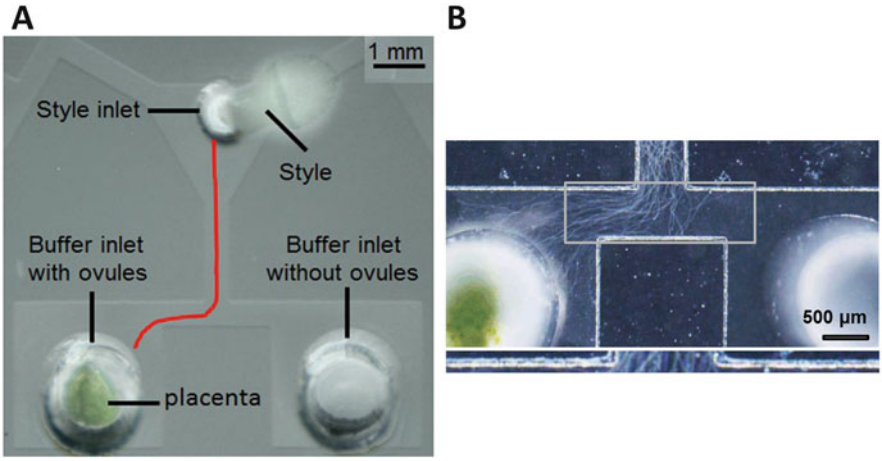

Fig. 8.3 Microfluidic device for studying pollen tube guidance. Photograph of the T-junction microfluidic device showing pollen tube attraction toward a reservoir with ovule. (**a**) A micro-device for quantitative analysis of chemoattraction in plants. (**b**) All pollen tubes are seen to be growing toward the reservoir (*left*) containing the ovule. Images taken from Sato et al. (2015) and Horade et al. (2013)

advance our understanding of the pollen tube growth process and also can serve as a platform for quantitative analysis of chemoattraction in plants.

Recent years has seen the development of a lab-on-a-chip (LoC) platform to understand and manipulate the growth of individual pollen tubes. This platform greatly relies on the use of a biocompatible polydimethylsiloxane (PDMS) chip and microfluidics to distribute pollen grains to serially arranged microchannels where their growth can be studied individually (Agudelo et al. 2012; see also Chap. 5). It also allows for studying the growth of pollen tubes toward the source of chemoattractants and the quantification of pollen tube attraction in response to guidance by female gametophyte tissue (Fig. 8.3). The planar form factor and optical transparency of micro-devices make them compatible with high-resolution and real-time microscopy (Duncombe et al. 2015). They are also extremely suitable for live-cell imaging as they provide controlled cellular microenvironments for long-term monitoring of growing pollen tubes.

Microfluidic cell culture is extensively used to study the development of small multicellular organisms (Sivagnanam and Gijs 2013; Choudhury et al. 2012). It is successfully employed to monitor detailed gene expression of several *Arabidopsis* transgenic reporter lines under different growth conditions (Busch et al. 2012). Microfluidics is also a powerful tool to understand cell mechanics and quantification of cellular penetrative forces generated in tip-growing cells including pollen tubes (Vanapalli et al. 2009; Nezhad et al. 2013). Microfluidic devices can provide a great advantage to study pollen tube guidance compared to the existing bioassays, e.g., semi-in vivo assays in petri dish, to monitor pollen tube behavior in response to

complex guidance cues. It is suggested that the reorientation of pollen tube growth is controlled by a gradient of guidance or attractant molecules (Palanivelu et al. 2003; Okuda et al. 2009). Pollen tubes grown in in vitro culture medium tend to grow toward the source of attractant in a concentration-dependent manner (Okuda et al. 2009). It is difficult to determine the concentration of attractant molecules near the pollen tube, as the attractants used in the assay are spotted on a solidified medium resulting in rapid diffusion. A narrow microfluidic channel-based pollen tube assay system can restrict the growth path of pollen tubes, can enable the monitoring of pollen tubes into the concentration gradients of chemoattractants for an extended duration, and also enables the generation of different micro-gradients of guidance cues (Horade et al. 2013). In vivo pollen tube guidance to the excised ovules is successfully recapitulated in *Arabidopsis* and *Torenia fournieri* using fabricated micro-devices (Yetisen et al. 2011; Horade et al. 2013; Sato et al. 2015). Thus, microfluidic devices can serve as a promising platform for the modeling of pollen tube behavior in response to guidance cues and searching individual molecules that serve as guidance cues, also allowing high-throughput screening of chemical libraries or purified molecules from female tissues. In future, the use of microfluidics can be extended to single-cell genome and transcriptome sequencing to study plant reproduction.

8.6 Conclusion and Perspective

Understanding directional growth of pollen tube through the pistil is essential to understand several aspects of plant reproduction, including the molecular and evolutionary basis of sexual plant reproduction. Research spanning several decades has shed light into the cellular dynamics that underlie the pollen tube growth process. Several molecules that enable the pollen tube to navigate through the pistil and precisely target the ovules for fertilization have been identified. The discovery of these molecules and their characterization has tremendously contributed to our understanding of the guidance signals. The detailed molecular mechanisms and signal transduction pathways that enable the pollen tube tips to reorient to these guidance cues are yet to be deciphered, and there are many questions still to be answered. Recent advances in molecular biology, functional genomics, bio-imaging, microfluidics, single-cell omics, and computational tools provide us with an improved spatiotemporal understanding of the cellular dynamics during pollen tube growth and navigation. Integration of all these tools and approaches would enable us to get a detailed understanding of the principles that regulate cell expansion and directional cell growth in future.

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Chapter 9
Molecular Mechanisms Regulating Root Hair Tip Growth: A Comparison with Pollen Tubes

Sébastjen Schoenaers, Daria Balcerowicz, and Kris Vissenberg

Abstract The developmental program of roots is constantly modified according to environmental signals and often includes an elevation in the density of root hairs, which increases the root's absorptive surface in an attempt to meet the ion and water demands of the plant. Root hairs emerge from certain epidermal cells and this depends on a complex genetic cascade. Once this has determined root hair cell fate, local wall loosening and turgor pressure initiate a bulge in the cell wall. The transition from root hair initiation to actual tip growth begins with the accumulation of secretory vesicles at the apical part of the bulge. A complex interplay between ion oscillations, cytoskeleton architecture, vesicle trafficking, cell wall metabolism and hormonal and environmental signals allows the root hair to maintain growth at the tip. This review summarizes the current knowledge on the core components regulating root hair tip growth, critically identifies challenges for future research and points to commonalities and differences with the current knowledge on pollen tube tip growth.

Keywords Arabidopsis • Calcium • Cell wall • Cytoskeleton • Elongation • Pollen tube • Root hair • ROPs • ROS • Tip growth

Abbreviations

ACA	autoinhibited Ca^{2+}-ATPase
ACT	ACTIN
ADF	actin-depolymerizing factor

S. Schoenaers • D. Balcerowicz
Biology Department, Integrated Molecular Plant Physiology Research, University of Antwerp, groenenborgerlaan 171, 2010 Antwerp, Belgium

K. Vissenberg (✉)
Biology Department, Integrated Molecular Plant Physiology Research, University of Antwerp, groenenborgerlaan 171, 2010 Antwerp, Belgium

Plant Biochemistry & Biotechnology Lab, Department of Agriculture, School of Agriculture, Food & Nutrition, UASC-TEI, Stavromenos, Heraklion, Crete, Greece
e-mail: kris.vissenberg@uantwerpen.be

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AFs	actin filaments
AHA	*Arabidopsis* H^+-ATPase
AIP1	AKT1 INTERACTING PROTEIN PHOSPHATASE 1
ANX	ANXUR
ARK1	ARMADILLO REPEAT-CONTAINING KINESIN 1
ARP2/3 complex	actin-related protein 2/3 complex
ATPase	adenosine triphosphatase
ATSFH1	ARABIDOPSIS THALIANA SHORT ROOT HAIR 1
AtSTP6	ARABIDOPSIS THALIANA SUGAR TRANSPORTER 6
BAPTA	1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid
CA	constitutively active
$[Ca^{2+}_{cyt}]$	cytoplasmic calcium concentration
$[Ca^{2+}_{ER}]$	endoplasmic reticulum calcium concentration
$[Ca^{2+}_{ext}]$	extracellular calcium concentration
CaM	calmodulin
cAMP	cyclic adenosine monophosphate
CAP1	$[CA^{2+}]$CYT-ASSOCIATED PROTEIN KINASE 1
CBLs	calcineurin B-like proteins
CBM3a	carbohydrate-binding module 3a
CDPK	calcium-dependent protein kinase
CESA	CELLULOSE SYNTHASE
CIPKs	CBL-interacting protein kinases
$[Cl^-_{cyt}]$	chloride concentration cytoplasmic
CMLs	calmodulin-like proteins
CMTs	cortical microtubules
CNGC	cyclic nucleotide-gated channel
cNMP	cyclic nucleotide
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CPK3	CALCIUM-DEPENDENT PROTEIN KINASE 3
CRIB	Cdc42- and Rac-interactive binding
CrRLK1Ls	*Catharanthus roseus* RLK1-like kinases
CSC	cellulose synthase complex
CSLD	CELLULOSE SYNTHASE-LIKE D
CytB	cytochalasin B
DACC	depolarization-activated calcium channel
DCB	2,6-dichlorobenzonitrile
DN	dominant negative
DPI	diphenyleneiodonium
ECA1	ER-type Ca^{2+}-ATPase
EGTA	ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid
EMTs	endoplasmic microtubules
EXP7	EXPANSIN7
F-actin	filamentous actin
FER	FERONIA
FH1 domain	formin homology 1 domain

FH2 domain	formin homology 2 domain
FRET	Förster resonance energy transfer
G-actin	globular actin
GAE6	UDP-D-GLUCURONATE 4-EPIMERASE 6
GAP	GTPase-activating protein
GDI	guanosine nucleotide dissociation inhibitor
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
GLR	glutamate receptor
GTP	guanosine triphosphate
GTPase	guanosine triphosphatase
HACC	hyperpolarization-activated calcium channel
HAK5	HIGH-AFFINITY K^+ TRANSPORTER 5
HEK	human embryonic kidney
HG	homogalacturonan
IP_3	inositol trisphosphate
LatB	latrunculin B
LePT1	*Lycopersicon esculentum* phosphate transporter 1
LRX1	LEUCINE-RICH REPEAT/EXTENSIN 1
MAPK/MPK	MITOGEN-ACTIVATED PROTEIN KINASE
MCA1/2	MID1-COMPLEMENTING ACTIVITY 1/2
MICU	mitochondrial Ca^{2+} uniporter
MOR1	MICROTUBULE ORGANIZATION 1
MRH2	MORPHOGENESIS OF ROOT HAIR 2
MSL2/3	MSCS-LIKE 2/3
MT	microtubules
MyoB1/2	myosin-binding proteins 1/2
NHX1-4	SODIUM HYDROGEN EXCHANGER 1-4
NOX	NADPH oxidase
ORF	open reading frame
OXI1	OXIDATIVE SIGNAL-INDUCIBLE1
PH	pleckstrin homology
pH_{cyt}	cytoplasmic pH
pH_{ext}	extracellular pH
PIN2	PIN-FORMED 2
PI-4Kβ1	PHOSPHATIDYLINOSITOL 4-OH KINASE β1
PI-4P	phosphatidylinositol 4-phosphate
$PI(4,5)P_2$	phosphatidylinositol 4,5-biphosphate
Plus(+) end	barbed actin filament end
PM	plasma membrane
PME	pectin methylesterase
PMEI	pectin methylesterase inhibitor
prf1	profilin 1
PRONE	plant-specific ROP nucleotide exchanger
PT	pollen tube

qRT-PCR	quantitative reverse transcriptase polymerase chain reaction
RabA4b	RAB GTPASE HOMOLOGUE A4B
RBOH	RESPIRATORY BURST OXIDASE HOMOLOGUE
RH	root hair
RHD2	ROOT HAIR DEFECTIVE 2
RHM1	RHAMNOSE BIOSYNTHESIS 1
RHS	root hair specific
RHS8	ROOT HAIR SPECIFIC 8
RIC	ROP-INTERACTIVE CRIB MOTIF-CONTAINING PROTEIN
RIP	ROP INTERACTIVE PARTNER
RLK	receptor-like kinase
RNAi	RNA interference
ROPs	Rho-like GTPases from plants
ROS	reactive oxygen species
SCN1	SUPERCENTIPEDE1
SIMK	STRESS-INDUCED MAPK
SLAH3	SLAC1 HOMOLOGUE 3
SOD	superoxide dismutase
TAIR	The Arabidopsis Information Resource
TCH2	TOUCH2
T-DNA	transfer-DNA
TPC1	TWO-PORE CHANNEL 1
UER1	UDP-4-KETO-6-DEOXY-D-GLUCOSE-3,5-EPIMERASE-4-REDUCTASE 1
VGD1	VANGUARD1
VLN	VILLIN
WER	WEREWOLF
XEH	XYLOGLUCAN ENDOHYDROLASE
XTH	XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE
XUT1	XYLOGLUCAN-SPECIFIC GALACTURONOSYLTRANSFERASE 1
XXT	XYLOGLUCAN XYLOSYLTRANSFERASE
XyG	xyloglucan
YC3.6	Yellow Cameleon 3.6 (cytosolic calcium sensor)
YFP	yellow fluorescent protein

9.1 Introduction

Two types of cell elongation, named diffuse and tip growth, are found in higher plants. Tip growth is a highly specific and conserved developmental process that governs both root hair (RH) and pollen tube (PT) growth. Following pollination,

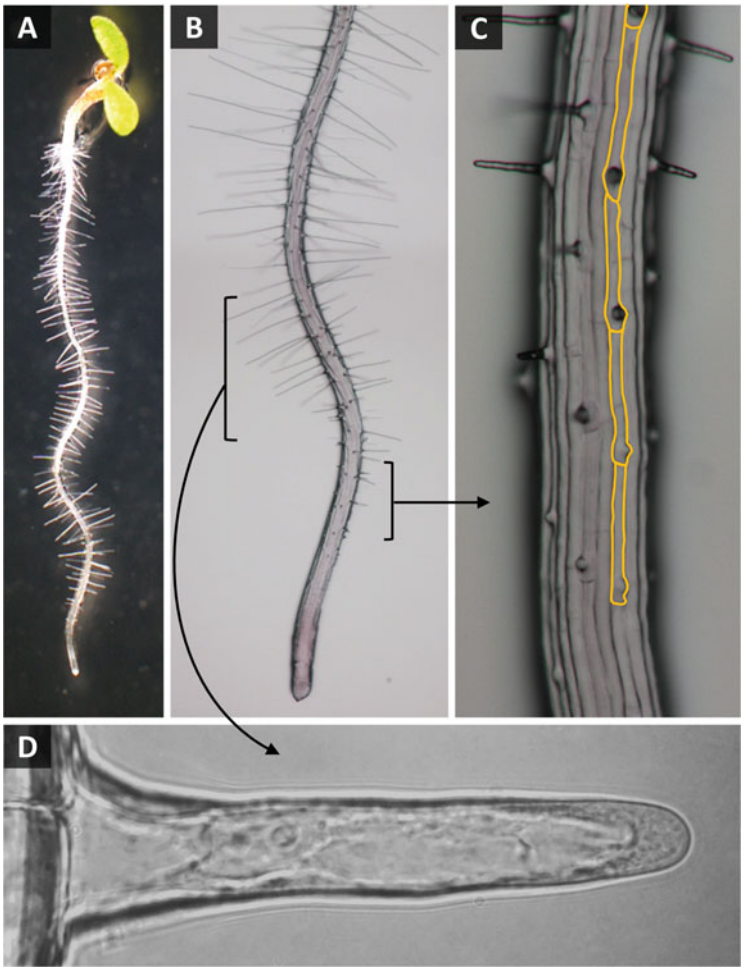

Fig. 9.1 Root hair growth pattern and morphology in *Arabidopsis*. (**a**) 5-day-old *Arabidopsis* seedling with root hairs decorating the primary root. (**b**) Close-up of the root hair growth zone showing the zone of root hair bulge emergence and the fast tip growth zone. (**c**) Close-up of the bulge formation zone, showing emerging root hairs at the basal side of single epidermal trichoblast cells (*orange* = outline of root hair cell file). (**d**) Bright-field microscopy image of a single tip-growing root hair

PTs emerge from the pollen grains and transport the male gametophytes towards the ovule to facilitate double fertilization. RHs on the other hand arise from the root's epidermis, where they drastically increase the root's absorption/contact area (Fig. 9.1). As such, tip growth lies at the basis of the plant's ability to reproduce, acquire nutrients and water, anchor to the soil and interact with soil microbiota. RHs are often exploited to study tip growth since many mutations exist, they are easy to visualize and especially since their presence is not a requirement for plant survival.

The root epidermis is typically built up by RH (trichoblasts) and non-RH cells (atrichoblasts). Cell fate specification and initiation site determination are the processes that regulate whether and where RH bulges are formed on epidermal cells. A genetic cascade involving intra- and intercellular position-dependent signalling and complex feedback loops determines cellular fate, and local wall weakening coupled to turgor pressure initiates a highly localized bulge (reviewed in Balcerowicz et al. 2015). Next, fast tip growth is initiated. Unlike diffuse cell elongation where growth occurs over the whole cell's surface, tip growth is restricted to the very apex of the tubular growing structure. The frequency and amplitude of PT and RH growth depends on extracellular conditions and a highly organized interplay between gene transcription, protein turnover and modification, the cytoskeleton, the cell wall, ion and reactive oxygen species (ROS) gradients and membrane-localized import and export proteins. At first sight, the process by which PTs and RHs grow appears to be highly similar. More so, comparing the RH and PT transcriptome revealed the existence of a common apical cell growth signature (Becker et al. 2014). However, several decades of research has shown that even though some central aspects are shared, multiple regulatory components differ between PT and RH development. Here, we review ROP GTPases, ROS, calcium and pH gradients, the actin and microtubule (MT) cytoskeleton and the cell wall, main molecular determinants controlling both PT and RH tip growth. We will not focus on the hormonal and genetic (mainly *root hair specific-like 4; RSL4*) regulation of these molecular players, but we will identify commonalities, differences and potential targets for future research based on the current knowledge available at the time of writing.

9.2 ROP GTPases as Master Regulatory Switches

9.2.1 ROPs Control Tip Growth

Plant Rho-like GTPases (ROPs) play a key role in the regulation of various developmental and cellular processes with emphasis on polarized growth of RHs and PTs. These small GTPases cycle between an inactive (GDP-bound) and an active (GTP-bound) state and thus act as molecular switches 'turning on and off' a wide range of signalling pathways (Fig. 9.2). ROP activity oscillates with the same frequency as tip growth oscillations (Monshausen et al. 2007; Hong et al. 2015) and depends on the action of several regulatory proteins. GTP-bound ROPs localize to the plasma membrane (PM) at the apex of elongating RHs and PTs (Kost et al. 1999; Molendijk et al. 2001; Jones et al. 2002), allowing them to relay extracellular signals from PM-associated receptors such as receptor-like kinases (RLKs; Nibau and Cheung 2011). ROPs interact with diverse effectors in order to mediate actin dynamics, MT organization, vesicle trafficking (reviewed in Nibau et al. 2006; Yalovsky et al. 2008; Yang 2008), ROS production (Carol et al. 2005) and the formation of a tip-focused Ca^{2+} gradient (Li et al. 1999).

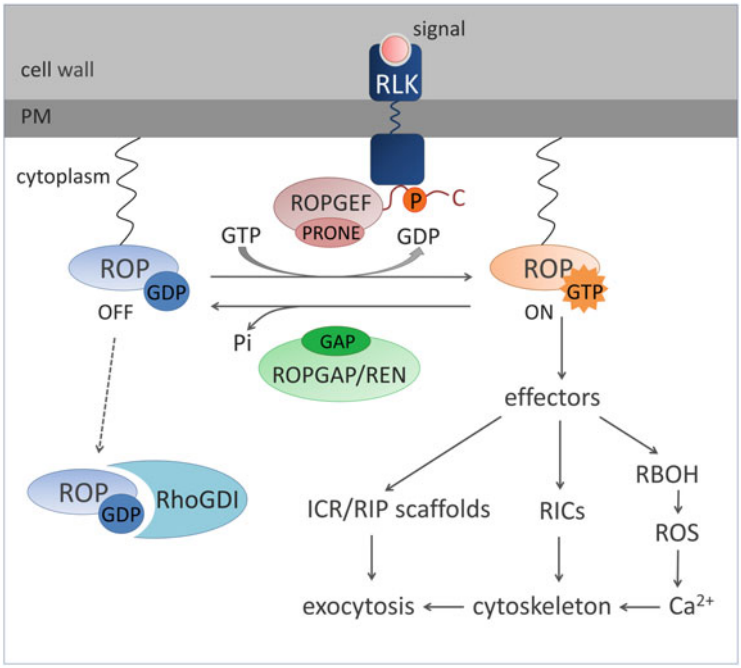

Fig. 9.2 Graphical representation of the ROP-GTPase signalling cascade during root hair growth. All abbreviations are referred to in Sect. 9.2

The *Arabidopsis* genome encodes 11 ROPs from which ROP1, 2, 4 and 6 have been implicated in tip growth of RHs. In general, expression of constitutively active (CA) forms of ROPs leads to non-polarized RH growth, whereas overexpression of dominant negative (DN) variants results in reduced RH elongation (Molendijk et al. 2001; Tao et al. 2002; Jones et al. 2002, 2007; Carol et al. 2005). Furthermore, ROP1, ROP3 and ROP5 have been shown to regulate PT growth and perturbation of their expression, and activity causes similar effects to those observed in RHs (Kost et al. 1999; Fu et al. 2001; Chen et al. 2003; Gu et al. 2003). Interestingly, transcription of a common set of ROP-related genes defines both RH and PT development in *Arabidopsis* (Becker et al. 2014).

9.2.2 ROP-Associated Proteins Regulate ROP-GTPase Activity

Activity of ROPs is mainly regulated by guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs) and guanine nucleotide dissociation inhibitors (GDIs) (Fig. 9.2; reviewed by Yang 2002; Nagawa et al. 2010).

9.2.2.1 Guanine Nucleotide Exchange Factors

GEFs are PRONE-domain-containing proteins which activate ROPs at the apical PM by catalysing the exchange of GDP to GTP (Fig. 9.2; Berken et al. 2005). In *Arabidopsis*, 3 of 14 members of the GEF family are implicated in RH development. The RH-specific ROPGEF4/RHS11 is involved in the RH elongation stage since loss of function causes a short RH phenotype (Won et al. 2009; Huang et al. 2013a), while knocking out ROPGEF10 mainly results in a reduced RH number. The fact that ROPGEF10 is expressed in all root epidermal cells (Won et al. 2009) and that no changes in the expression of RH-specific genes were found in *gef10* plants indicates that ROPGEF10 rather plays a role during RH initiation. Truncation and domain-swapping experiments revealed that the distinct functions of ROPGEF4 and ROPGEF10 result from their noncatalytic domains (Huang et al. 2013a). On the other hand, both ROPGEFs are interacting partners for RLK FERONIA (FER) and seem to participate in a common pathway for ROS production which involves activation of ROP2 and ROP6 (see Sect. 9.3; Huang et al. 2013a). Shin et al. (2010) reported that overexpression of the third member, ROPGEF11/phytochrome-interacting ROPGEF1 (PIRF1), affects RH polarity and RH density. Furthermore, growing PTs, similarly to RHs, express seven members of the ROPGEF family and also require the action of ROP activators (Gu et al. 2006; Zhang and McCormick 2007). It has been demonstrated that the GFP-tagged RopGEF1, RopGEF8, RopGEF9, RopGEF12 and RopGEF14 localize to the PM of the PT apex and that overexpression of these GEFs causes loss of polar PT growth with the most severe phenotype seen for RopGEF1 (Gu et al. 2006). Notably, overexpression of RopGEF12 leads to dramatic changes in PT morphology only if RopGEF12 is C-terminally truncated, suggesting that the C-terminal region is auto-inhibitory to GEF activity (Zhang and McCormick 2007). According to more recent findings, the C-terminus of RopGEF1 is directly phosphorylated by the pollen RLK pollen receptor-like kinase 2 (PRK2) (Chang et al. 2013). The authors proposed a model in which PRK2 activates RopGEF1 through releasing its auto-inhibition by phosphorylation, which in turn leads to the activation of ROP1. In addition, interactions with ROPGEFs have also been reported for the RLKs from the *Catharanthus roseus* receptor-like kinase 1-like (CrRLK1L) subfamily (Duan et al. 2010).

9.2.2.2 GTPase-Activating Proteins

Activated ROPs are ‘switched off’ by GTPase-activating proteins (GAPs) which accelerate GTP hydrolysis. In *Arabidopsis*, nine genes have been found to encode for proteins with a GAP catalytic domain (Wu et al. 2000; Hwang et al. 2008). Among these, six members, termed ROPGAP1–ROPGAP6, contain a CRIB motif that is required for binding to ROPs and acts as a positive regulator of GAP activity (Wu et al. 2000). So far nothing is known about the role of ROPGAPs in RH growth. However, according to the *Arabidopsis* eFP Browser (Winter et al. 2007),

ROPGAP1 and ROPGAP6 are specifically expressed in RH cell files. ROPGAP1 has been shown previously to regulate ROP1 activity in PTs since coexpression of this GAP restores polarized PT growth in ROP1-overexpressing plants (Hwang et al. 2010). Consistently, NtRhoGAP1, a tobacco homologue of ROPGAP1, suppresses a phenotype caused by NtRAC5 (a tobacco ROP) overexpression (Klahre and Kost 2006). ROPGAP6 is annotated as a pseudogene although it has an intact ORF and a normal structure. In addition, the ROPGAP6 protein lacks part of a conserved GAP motif (Kost 2010). Hence, it remains unclear whether ROPGAP6 encodes a functional ROPGAP. Furthermore, Hwang et al. (2008) have identified novel structurally distinct *Arabidopsis* ROPGAPs, termed REN1-3, which contain a conserved GAP motif, an N-terminal pleckstrin homology (PH) domain and C-terminal coiled-coil regions. It is likely that a PH domain regulates catalytic activity and/or localization of RENs through binding to phospholipids, as it was shown for non-plant GAPs (Harlan et al. 1994; Lemmon 2008). To date, REN1 is the only PH-type ROPGAP characterized in detail. Knocking out REN1 leads to formation of balloon-shaped PTs indicating that REN1 plays a major role in controlling PT growth polarity. The authors suggested a self-organizing mechanism in which REN1 associates with exocytic vesicles via the coiled-cold part and is targeted to the apical PM where it globally inhibits ROP1 activity. Vesicle trafficking is stimulated by ROP1 signalling which creates a negative feedback loop and prevents excessive ROP activation. It would be interesting to verify whether a similar mechanism involving REN1 exists in RHs.

9.2.2.3 Guanine Nucleotide Dissociation Inhibitors

Spatial control of ROP signalling also depends on RhoGDIs, small cytoplasmic proteins characterized by the presence of a C-terminal immunoglobulin-like domain (IG-like) and a regulatory arm (RA). The IG-like domain is responsible for transferring inactive ROPs from the PM to the cytoplasm, followed by the formation of ROP/RhoGDI heterodimers (Klahre et al. 2006; Kost 2010). The RA sequesters ROPs in their inactive state by preventing GDP dissociation and blocking interactions with regulators and effectors (DerMardirossian and Bokoch 2005). The *Arabidopsis* RhoGDI family consists of three members (RhoGDI1, 2a and 2b) that share high similarity with mammalian RhoGDIs (Bischoff et al. 2000). RhoGDI1/SUPERCENTIPEDE1 (SCN1) is involved in RH growth through promoting the tip-focused production of ROS by ROOT HAIR DEFECTIVE 2 (RHD2)/RBOHC (see Sect. 9.3; Carol et al. 2005). Yeast two-hybrid assay and FRET analysis revealed that RhoGDI1 interacts with ROP2, ROP4 and ROP6 (Bischoff et al. 2000; Wu et al. 2013). Further, it has been demonstrated that the phosphorylation of RhoGDI1 by calcium-dependent protein kinase 3 (CPK3) has an effect on the binding ability to ROPs (Wu et al. 2013). RhoGDI1 might regulate subcellular localizations of ROP2 since ROP2::YFP was ectopically localized at the trichoblast surface in *scn1* (Carol et al. 2005). Similarly, depolarized localization of ROPs has also been observed in *gdi2a-RNAi* PTs (Hwang et al. 2010). Very recently,

Feng et al. (2016) reported that all three RhoGDIs play redundant roles in sustaining cellular homeostasis during PT growth. In addition, RhoGDI2a and RhoGDI2b are mainly expressed in the male gametophyte suggesting that PTs have higher demands for GDI-mediated ROP signalling in comparison to RHs (Feng et al. 2016).

9.2.3 ROP Effector Proteins

In addition to ROP regulators, several ROP effector proteins such as ROP-INTERACTIVE CRIB MOTIF-CONTAINING PROTEINS (RICs), INTERACTORS OF CONSTITUTIVE ACTIVE ROPS (ICRs)/ROP INTERACTIVE PARTNERS (RIPs), receptor-like cytoplasmic kinases and cysteine-rich receptor kinase have been identified (reviewed in Nagawa et al. 2010).

9.2.3.1 ROP-Interactive CRIB Motif-Containing Proteins (RICs)

The *Arabidopsis* genome encodes 11 RICs, which interact with GTP-bound ROPs (Wu et al. 2001). To date, most of our knowledge about the involvement of RICs in tip growth comes from studies on PTs, where ROPs recruit several RICs to coordinate PT growth. Overexpression of RIC1, RIC2, RIC5, RIC6 and RIC7 inhibits, while overexpression of RIC10 promotes elongation of tobacco PTs (Wu et al. 2001). Moreover, elevated expression of RIC3 and RIC4 leads to non-polar PT growth and causes formation of bulbous tips. Phenotypes of RIC3 and RIC4 overexpression lines are associated with antagonist actions of these two effectors on F-actin dynamics, and co-overexpression of both RICs restores proper tip growth of PTs (Gu et al. 2005). RIC4 promotes actin assembly which is required for vesicle accumulation at the tube apex, whereas RIC3 mediates actin disassembly via activation of Ca^{2+} signalling which induces exocytosis in the growing tip. Importantly, both RICs are downstream components of ROP1 signalling demonstrating that a single ROP can act through activation of antagonist pathways (Gu et al. 2005; Lee et al. 2008). According to a recent report, regulation of F-actin dynamics in PTs also depends on the action of RIC1 (Zhou et al. 2015b). In vitro studies revealed that RIC1 has an ability to sever and cap F-actin in the presence of Ca^{2+}. In addition, RIC1 localizes to the apical PM of PTs and its distribution oscillates together with growth oscillations. Noteworthy, previous studies have shown that ROP6 activates RIC1 in order to promote katanin-mediated MT severing in leaf pavement cells (Fu et al. 2005, 2009; Lin et al. 2013) and to inhibit PIN2 internalization through stabilization of AFs in roots (Lin et al. 2012). Thus, the action of RIC1 on the cytoskeleton seems to differ between tissues or cell types. It would be interesting to determine whether RICs also contribute to RH growth by regulating the actin and/or MT cytoskeleton (Jones et al. 2006; Cole and Fowler 2006).

9.2.3.2 Interactors of Constitutive Active ROPS (ICRs)/ROP Interactive Partners (RIPs)

Activated ROPs also interact with a novel class of plant-specific effector proteins known as ICRs/RIPs. All five members of the *Arabidopsis* ICR/RIP family are characterized by the presence of a highly conserved QWRKAA motif in their C-terminal region which is required for binding to ROPs (Li et al. 2008). Until now, the role of these effectors in RH growth remains unknown. ICR1/RIP1, the most extensively studied member of the group, has been identified from two independent yeast two-hybrid screens using CA forms of ROPs as bait (Lavy et al. 2007; Li et al. 2008). GFP-tagged ICR1/RIP1 localizes to the apical cortex of growing PTs and its distribution depends on the activity of ROP1. At the same time, overexpression of ICR1/RIP1 enhances recruitment of GFP-ROP1 to the PM what suggests that ICR1/RIC1 might participate in a positive feedback loop to ensure polar localization of ROP1 to the PM (Li et al. 2008). Furthermore, Lavy et al. (2007) have demonstrated that ICR1/RIP1 form complexes with the exocyst complex subunit SEC3A and that these complexes can interact with ROPs in vivo. This indicates that ICR1/RIP1 acts as a scaffold mediating interactions between different proteins. More recently, ICR1/RIP1 has also been shown to control polar localization of PIN auxin transporters in roots and embryos (Hazak et al. 2010). Thus, ICR1/RIP1 forms a link between auxin, ROPs and exocytosis. In addition, RIP3, which localizes to MTs, has been found to interact with active ROPs and the plant-specific kinesin-13A, thereby linking ROPs with the MT cytoskeleton (Mucha et al. 2010).

9.3 ROS as Diverse Signalling Molecules

9.3.1 ROS Production at the Tip

It is well established that RHs and PTs require production and accumulation of ROS at the tip in order to maintain polarized growth (Fig. 9.3). This process involves the activity of membrane-bound NADPH oxidases (NOX) which catalyse the reduction of molecular oxygen to the superoxide anion ($O_2^{\bullet -}$), a form of ROS (Sagi and Fluhr 2001). Monshausen et al. (2007) have demonstrated that *Arabidopsis* RHs exhibit oscillating increases in NOX-derived extracellular ROS levels which follow peaks in growth rate by approximately 7 s. *Arabidopsis* NOX, also named RBOH for respiratory burst oxidase homologues, are encoded by 10 genes (*RBOHA-RBOHJ*) (Torres and Dangl 2005). Generation of ROS in RHs is linked to RBOHC/RHD2 since mutations in *RHD2* greatly reduce ROS levels and arrest RH development at the bulge formation stage. A similar effect on RH growth can be observed after treatment of wild-type roots with diphenyliodonium chloride (DPI), a NOX inhibitor (Foreman et al. 2003). Two other members, RBOHH and RBOHJ, share

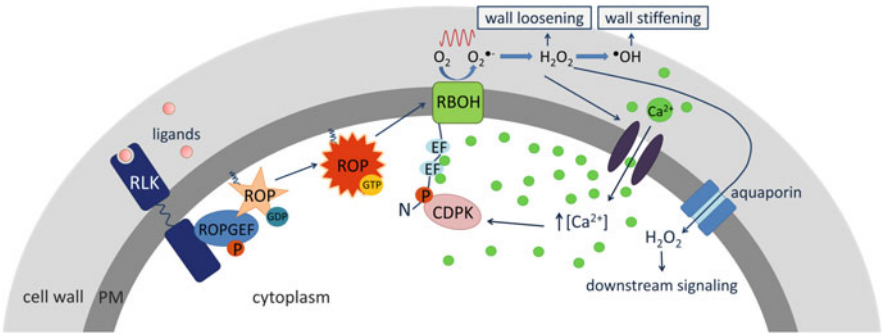

Fig. 9.3 Simplified model showing interactions between ROS and other key regulators at the tip of growing root hairs. All abbreviations are referred to in Sect. 9.3

81% amino acid identity and display partial redundancy in the regulation of PT growth. In vitro and in vivo studies have shown that *rbohH rbohJ* double mutants produce short and bursting PTs and exhibit reduced male fertility (Boisson-Dernier et al. 2013; Kaya et al. 2014). In *Nicotiana*, transfection of PTs with antisense NtNOX nucleotides downregulates the NtNOX level, decreases ROS formation and consequently inhibits tube growth (Potocký et al. 2007).

9.3.2 ROS Interacts with Calcium Signalling

Several lines of evidence support the hypothesis that NOX-derived ROS are involved in the activation of Ca^{2+} channels and Ca^{2+} flux into the cytoplasm at the tips of RHs and PTs (Fig. 9.3; reviewed in Wudick and Feijó 2014). First, *rhd2* plants lack the tip-focused Ca^{2+} gradient in RHs, and exogenous application of ROS increases the level of cytosolic Ca^{2+} and partially restores RH growth (Foreman et al. 2003). Further, knocking out *RBOHH* and *RBOHJ* disrupts Ca^{2+} homeostasis in PTs although the effect is less severe than that caused in the *rhd2* mutant. PTs of *rbohH rbohJ* retain the ability to form the tip-focused Ca^{2+} gradient, but this gradient is unsteady over time (Boisson-Dernier et al. 2013). In addition, it has been demonstrated that ROS activates Ca^{2+} channels in the PM of guard cells in response to abscisic acid (ABA; Pei et al. 2000). Furthermore, all *Arabidopsis* NOX proteins contain two putative EF-hand motifs in their N-terminal cytosolic part that are helix-loop-helix Ca^{2+}-binding domains (Keller et al. 1998; Torres et al. 1998). Takeda et al. (2008) have reported the existence of a positive feedback loop between Ca^{2+} and ROS which results from binding of Ca^{2+} to EF-hand motifs within RHD2 and the Ca^{2+}-dependent phosphorylation of RHD2. The authors demonstrated that point mutations in the EF-hands affect RH growth and that these mutations in both EF-hands and phosphorylation sites disrupt ROS formation in vitro. Ca^{2+}-stimulated ROS production has also been confirmed for other NOX proteins including pollen-

specific RBOHH and RBOHJ (Ogasawara et al. 2008; Kimura et al. 2012; Kaya et al. 2014).

9.3.3 ROS Regulates Cell Wall Properties

Given the fact that RHs of *rhd2-1* burst when switching to tip growth, RHD2-generated ROS are proposed to stabilize the cell wall at the expanding tip. In this context ROS might act in a complementary manner with extracellular pH since increasing the growth medium pH to ≥6.0 rescues the *rhd2-1* phenotype (Monshausen et al. 2007). Several studies indeed have shown that ROS affects cell wall properties although different forms of ROS may play opposite roles (Fig. 9.3). Extracellular superoxide produced by NOX can give rise to hydrogen peroxide (H_2O_2), a more stable type of ROS, either spontaneously or in the presence of superoxide dismutase (SOD; Halliwell and Gutteridge 1999). It has been shown that H_2O_2 causes cell wall stiffening by increasing cross-linking of wall polymers (Hohl et al. 1995; Schopfer 1996). Moreover, H_2O_2 can readily diffuse across the PM through aquaporins (Bienert et al. 2007; Hooijmaijers et al. 2012). Interestingly, a ROS gradient exists in the apical RH cytoplasm (Foreman et al. 2003; Bai et al. 2014b). However, no direct evidence exists for aquaporin-mediated transport of H_2O_2 at the tip of growing RHs. Most interestingly however, Di Giorgio et al. (2016) recently showed that the two aquaporins *NOD26-LIKE INTRINSIC PROTEIN 4;1/2 (NIP4;1/2)* are specifically expressed in PTs and that NIP4;1 can transport H_2O_2. Could aquaporins govern transmembrane H_2O_2 transport in tip-growing cells? In the presence of transition metals such as copper or iron, H_2O_2 can further be converted to hydroxyl radicals (•OH), the most reactive type of ROS (Fry 1998; Halliwell and Gutteridge 1999), which, in contrast, are involved in cell wall loosening through breakdown of wall polysaccharides (Fry 1998; Schopfer 2001; Liszkay et al. 2004). Thus, the proper balance between ROS species may be crucial for regulation of tip growth oscillations.

9.3.4 ROS and Kinase Signalling

Apart from the above-mentioned functions, ROS might also be involved in tip growth by participating in signal transduction pathways. Work by Rentel et al. (2004) has demonstrated that expression of *OXIDATIVE SIGNAL-INDUCIBLE1* (*OXI1*), which encodes a serine/threonine protein kinase, is induced in response to H_2O_2 administration. In addition, H_2O_2 has an ability to stimulate OXI1 kinase activity in vivo. Phenotypic analysis revealed that loss-of-function mutation in *OXI1* results in a slightly reduced RH length. Furthermore, OXI1 has been found to work upstream of mitogen-activated protein kinases (MAPKs) MPK3 and MPK6 since in the *oxi1* mutant both MAPKs showed reduced activation after treatment with

H_2O_2 and cellulase. Hence, it is likely that ROS may act as signalling molecules regulating activity of MAPKs by the intermediate of OXI1. The role of MAPK in RH growth has already been reported in *Medicago sativa* (Šamaj et al. 2002). SIMK, the homologue of MPK6, localizes to RH tips, and overexpression of its gain-of-function form has a stimulatory effect on RH growth. Besides, more recently, MPK3 and MPK6 have been implicated in funicular guidance of PTs (Guan et al. 2014).

ROS are downstream components of *Catharanthus roseus* receptor-like kinase (CrRLK1L) subfamily signalling pathways (Fig. 9.3). CrRLK1Ls are major regulators of cell expansion in response to extracellular signals and are likely to act as cell wall integrity sensors. In a current model, ligand-bound CrRLK1Ls interact with ROPGEFs in order to activate ROP GTPases. ROPs, in turn, stimulate NOX-dependent ROS production leading to the activation of Ca^{2+} channels and changes in cell wall properties (reviewed by Nibau and Cheung 2011; Wolf and Höfte 2014; Nissen et al. 2016). In RHs, ROS formation is controlled by PM-localized CrRLK1L FERONIA (FER) and *fer* mutants show RH defects similar to those seen in *rhd2* plants (Duan et al. 2010). The FER signalling pathway in RHs involves ROPGEF1, ROPGEF4, ROPGEF10 and ROP2 and ROP6 (Duan et al. 2010; Huang et al. 2013a). Interestingly, FER is also expressed in the female gametophyte where it induces ROS-mediated PT rupture (Escobar-Restrepo et al. 2007; Duan et al. 2010, 2014). Furthermore, ERULUS/$[Ca^{2+}]_{cyt}$-ASSOCIATED PROTEIN KINASE 1 (CAP1), which unlike other CrRLK1Ls is localized at the tonoplast, also contributes to ROS-mediated RH growth since abnormal RH growth in the *cap-1* mutant coincides with the absence of a ROS gradient in the hair tips (Haruta et al. 2014; Bai et al. 2014a, b). The ROS gradient in *cap-1* can be restored in NH_4^+-free growth medium suggesting a possible interplay between ROS and ammonium (Bai et al. 2014b). Besides, RHD2 activity also depends on the action of ROPGDI1/SCN1, a ROP regulatory protein. In *scn1* mutants RH bulges are formed at ectopic positions on the trichoblast, which results from delocalized RHD2 accumulation and ROS production (Carol et al. 2005). Proper distribution of RHD2 relies on actin microfilaments because functional loss of *ACTIN2* and treatment with microfilament-disrupting drug cytochalasin D (CytD) both lead to RHD2 accumulation in cytoplasmic clumps (Takeda et al. 2008). The fact that actin microfilament dynamics in RHs are regulated by ROP2, which is a target of SCN1, suggests that SCN1 might spatially control RHD2 localization via this ROP (Jones et al. 2002; Takeda et al. 2008). Similarly to RHs, ROS production in PTs is also controlled by CrRLK1L members. Pollen-specific ANXUR1 (ANX1) and ANXUR2 (ANX2) act redundantly to maintain PT growth within female tissue since PTs of *anx1 anx2* double mutant burst after germinating in vitro and fail to reach the ovules in vivo (Boisson-Dernier et al. 2009). Given the fact that the phenotype of *anx1 anx2* resembles that of *rbohH rbohJ* and that overexpression of *ANX*s and knockout of *RBOHH* and *RBOHJ* enhances exocytosis, it is proposed that ANXs regulate RBOHs to synchronize growth rate with cell wall exocytosis (Boisson-Dernier et al. 2009; Kaya et al. 2014; Lassig et al. 2014; Nissen et al. 2016).

9.4 Ion Oscillations Integrate Extra- and Intracellular Signalling

9.4.1 Calcium

9.4.1.1 Introduction

Calcium ions function as important second messenger in eukaryotic cells. In plants, Ca^{2+} is of crucial importance for signal transduction in processes such as long-distance propagation of electrical signals (reviewed in Steinhorst and Kudla 2014), the response to salt stress (Choi et al. 2014), regulation of exocytosis (Zorec and Tester 1992; Battey et al. 1999) and actin cytoskeleton dynamics (Battey and Blackbourn 1993; Chen et al. 2002; Braun et al. 2004), cell wall remodelling (Holdaway-clarke et al. 1997; Rounds et al. 2011), phosphoinositide signalling (Franklin-Tong 1999), stomatal aperture (Allen et al. 2001; Evans et al. 2001), mechanosensing (Monshausen et al. 2009) and gravitropism (Plieth and Trewavas 2002; Toyota et al. 2007).

Given the low diffusion constant of Ca^{2+} in the cytoplasm (10^{-7} cm^{-2} s^{-1}; Thomas 1982), local Ca^{2+} concentration maxima can be maintained for a prolonged period of time. Ca^{2+} concentration peaks are often keys for local regulation of specific signalling pathways in the cell. For instance, in living systems, integration of biotic and abiotic stimulus perception often occurs through Ca^{2+} signalling by local elevation of the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$; Hetherington and Brownlee 2004; Trewavas 1999).The existence of Ca^{2+} maxima relies on a well-coordinated release and sequestration from and to inter- and intracellular Ca^{2+} stores, through a collection of Ca^{2+} transporters. Such elevations in $[Ca^{2+}]_{cyt}$ can consist of a single transient peak, but often exhibit a more complex wave-like pattern. Highly specific differences in the temporal and spatial nature of these Ca^{2+} dynamics are often referred to as 'Ca^{2+} signatures' (Dodd et al. 2010). The characteristics (duration, frequency and amplitude) of these signatures determine downstream signal perception and propagation through a number of Ca^{2+}-sensing and Ca^{2+}-relaying proteins. Final perception of the Ca^{2+} signal steers multiple developmental pathways, often related to plant morphogenesis, including RH and PT development.

A central role for Ca^{2+} signalling in tip-growing cells was established over 20 years ago. For instance, both RHs and PTs rely on optimal extracellular Ca^{2+} concentrations ($[Ca^{2+}]_{ext}$) for successful tip growth. Generally, a lack of Ca^{2+} ions results in decreased RH growth in *Arabidopsis* (Schiefelbein et al. 1992). Also for PTs, a lack or excess of Ca^{2+} in the growth medium results in abnormal, stunted tubes (Boavida and McCormick 2007). Recently, advancements in spatial and temporal visualization of in vivo Ca^{2+} dynamics have further contributed to unraveling the complexity of Ca^{2+} signalling in RH and PT development. Whereas both cell types grow by the process of tip growth, some remarkable differences between PT and RH exist in the Ca^{2+}-related machinery. More so, while our

understanding has greatly improved, it is clear that the current knowledge is only scratching the surface, and many discoveries are yet to be made.

9.4.1.2 An Oscillating Tip-Focused Calcium Gradient Controls RH and PT Growth

Growing RHs and PTs exhibit a clear zone at their extreme apex. The clear zone delineates the region to which all tip growth-related machinery is concentrated. Numerous studies have now shown that the clear zone coincides with a tip-focused cytosolic Ca^{2+} gradient in both RHs (Fig. 9.4) and PTs of multiple species

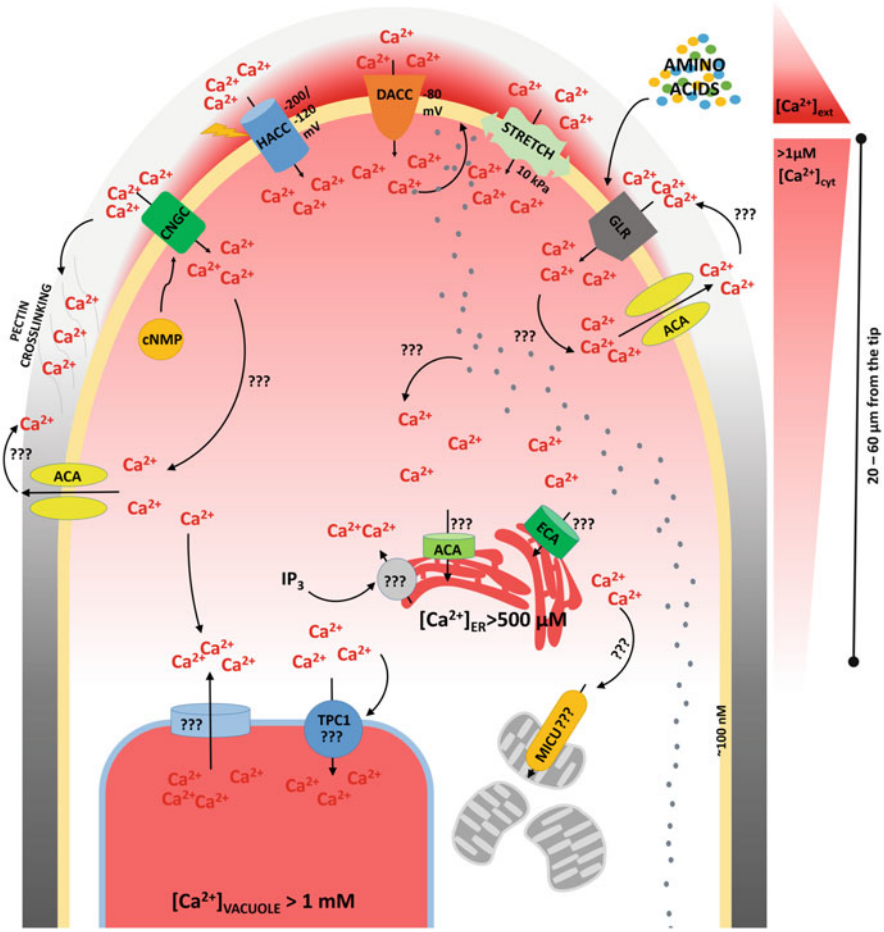

Fig. 9.4 Graphical representation of calcium transport and storage systems governing root hair tip growth. *Question marks* refer to hypothetically involved components and mechanisms. *Red colours* represent calcium concentrations. *Grey outline* = cell wall, *yellow outline* = plasma membrane, *grey spheres* = exocytic vesicles. All abbreviations are referred to in Sect. 9.4.1

(Schiefelbein et al. 1992; Pierson et al. 1996; Holdaway-clarke et al. 1997; Felle and Hepler 1997; Michard et al. 2008; Fan et al. 2011). Relative to the RH tip, this gradient extents inward for 20–60 μm (Felle and Hepler 1997; Monshausen et al. 2008). In rapidly growing RHs, the $[Ca^{2+}]_{cyt}$ ranges from 100 nM at the basal part (RH shank) to more than 1 μM at the apex (Wymer et al. 1997; Felle and Hepler 1997). In PTs, $[Ca^{2+}]_{cyt}$ higher than 3 μM have been reported at the tip (Pierson et al. 1996).

Several pharmacological experiments showed that the existence of this tip-focused Ca^{2+} gradient is crucial for proper elongation of tip-growing cells. For instance, treatment of RHs with Ca^{2+} channel blockers such as verapamil, nifedipine or La^{3+} or the Ca^{2+} chelator EGTA resulted in dissipation of the Ca^{2+} gradient and subsequent RH growth inhibition or a sudden increase in growth rate followed by bursting of the RH tip (Herrmann and Felle 1995; Bates and Lynch 1996; Wymer et al. 1997; Monshausen et al. 2008).

The localization of the Ca^{2+} gradient also determines the directionality of growth in RHs and PTs. Using a caged Ca^{2+} ionophore (A23187) approach, a local and transient Ca^{2+} increase can be induced extracellularly, which stops RH growth immediately (Monshausen et al. 2008). Conversely, it was also shown that both RHs and PTs direct their growth towards a transiently induced $[Ca^{2+}]_{ext}$ maximum (Malhó and Trewavas 1996; Bibikova et al. 1997). Curiously, whereas RHs redirect towards the original growth direction after reestablishment of the normal $[Ca^{2+}]_{ext}$ distribution, PTs do not. At the time of writing, the mechanisms that lie at the basis of this difference between RH and PT development remain unidentified.

Together, these results show that the Ca^{2+} gradient is a key component of the tip growth machinery. Importantly however, tip growth does not occur as a linear process. Instead, RH and PT growth rates oscillate, with alternate periods of slower growth followed by periods of fast expansion. In RHs, growth rates range between 1 and 3.2 μm min^{-1} (Monshausen et al. 2008; Cárdenas 2009). PTs grow considerably faster, with growth rates ranging between 6 and 30 μm min^{-1} (Messerli et al. 1999, 2000). The tip-focused $[Ca^{2+}]_{cyt}$ oscillates at the same frequency as the growth rate, but with a phase delay of several seconds. For instance, *Arabidopsis* RH growth rates and apical $[Ca^{2+}]_{cyt}$ oscillate at a frequency of 2–4 min^{-1}. By using the ratiometric calcium marker Yellow Cameleon 3.6 (YC3.6), Monshausen et al. (2008) demonstrated that the peak Ca^{2+} level lags the growth rate peak by approximately 5 s. It seems that the timing of these events is conserved between PT and RH development and among species. For instance, whereas the growth rate oscillations of PTs (period of 15–50 s) occur much faster compared to RHs, the phase lag in lily PTs was also found to be approximately 4–5 s (Pierson et al. 1996; Messerli et al. 2000).

9.4.1.3 Ca^{2+} Fluxes

An Oscillating, Tip-Focused Influx of Extracellular Ca^{2+}

The presence of an oscillating tip-focused Ca^{2+} gradient in RHs and PTs relies on active Ca^{2+} transport of extracellular Ca^{2+} across the apical PM. Using non-invasive ion-selective vibrating probe analysis, several independent experiments reported on the existence of an apical Ca^{2+} influx both in RHs and PTs of several species. In RHs, the Ca^{2+} influx ranged from approx. 0.01 to 0.04 amol μm^{-2} s^{-1} at the tip, until reaching basal levels at a distance of ± 20 μm from the apex (Schiefelbein et al. 1992; Jones et al. 1995; Felle and Hepler 1997). In growing PTs, inward Ca^{2+} fluxes ranged from 0.003 to 0.5 amol μm^{-2} s^{-1} (Kuhtreiber and Jaffe 1990; Pierson et al. 1996; Holdaway-clarke et al. 1997; Messerli et al. 1999; Michard et al. 2008, 2011). The study of Messerli et al. (1999) revealed that PTs grew at a steady rate (0.2 μm s^{-1}) until they reached a certain length (approx. 1 mm); after that PT growth rate and Ca^{2+} influx switched to an oscillating pattern. Similarly, Michard et al. (2011) observed strong Ca^{2+} influx oscillations at the tip of tobacco PTs (fluctuating between 0.06 and 0.5 amol μm^{-2} s^{-1}). The rate of Ca^{2+} influx oscillations seemed to be correlated to the PT growth rate (Pierson et al. 1996) and is sensitive to extracellular stimuli such as D-serine (Michard et al. 2011).

Similar to the $[Ca^{2+}]_{cyt}$, the tip-focused Ca^{2+} influx also exhibits the same period, but cross-correlation analysis showed that the peak Ca^{2+} influx followed the peak growth rate with a lag time of approx. 15 s (Holdaway-Clarke et al. 1997; Messerli et al. 1999; Holdaway-Clarke and Hepler 2003).

The structural properties of the cell wall largely depend on the presence of Ca^{2+} (see Sect. 9.4.1.4). As such, the observed Ca^{2+} influx at the tip of growing RHs and PTs is expected to simultaneously provide Ca^{2+} for the cell wall and Ca^{2+} for active transport into the cytosol. Recently, Hepler et al. (2012) calculated the species-specific Ca^{2+} influx in growing PTs that would be needed to provide the required amount of structural Ca^{2+} to the cell wall (Hepler et al. 2012). Their findings implicate that the majority of vibrating microelectrode measurements likely underestimate the actual fluxes involved in tip growth (Table 9.1). Holdaway-Clarke et al. (1997) performed a similar analysis for *Lilium* PTs and found that, in perfect agreement with their experimental data, a flux of approx. 0.35 amol μm^{-2} s^{-1} would be needed to supply the cell wall with the required amount of Ca^{2+} (Holdaway-Clarke et al. 1997).

Ca^{2+} Transporters

Plants have evolved specific Ca^{2+} transport proteins, different from those found in other eukaryotic organisms (Steinhorst and Kudla 2014). Both in PTs and RHs, a Ca^{2+} influx is maintained by a specialized set of Ca^{2+} transport proteins (Fig. 9.4). Given the heterogeneity of the extracellular environment, it is expected that

Table 9.1 Observed and predicted Ca^{2+} flux densities to sustain pollen tube growth (Adopted from Hepler et al. 2012)

Species	PT diameter (μm)	Growth rate (μm min^{-1})	Observed flux (amol μm^{-2} s^{-1})	Predicted flux needed for cell wall (amol μm^{-2} s^{-1})	Predicted flux needed for 10 μM $[Ca^{2+}]_{cyt}$ (amol μm^{-2} s^{-1})
Arabidopsis	4	0.3–1.5	0.01–003	0.03–0.16	0.002
Nicotiana	10	2–10	0.05–0.5	0.09–0.43	0.006
Lilium	18	6–30	0.02–0.15	0.14–0.72	0.005

tip-growing cells contain several types of Ca^{2+} transporters that would allow for Ca^{2+} influx in different conditions. Great scientific effort has gone into identifying the precise characteristics of the Ca^{2+} influx, in order to narrow the search for the involved Ca^{2+} channels in both RH and PT development. The ability to perform patch-clamp analysis on either isolated protoplasts or intact tip-growing cells leads to the identification of a series of candidates, some of which are yet to be confirmed and others which are now believed to be crucial tip growth regulators.

Cyclic nucleotide-gated channels (CNGCs) are non-specific, Ca^{2+}-permeable cation channels regulated by cyclic nucleotides such as cAMP (Maser et al. 2001; Talke et al. 2003; Ward et al. 2009). CNGCs also have a binding site for calmodulin (Kohler and Neuhaus 2000). As such, they can form a direct relay system between cNMP and Ca^{2+} signal transduction pathways. The *Arabidopsis* genome contains 20 CNGCs, some of which have been shown to be involved in Ca^{2+}-mediated PT growth. The latter is consistent with the fact that cNMP signals in pollen can affect growth in a Ca^{2+}-dependent manner (Moutinho et al. 2001; Rato et al. 2004; Wu et al. 2011). Publicly available transcriptomics data shows that at least six *Arabidopsis* CNGCs are strongly expressed in in vivo grown PTs and nine CNGCs show strong expression in trichoblast cell files (Table 9.2). Alterations in expression levels of the PT-expressed CNGCs resulted in dramatic growth defects and reduced male fertility (Tunc-Ozdemir et al. 2013; Frietsch et al. 2007; Gao et al. 2016). The transcription of several CNGC genes is altered in a number of well-characterized RH developmental mutants (Bruex et al. 2012; Simon et al. 2013). Most notably, CNGC6 and CNGC9 show very high and specific transcription in RH cell files, and their transcription is strongly affected in several RH developmental mutants. At the time of writing, however, no studies have focused on the role of CNGCs in RH development.

Hyperpolarization- and depolarization-activated Ca^{2+} channels (HACCs and DACCs) have been identified in the RH and PT apical PM. Over a decade ago, Véry and Davies identified a hyperpolarization-activated Ca^{2+} conductance in growing RH protoplasts, operative specifically at membrane potentials equal to and lower than the resting potential of growing *Arabidopsis* RHs (between −160 and −200 mV; Lew 1996), and within the range of physiological $[Ca^{2+}]_{cyt}$ (Wymer et al. 1997; Felle and Hepler 1997; Véry and Davies 2000). Consistent with the model of tip-localized Ca^{2+} influx, this hyperpolarization-activated Ca^{2+} conductance was found to be much lower in the subapical RH region. Interestingly, the conductance increased in the presence of higher physiologically relevant $[Ca^{2+}]_{cyt}$ (100–900 nM). The latter suggest that Ca^{2+} influx across the PM and subsequent Ca^{2+} accumulation in the cytosol could induce the Ca^{2+} influx even further. This mechanism might be part of a feedback loop, regulating Ca^{2+} oscillations. In 2003, Foreman et al. found a ROS-dependent HACC conductance in RHs, related to ROS originating from NADPH oxidase activity (RHD2; Foreman et al. 2003). The mechanism of ROS-controlled Ca^{2+} influx was also found in PTs (e.g. Wu et al. 2010), and is now considered to be a pivotal part of tip growth in general

Table 9.2 Transcription level of *Arabidopsis* cyclic nucleotide-gated calcium channels in tip-growing cells (no < yes < strong < very strong)

CNGC ID	Transcription in PTs (Qin et al. 2009)	Transcription in trichoblasts (Brady et al. 2007)	Transcription in atrichoblasts (Brady et al. 2007)	Altered transcription in RH developmental mutants
1				
2				
3		Yes		No
4				
5				
6		Very strong		Yes
7	Yes	Yes		No
8	Yes		Yes	No
9	Yes	Very strong		Yes
10	Yes	Yes		Yes
11		Yes		No
12			Yes	No
13				
14		Strong		Yes
15			Yes	Yes
16	Yes			
17		Yes		Yes
18	Yes	Yes		No
19				
20				

(see Sect. 9.3.2). HACCs have also been identified in PT protoplasts of several species, indicating a voltage-gated Ca^{2+} influx as a central player in establishing tip growth-related Ca^{2+} dynamics (Shang et al. 2005; Qu et al. 2007; Wu et al. 2007, 2011). Comparable to RHs, these channels typically open at a threshold voltage of approximately −100 mV, similar to the resting potential of PTs (Messerli et al. 1999). Next, a DACC conductance was identified in the apical region of *Arabidopsis* RHs (Miedema et al. 2008). DACCs are typically most active at −80 mV (in the presence of 30 mM external Ca^{2+}; Thion et al. 1998, 1996).

Given the known membrane potential observed in growing RHs, HACCs seem to be the main component for tip growth-related Ca^{2+} influx. However, the co-occurrence of CNGCs, HACCs and DACCs could provide RHs with a mechanism for Ca^{2+} influx at a broad range of membrane potentials and intra- as well as extracellular Ca^{2+} concentrations. For instance, increase of the tip-focused $[Ca^{2+}]_{cyt}$ by CNGC-mediated Ca^{2+} influx at moderately negative voltage values would lower the activation voltage needed for HACC activity (Demidchik et al. 2002). Despite the identification of HACC conductances in RHs, actual identification and thus functional characterization of the genes coding for these channels is still lacking.

Notably, the presence of a cAMP-activated HACC was shown in *Pyrus pyrifolia* PTs using patch-clamp analysis (Wu et al. 2011). This finding might hint towards the family of CNGCs in the quest for HACCs.

Mechanosensitive calcium channels are also involved in regulating Ca^{2+} fluxes in PT growth. *Tradescantia virginiana* PTs were found to reorient upon touch or relocalization of the Ca^{2+} maximum, strongly suggesting that PTs contain mechanosensitive regulation of Ca^{2+} fluxes (Bibikova et al. 1997). Indeed, a mechanosensitive Ca^{2+} conductance was identified in protoplasts of *Lilium longiflorum* PTs (Dutta and Robinson 2004). Upon application of 10 kPa suction force, these channels would open and preferentially allow Ca^{2+} to enter the cytosol. Interestingly, Bibikova et al. (1997) found that in RHs and PTs, the Ca^{2+} gradient maximum is reoriented away from a mechanical obstacle or a touch induction site. More so, local mechanical perturbation of RH cells elicits a transient Ca^{2+} peak 1–18 s after application of the stimulus (Monshausen et al. 2009). Finally, Wang and colleagues found that impalement of RH cells with a glass microelectrode results in a similar transient increase of the $[Ca^{2+}]_{cyt}$ (Wang et al. 2015b). These data strongly suggest that also in RHs, despite the lack of further evidence, a mechanosensitive Ca^{2+}-linked mechanism is in place. Identification of the possible gene(s) related to this mechanosensitive Ca^{2+} conductance in PTs and RHs is still lacking.

The consensus regarding Ca^{2+} dynamics and growth rate oscillations states that the phase of Ca^{2+} oscillations follows that of growth oscillations with a lag of approximately 4–5 s. It is therefore easy to speculate that a growth spurt would result in subsequent opening of mechanosensitive Ca^{2+} channels due to mechanical deformation of the PM at the tip.

The search for identification of mechanosensitive channels involved in tip growth is progressing. Recently, Hamilton and colleagues identified an anion-selective mechanosensitive channel named MSCS-LIKE 8 (MSL8), with a crucial role in male fertility through regulation of osmotic forces during pollen development (Hamilton et al. 2015). MID1-COMPLEMENTING ACTIVITY 1 and 2 (MCA1, MCA2) were shown to be Ca^{2+} conducting mechanosensitive channels involved in root growth (Nakagawa et al. 2007; Yamanaka et al. 2010). Transcriptomics data shows that at least MCA1 is expressed in RH cell files (Brady et al. 2007). More so, transcription of MSL2 and MSL3 was detected in both RHs and PTs, making these genes worthwhile candidates for further investigation.

Glutamate receptor-like channels (GLRs) are other candidates for generating the Ca^{2+} influx in PTs and RHs. In *Arabidopsis*, 20 genes code for proteins with strong similarity to the mammalian Ca^{2+} conducting glutamate receptor (Ward et al. 2009). Pollen transcriptome data revealed that at least six *GLRs* are preferentially expressed in pollen and one in PTs. Interestingly, at least four *GLRs* are preferentially expressed in trichoblast cell files (Brady et al. 2007). Of these four genes, three (*GLR3.5*, *GLR3.7* and *GLR2.1*) are differentially expressed in several RH developmental mutants (Bruex et al. 2012; Simon et al. 2013). Despite the implications for these genes in their involvement in tip growth, current knowledge on their function is still mostly lacking. Only *GLR1.2* and *GLR3.7* were shown to be

important in PT growth (Michard et al. 2011). Loss-of-function mutations in both genes resulted in shorter PTs and reduced fertility. Moreover, *glr1.2* mutant pollen resembled the morphology associated with GLR inhibition by CNQX treatment, a well-known inhibitor of mammalian glutamate receptors. Crucially, the results of Michard and colleagues show that GLRs are responsible for perception of D-serine and subsequent Ca^{2+} influx in PTs followed by growth stimulation. Upon exogenous application of D-serine (a rare amino acid present in the pistil and ovules) to growing PTs, YC3.6 monitoring of the $[Ca^{2+}]_{cyt}$ revealed an increase in the amplitude of Ca^{2+} oscillations in the tip. This effect of D-serine was not seen in the presence of GLR inhibitors, and the Ca^{2+} signature was not detected in response to other amino acids such as glutamate. These data suggest that GLRs form another component of the apical Ca^{2+} influx machinery. Importantly, it also shows that the specificity of GLRs is diverse and not limited to glutamate. The latter is supported by the finding that GLR3.4 expressed in HEK cells shows highly selective Ca^{2+} transport in response to the amono acids Asn, Gly and Ser (Vincill et al. 2012).

Calcium-efflux transporters have been largely neglected when it comes to studying tip growth. This is largely due to the fact that no outward Ca^{2+} currents have been observed at the PT or RH tip. However, Schiott and colleagues identified the autoinhibited Ca^{2+}-ATPase efflux pump ACA9 as a crucial component of PT tip growth (Schiott et al. 2004). *Aca9* knockout PTs grew slower in vivo and in vitro, eventually resulting in shorter PTs (75% reduction in length) and a strong reduction in fertilization efficiency. Given the fact that ACA9 is localized at the PT PM, Schiott et al. (2004) suggest that it might have a role in recycling cytosolic Ca^{2+} towards the cell wall during tip growth. Interestingly, *ACA9* is strongly expressed in RH cell files in *Arabidopsis*. It would be interesting to see what in-depth analysis of Ca^{2+} dynamics in *aca9* mutant RHs and PTs could reveal. In total, ten autoinhibited efflux Ca^{2+}-ATPases are found in the genome of *Arabidopsis thaliana*. Besides ACA9, ACA7 is also localized to the PM and expressed (albeit relatively low) in PTs and RHs. Current knowledge states that ACA7 is involved in microsporogenesis (Lucca and León 2012), but no experiments have been performed on PT and RH development. Finally, ACA12 was recently found to be localized to the plant PM (Limonta et al. 2014). Ectopic expression of ACA12 in *aca9* plants led to the rescue of the *aca9* male sterility phenotype, showing that ACA12 is a functional PM-localized Ca^{2+}-efflux pump. No *ACA12* transcription has been documented in in vivo grown PTs. However, in roots, *ACA12* is preferentially expressed in trichoblast cells (Brady et al. 2007).

9.4.1.4 Ca^{2+} Sources and Sinks

A large influx of Ca^{2+} is in place during RH and PT growth; however the subsequent fate of incoming Ca^{2+} is unknown. The nature of fluctuating Ca^{2+} dynamics in tip-growing cells inherently relies on active transport and sequestration of Ca^{2+} ions from and to Ca^{2+} sources and sinks (Fig. 9.4). Solely based on the major

concentration differences observed between the cytosol, cell wall and subcellular organelles, it is expected that inter-compartment Ca^{2+} allocation takes place inside the cell. Illustratively, the typical resting $[Ca^{2+}]_{cyt}$ is kept around 100–200 nM, whereas the free Ca^{2+} concentration in some of the major subcellular Ca^{2+} stores and the cell wall can easily reach 10 mM (Bush 1995). So which compartments are known to or could contribute to Ca^{2+} dynamics in tip growth?

The cell wall has been considered the main source for tip-focused cytosolic Ca^{2+} in tip-growing cells. In support of this consensus is the presence of multiple Ca^{2+} channels and Ca^{2+} conductances, together with the discovery of a growth-correlated oscillating inward Ca^{2+} influx. More so, already very early in the research of PT tip growth, it was shown that radioactive $^{45}Ca^{2+}$ incorporates mostly in the PT cell wall (Kwak 1967). The extracellular environment is considered to form the main source for cell wall accumulating Ca^{2+} in both RHs and PTs. This is illustrated by the fact that in vitro growth of RHs and PTs relies on a $[Ca^{2+}]_{ext}$ optimum. Importantly, instead of simply diffusing through the cell wall, a large quantity of Ca^{2+} is used as a means of providing dynamic structural cell wall support during tip growth. As such, normal cell wall dynamics at the tip of growing PTs and RHs inherently rely on the maintained presence of Ca^{2+}. Thus, in order to provide sufficient Ca^{2+} to the cytosol while maintaining proper Ca^{2+} levels in the cell wall, an intricate balance must be kept between Ca^{2+} export and import from and to the cell wall. An array of Ca^{2+} transport proteins provides tight control over the Ca^{2+} efflux (cytosolic influx) from the cell wall (see Sect. 9.4.1.3). But how is Ca^{2+} retained and released from the cell wall at the tip? The RH and PT cell wall consists partly of pectins which alternate between their methylesterified and de-esterified form during tip growth (Bosch and Hepler 2005). The Ca^{2+}-binding capacity of pectins changes accordingly, as does the availability of Ca^{2+} for maintaining the influx into the cytoplasm. An autoregulatory feedback loop thus links oscillations in Ca^{2+} influx, Ca^{2+} cell wall cross-linking and $[Ca^{2+}]_{cyt}$ (Franklin-Tong 1999). As such, experimental evidence suggests that a self-regulating interplay between cytosolic and extracellular Ca^{2+} controls Ca^{2+} fluxes from and to the cell wall and into the cytosol. Hepler et al. (2012), however, calculated that in theory most of the Ca^{2+} flux at the PT tip is used for structural cell wall support and only a small quantity can be used for cytosolic influx.

Hereafter, the potential role of subcellular compartments in Ca^{2+} release and sequestration is discussed. However, contrary to the belief that the cell wall acts solely as a source of cytosolic Ca^{2+}, it is rarely considered that the cell wall might also act as a sink, receiving Ca^{2+} from the intracellular environment through active transport. As discussed earlier, a Ca^{2+}-efflux pump was shown to be involved in regulating PT tip growth (Schiott et al. 2004). The authors hypothesized that ACA9 could be responsible for recycling Ca^{2+} back to the cell wall, thereby regulating peak/trough Ca^{2+} levels at the tip.

The vacuole extents throughout tip-growing cells and is thought to be crucial for the generation of an outward-directed osmotic pressure that drives cell expansion (Schiefelbein et al. 1993). In general the vacuole maintains a much higher Ca^{2+}

concentration than that found in the cytoplasm (Bush 1995). The role of the vacuole in regulating Ca^{2+} release/sequestration in plants has been questioned several times (Lommel and Felle 1997; Britto and Kronzucker 2002; Rocha and Vothknecht 2012; Schönknecht 2013; Nomura and Shiina 2014). Also in RH and PT tip growth, a prominent role might exist for vacuolar Ca^{2+} release and sequestration (Carol and Dolan 2002). However, given the distance between the vacuole and the tip-focused clear zone, it is unlikely that the vacuole would directly contribute to the fine regulation of Ca^{2+} oscillations in the tip. However, massive vacuolar Ca^{2+} release could have a pivotal role in Ca^{2+}-induced Ca^{2+} release and subsequent intracellular signal transduction. Unfortunately, direct Ca^{2+} dynamics within the vacuolar lumen have not been observed at the time of writing, due to the fact that the vacuolar pH interferes with Ca^{2+} reporters. A hopeful study came from Wang and colleagues, where they describe vacuolar ion conductance and channel activity in response to cytosolic Ca^{2+} alterations (Wang et al. 2015b). Their findings suggest a vacuolar Ca^{2+} release in RHs following a cytosolic Ca^{2+} peak. Wang and colleagues found that a BAPTA- or FURA-2 induced $[Ca^{2+}]_{cyt}$ peak evoked a transient 2.5-fold increase in tonoplast ion conductance, whereas slow and small Ca^{2+} peaks did not elicit this response. The Ca^{2+} induced increase in tonoplast ion conductance is thought to be due to activation of voltage-independent channels.

TWO-PORE CHANNEL 1 (TPC1), a tonoplast localized depolarization-activated Ca^{2+} influx channel, was shown to be important for salt stress-induced Ca^{2+} signal propagation through the root (Choi et al. 2014). TPC1 is activated by high $[Ca^{2+}]_{cyt}$ and is sensitive to both cytosolic and vacuolar Ca^{2+} levels, which suggests that it might be involved in regulating the Ca^{2+} balance across the vacuolar membrane (Dadacz-Narloch et al. 2011). It might, however, act indirectly through the generation of a vacuolar membrane potential which in turn would activate other voltage-gated Ca^{2+} channels (Choi et al. 2014). The role of TPC1 in tip growth remains unexplored, but transcript levels are selectively high in trichoblast cell files in *Arabidopsis* (Brady et al. 2007). As such, it will be interesting to see if TPC1 might be involved in regulating Ca^{2+} dynamics in RH cells (Konrad et al. 2011).

Finally, patch-clamp measurement of ion conductance on intact vacuole has revealed the existence of several types of Ca^{2+}-regulated cation channels in the tonoplast (Allen and Sanders 1996; Isayenkov et al. 2010; Hedrich and Marten 2011). At the time of writing, no data exists on their involvement in RH or PT development.

Elements of the endoplasmic reticulum (ER) are continuously replenished through the clear zone, at the tip of growing PTs and RHs (Lovy-Wheeler et al. 2007). They move along the 'reverse fountain' cytoplasmic streaming typical of tip-growing cells. This finding, together with several implications that the ER is able to sequester vast amounts of Ca^{2+}, makes it an ideal candidate organelle for a Ca^{2+} source/sink in tip-growing cells. The ER has been hypothesized to function as a capacitive Ca^{2+} store (Trewavas and Malho 1997) and is one of the possible organelles that could contribute to the oscillating Ca^{2+} dynamics observed in the apical cytoplasm. Following a $[Ca^{2+}]_{cyt}$ peak, Ca^{2+} sequestration in the ER could bring the Ca^{2+}

level back to the basal level. The ER in *Arabidopsis* PTs sequesters up to 500 μM of Ca^{2+} (Iwano et al. 2009) and contains a protein called calreticulin which is able to bind 25 mol of Ca^{2+} per mol of protein (Michalak et al. 2009). Even more, the dissipation of tip-localized Ca^{2+} is thought to be regulated by ER-localized Ca^{2+}-ATPases which would allow Ca^{2+} ions to move against the concentration gradient into the ER lumen (Obermeyer and Weisenseel 1991; Sze et al. 1999; Franklin-Tong 1999). Using an ER-targeted YC3.6, it was shown that cyclopiazonic acid (an inhibitor of ER-type Ca^{2+}-ATPases) decreased the $[Ca^{2+}]_{ER}$ and inhibited the growth of PTs (Iwano et al. 2009). Several Ca^{2+}-transporting proteins such as ACA2 (autoinhibited Ca^{2+}-ATPase) and ECA1 (ER-type Ca^{2+}-ATPase) are expressed in PTs (Sze et al. 2006).

Finally, despite the strong influx of Ca^{2+} from the cell wall, the ER might also release Ca^{2+} at the tip. A possibility is that elements in the ER would facilitate IP_3-induced Ca^{2+} release (Malhó 1998; Franklin-Tong 1999). A role for IP_3-regulated Ca^{2+} release is well established in PTs (Malhó 1998; Monteiro et al. 2005). More so, the ER membrane contains inositol triphosphate (IP_3) receptors which, in response to low $[Ca^{2+}]$, relay information to the PM ultimately resulting in an influx of extracellular Ca^{2+} (Putney et al. 2001).

Vesicles are densely packed in the tip of growing RHs and PTs and occupy the entire clear zone. Vesicle fusion relies strongly on a high $[Ca^{2+}]_{cyt}$ micro-environment (Zorec and Tester 1992). However, despite their obvious role in the exo- and endocytic pathway, they might also function as a Ca^{2+} store. In neuroendocrine cells, some vesicles were shown to harbor Ca^{2+} that can be exchanged in a cyclic ADP ribose-dependent manner (Mitchell et al. 2001). In plants, another hint towards a role as Ca^{2+} stores for vesicles is the fact that a calcium pump (ECA3) was shown to be localized to the post-Golgi endomembranes, which give rise to secretory vesicles (Sze et al. 2006).

Mitochondria are not found in the clear zone of growing RHs and PTs. The mitochondrial proteome contains several Ca^{2+}-binding, EF-hand-containing proteins and Ca^{2+}-dependent protein kinases (Day et al. 2002; Heazlewood et al. 2004). Mitochondria have been shown to release Ca^{2+} to the cytosol, however, in a way that is too slow to relate to the rapid Ca^{2+} spikes observed in tip-growing cells (Loro et al. 2012).

Contrastingly, accumulated evidence implicates that mitochondria might have a pivotal role in Ca^{2+} sequestration instead. Plant mitochondria were shown to contain a Ca^{2+} uptake system (Hanson et al. 1965; Dieter and Marmé 1980; Akerman and Moore 1983; Zottini and Zannoni 1993). For Ca^{2+} to be pumped into the mitochondrial matrix, the Ca^{2+} acquisition system must be able to function at an inner-membrane potential of approx. 180 mV. Using targeted YC3.6 Ca^{2+} sensors to simultaneously monitor Ca^{2+} dynamics in the cytoplasm and mitochondria of *Arabidopsis* root cells, it was shown that a cytoplasmic Ca^{2+} increase related directly to mitochondrial Ca^{2+} accumulation (Loro et al. 2012). Importantly, the observed Ca^{2+} dynamics were particularly different from those in the cytosol (Logan and Knight 2003; Loro et al. 2012). Ca^{2+} dynamics also responded to

exogenous application of ATP, which has also been implicated in the regulation of RH growth. A hint towards the protein(s) that might regulate mitochondrial Ca^{2+} dynamics came from the functional characterization of the *Arabidopsis* MICU protein, an EF-hand mitochondrial Ca^{2+} uniporter (Wagner et al. 2015). MICU is involved in fine-tuning the Ca^{2+} influx into the mitochondrial lumen. The *micu* loss-of-function mutations resulted in a higher than normal mitochondrial Ca^{2+} resting concentration and a reduced ability to dampen auxin and ATP-triggered Ca^{2+} influx into the mitochondrial matrix. Most notably, *MICU* is expressed in in vivo grown PTs and throughout the root (Brady et al. 2007; Qin et al. 2009).

The hereby mentioned Ca^{2+} sources and sinks do not function separately. It is important to acknowledge that Ca^{2+} dynamics in tip-growing cells are the result of a complex pathway that connects all involved Ca^{2+} stores and transport mechanisms. Localized Ca^{2+} concentrations depend on diffusion through the cell wall; retention in the cell wall; active inward transport; uptake into the vacuole, ER, mitochondria and vesicles; and transport outward towards the apoplast.

9.4.1.5 Direct Targets of Calcium Signalling

The intrinsic properties of Ca^{2+} signatures are interpreted by the action of several Ca^{2+}-sensing proteins. The Ca^{2+} signal is then 'decoded', resulting in a specific cellular response (e.g. long-distance Ca^{2+}-mediated signal transduction from root to shoot leads to changes in shoot gene transcription; Choi et al. 2014). In tip growth, Ca^{2+} signal transduction has been shown to ultimately regulate most of the key components related to the tip growth machinery (Fig. 9.5). It has been found that Ca^{2+} may regulate membrane trafficking since loss of the Ca^{2+} gradient disrupts the tip-focused accumulation of RabA4b and affects RH growth (Preuss et al. 2006). In addition, cytosolic Ca^{2+} can modulate F-actin dynamics by controlling the activity of diverse actin-binding proteins (ABPs) such as profilins, villins and actin-depolymerizing factors (Hussey et al. 2006), thereby indirectly influencing vesicle movement. Moreover, Ca^{2+} has been implicated as a crucial component of several feedback loops regulating ROP-GTPase activity (Yan et al. 2009) and ROS production at the tip (Takeda et al. 2008).

Multiple Ca^{2+}-sensing/-binding proteins are expressed in PTs and RHs (Fig. 9.5). Upon Ca^{2+} binding, a conformational change is induced leading to signal transduction. Signal transduction is often the result of consecutive phosphorylation cascades initiated by Ca^{2+}-dependent protein kinases (CDPKs). Such CDPKs containing an enzymatic domain are commonly referred to as Ca^{2+} responders, whereas proteins that lack direct Ca^{2+}-dependent catalytic activity (calmodulin proteins (CaMs), calmodulin-like proteins (CMLs), calcineurin B-like proteins (CBLs) and CBL-interacting protein kinases, (CIPKs)) are designated sensor relays (Sanders et al. 2002). The latter 'relay' the Ca^{2+} signal by conformation-induced

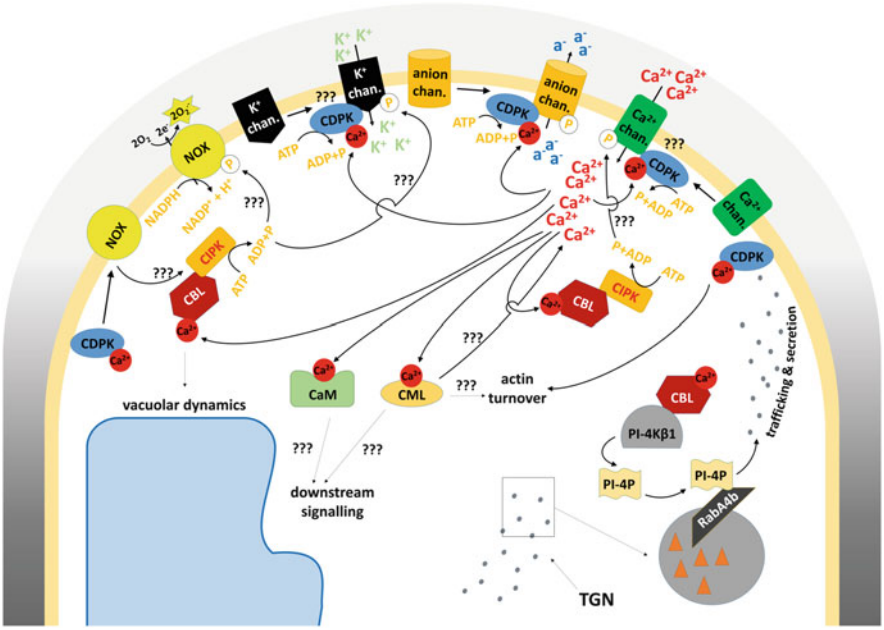

Fig. 9.5 Graphical representation of direct targets of calcium signalling during root hair tip growth. *Question marks* refer to hypothetically involved components and mechanisms. *Grey outline* = cell wall, *yellow outline* = plasma membrane, *grey spheres* = exocytic vesicles, a^- = anion, e^- = electron. All abbreviations are referred to in Sect. 9.4.1

protein-protein interactions. We will shortly discuss these proteins in more detail, in relation to RH and PT development.

Calcium-dependent protein kinases have both a CaM and kinase domain, allowing them to 'sense' Ca^{2+} and immediately relay the Ca^{2+} signal through phosphorylation of one or more target proteins. The CDPK family consists of 34 members, most of which contain N-myristoylation and/or N-palmitoylation sites. The latter reflects a probable membrane association, consistent with their role as Ca^{2+}-sensing proteins. Importantly, one fourth of the known *Arabidopsis CDPK*s is preferentially expressed in pollen (Pina et al. 2005), suggesting a major role in tip growth. Moreover, CDPKs are considered active at Ca^{2+} concentrations in the physiological range detected at the tip of growing RHs and PTs (100–1000 nM; Myers et al. 2009). Multiple studies have indeed shown that CDPKs are important regulators of tip growth. For instance, Myers et al. (2009) have shown that the Ca^{2+}-dependent protein kinases 17 and 34 (CPK17 and CPK34) are localized at the PM at the tip of growing PTs and that they act redundantly to regulate PT elongation and fertilization. Most interestingly, a recent study found that a CDPK-regulated negative anion gradient exists at the tip of growing RHs (Gutermuth et al. 2013). More specifically, CDPK activated anion efflux at the tip resulted in the occurrence

of an anion decrease parallel with each Ca^{2+} peak. These anion oscillations are due to the interaction of CPK2 and CPK20 and their ability to phosphorylate SLAC1 HOMOLOGUE 3 (SLAH3) in growing PTs. Importantly, *CPK2* is transcribed in trichoblast cell files in *Arabidopsis* roots (Brady et al. 2007). More evidence showing that CDPKs can couple different ion dynamics comes from Zhao et al. (2013a). The PM-localized proteins CPK11 and CPK24 negatively regulate PT growth through Ca^{2+}-mediated control of the SHAKER POLLEN INWARD K^+ CHANNEL (SPIK; Zhao et al. 2013a). Interestingly, CPK32 was shown to interact with CNGC18, a crucial Ca^{2+} channel in PTs (Zhou et al. 2014). Furthermore, several CDPKs were shown to regulate actin cytoskeleton organization, polarity and even self-incompatibility in *Zea mays, Nicotiana alata* and *Petunia hybrid* (Estruch et al. 1994; Kunz et al. 1996; Allwood et al. 2001; Yoon et al. 2006).

Clearly, most research about the role of CDPKs in tip growth has focused on PTs. However, in *Medicago truncatula* CDPK1 was shown to control RH growth and morphology (Ivashuta et al. 2005). MtCDPK1 loss of function leads to dissipation of the tip-focused ROS gradient and disorganization of the actin cytoskeleton. Interestingly, Dubiella et al. (2013) showed that in response to a Ca^{2+} peak, CPK5 phosphorylates the NADPH oxidase RBOHD, thereby leading to apoplastic ROS production and cell-to-cell Ca^{2+}-ROS signal transduction (Dubiella et al. 2013). Publicly available transcriptomics data shows that *CPK5* is also expressed in RH cell files. Could it be involved in trichoblast-to-trichoblast Ca^{2+} signal transduction? Similarly, *CPK32* and its interaction partner *CNGC18* in PTs were also found to be expressed in RHs. Could both proteins be working together in RHs to regulate Ca^{2+} dynamics at the tip? Finally, *CPK11* but not *CPK24* (which together regulate K^+ channel activity in PTs) was found to be highly expressed in RH cells in *Arabidopsis*. Investigating RH growth and morphology in *cpk11* knockout plants could be an interesting avenue in identifying novel players in Ca^{2+}-regulated RH development.

Calmodulin (CaMs), calmodulin-like (CMLs), calcineurin B-like (CBLs) and CBL-interacting protein kinases (CIPKs) are also likely to have a prominent role in tip growth regulation. However, little is known about these proteins and their involvement in RH and PT development. The *Arabidopsis* genome contains seven *CaM*-coding sequences, three of which code for the same protein (CaM2, CaM3 and CaM5). Besides a potential role for CaM2 in pollen development and fertilization efficiency (Landoni et al. 2010), none of the CaM proteins have been functionally characterized in relation to tip growth. Based on their preferential expression in RHs and PTs, *CaM3* and potentially *CaM7* could be involved in regulating the tip growth process.

The family of CMLs contains 50 members in *Arabidopsis*. However, most of them remain uncharacterized at the time of writing. Two CML proteins have been shown to be involved in pollen development. *CML24/TOUCH2* loss of function leads to delayed pollen germination, slower PT growth and a shorter final PT length (Yang et al. 2014). *Cml24/touch2* PTs exhibit a higher $[Ca^{2+}]_{cyt}$ and a disorganized actin cytoskeleton. Interestingly, *CML24/TOUCH2* transcription is

induced by touch, suggesting it might function in the pathway of mechanosensitive Ca^{2+} channels. In the root, *TCH2* is specifically expressed in atrichoblast cell files. *CML25* knockout plants have a similar phenotype to that of *cml24* plants (Wang et al. 2015a). In addition, *cml25* plants were shown to regulate K^+ influx into PTs. Importantly, *CML25* is also specifically expressed in RH cell files, implying a general involvement in tip growth. Lin and colleagues showed that *cml25* plants grow longer RHs under phosphate starvation (Lin et al. 2011b). However, no additional information is known on the role of this protein in RH development. Public transcriptomics data shows that at least eight CML proteins could be involved in RH development. The transcription of *CML4*, *CML7*, *CML12*, *CML18*, *CML25*, *CML32*, *CML37* and *CML48* is high and specifically directed towards RH cells (Brady et al. 2007). As such, the current knowledge of CMLs might heavily underestimate their role in RH tip growth.

Ten *CBL*s are found in the *Arabidopsis* genome. CBL1 is the only CBL protein with an established role in RH development. Preuss et al. (2006) found that CBL1 interacts in vivo with PI-4Kβ1 (phosphatidyl-inositol 4-OH kinase which generates the membrane-trafficking regulator phosphoinositide PI-4P), which in turn interacts with RabA4b. RabA4b is found on apically localized trans-Golgi-derived vesicles which are thought to transport new cell wall material to the tip. As such, the authors provided a link between CBL1-mediated Ca^{2+} signalling and cell wall delivery at the tip. Despite the fact that *CBL3*, *CBL4*, *CBL6* and *CBL9* show preferential transcription in RH cell files, none of these have been investigated for a role in RH tip growth. However, interesting results have highlighted an important role for CBL1, *CBL2*, *CBL3* and *CBL9* in PT development. Knowing that CBL3 and CBL9 might also be present in RH cells, it could be very interesting to see if these findings also apply for RH development. CBL2 and CBL3 regulate vacuolar dynamics in PTs through interaction with CIPK12 (Steinhorst et al. 2015). Overexpression of *CBL2* and *CBL3* results in lower pollen germination frequency and slower PT growth, whereas single- or double-knockout mutant exhibits impaired PT growth in vivo and in vitro. Upon interaction of the CBLs with CIPK12, the latter protein relocalized from the cytosol to the vacuole. Overexpression of *CIPK12* results in a marked vacuolar phenotype, whereas loss of function results in complete loss of polar PT growth.

Similarly, *CBL1* and *CBL9* overexpression leads to impaired PT growth (Mähs et al. 2013). This phenotype was shown to be related to overexpression-induced hypersensitivity to high K^+ levels. As such, in knockout PTs, low K^+ availability resulted in a reduction of PT growth. Most interestingly, Drerup et al. (2013) subsequently showed that CBL1 and CBL9 interact with CIPK26 which in turn phosphorylates and activates the NADPH oxidase RBOHF, leading to induction of ROS production. Notably, *CBL9* and *RBOHF* are also expressed in RHs, implying that a similar mechanism might act during RH growth.

Twenty-six *CIPK* genes have been identified in the genome of *Arabidopsis*. Again, little is known about the function of these genes in plant development. However, recent effort has identified CIPKs as important regulators of PT growth. As highlighted before, CIPK12 was shown to be involved in PT growth through

interaction with CBL2 and CBL3 (Steinhorst et al. 2015). CIPK19 is likely involved in regulating the Ca^{2+} influx at the tip of growing PTs (Zhou et al. 2015a). Overexpression of *CIPK19* led to higher apical $[Ca^{2+}]_{cyt}$, the formation of bulged PT tips, loss of PT polarity and decreased fertilization competitiveness. Treatment with La^{3+} (a calcium channel blocker) rescued the phenotype, showing that CIPK19 affect tip-focused Ca^{2+} concentration through regulation of the apical Ca^{2+} influx. In the *Arabidopsis* root, *CIPK19* is specifically, albeit mildly transcribed in RH cell files, implying a common role in RH development. *CIPK23*, which is expressed in RH cells, was shown to be involved in HIGH-AFFINITY K^+ TRANSPORTER 5/K^+ TRANSPORTER 1 (HAK5/AKT1)-mediated K^+-uptake through interaction with CBL1/CBL9 in roots (Ragel et al. 2015; Wang et al. 2016). A possible role for CIPK26 was also discussed previously. Despite these efforts to characterize the role of CIPKs in tip growth, no studies have focused on RH growth. However, at least 12 out of 25 *CIPK*s are strongly expressed in RH cell files (*CIPK2*, *CIPK5*, *CIPK6*, *CIPK8*, *CIPK9*, *CIPK10*, *CIPK13*, *CIPK19*, *CIPK21*, *CIPK22*, *CIPK23*, *CIPK24*), suggesting a major role in RH development.

9.4.2 Proton Oscillations Regulate Tip Growth

9.4.2.1 Proton Concentration

Intra- and extracellular pH regulation is of crucial importance for several developmental processes. Cytosolic and apoplastic pH homeostasis allows nutrient and sugar transport across the PM, organ development and cell elongation (Gjetting et al. 2012). More so, the existence of local pH gradients controls secondary transport in plant cells (Sze et al. 1999; Felle 2001), enzymatic reactions mostly rely on a pH optimum, differences in pH allow for compartmentalization (e.g. cytosol vs. vacuolar lumen) and pH differences across membranes lay the basis of hyperpolarization-driven signal transduction.

Local pH alterations depend on (1) the buffering capacity of the considered compartment, (2) active transport of protons (H^+) across plant membranes and (3) local H^+ production or consumption by metabolic processes. Scientific research has led to the understanding that well-coordinated interaction between these processes can lead to the formation of local H^+ accumulation or depletion. As a result, intracellular pH gradients are formed, which have been shown to function as secondary messengers in plant development. Much like the characteristics of Ca^{2+} signatures, pH differences often occur in a highly spatially and temporally organized manner. Quantitative analyses of these pH signatures has been challenging for long. Consecutively, our understanding of pH dynamics is still limited compared to what has been observed for Ca^{2+}. However, the recent development of several genetically encoded ratiometric pH sensors (Choi et al. 2012; Zhang et al. 2012; Gjetting et al. 2012) is promising and will most definitely further contribute to our understanding.

H^+ translocation across membranes occurs in two directions, depending on specific transporters. As such, transient acidification or alkalinization has been observed both in the cytosol and the apoplast. With regard to cell growth, H^+ translocation between the intra- and extracellular environment is key to the 'acid growth' theory which states that cells are able to expand faster following acidification of the cell wall (Rayle and Cleland 1992). This finding is now largely attributed to the acidic pH optimum of cell wall loosening expansins (McQueen-Mason et al. 1992; McQueen-Mason and Cosgrove 1995).

Crucially, the extracellular pH (pH_{ext}) has a pivotal role in tip growth, too. For instance, when placed in different pH buffers, the growth of PT can be inhibited (Feijó et al. 1999). Similarly, a sudden increase in the pH_{ext} led to cessation of RH growth, whereas a sudden decrease caused RH bursting (Monshausen et al. 2007). Consistent with the acid growth theory, upon RH initiation the cytosolic pH (pH_{cyt}) locally increases, while the cell wall locally acidifies (Bibikova et al. 1998). Until the switch to tip growth is made, this configuration is maintained. Next, during fast tip growth, a tightly controlled mechanism regulates cytosolic and apical apoplastic H^+ dynamics (Fig. 9.6). The resulting spatial and temporal pH changes are pivotal for controlling oscillating tip growth.

9.4.2.2 A Tip-Focused Oscillating H^+ Gradient Controls PT and RH Growth

The diffusion coefficient is much higher for H^+ compared to that of Ca^{2+}. Moreover, experimental evidence has shown that a change in H^+ concentration of 25–90 mM is required for a one unit pH_{cyt} increase (Plieth et al. 1997; Plieth and Hansen 1998; Oja et al. 1999). Despite H^+ diffusion and the high passive buffering capacity of the cytosol (Felle 2001), a pH_{cyt} gradient exists at the tip of growing PTs and RHs (Fig. 9.6). In RHs, the tip-localized pH is slightly alkaline (pH 7.4–7.6) compared to the rest of the cytoplasm (Monshausen et al. 2007; Bai et al. 2014a). In PTs on the other hand, the pH at the tip is slightly acidic, followed by the presence of a subapical alkaline band (Feijó et al. 1999; Michard et al. 2008, 2009). As such, the maintained presence of alkaline micro domains suggests that in both RHs and PTs a mechanism must be in place that actively removes H^+ from the apical region. Indeed, both in RHs and PTs, scientific evidence points towards polarized H^+ efflux in the subapical regions (Fig. 9.6). More specifically, Feijó et al. (1999) reported on a H^+ efflux (0.004–0.01 amol μm^{-2} s^{-1}) at the base of the clear zone in PTs. Similarly, a H^+ efflux (0–0.02 amol μm^{-2} s^{-1}) was detected starting 20 μm from the tip in tobacco PTs (Certal et al. 2008). Further away from the tip, this pattern would revert to a continuous small influx with a peak close to the pollen grain. At the surface of the pollen grain itself, a strong H^+ efflux was again present. Certal et al. (2008) showed that, considering the entire process ranging from early pollen germination (10 μm) to prolonged PT growth (>500 μm), flux dynamics are much more complex than previously thought. Originally, a strong H^+ efflux is present

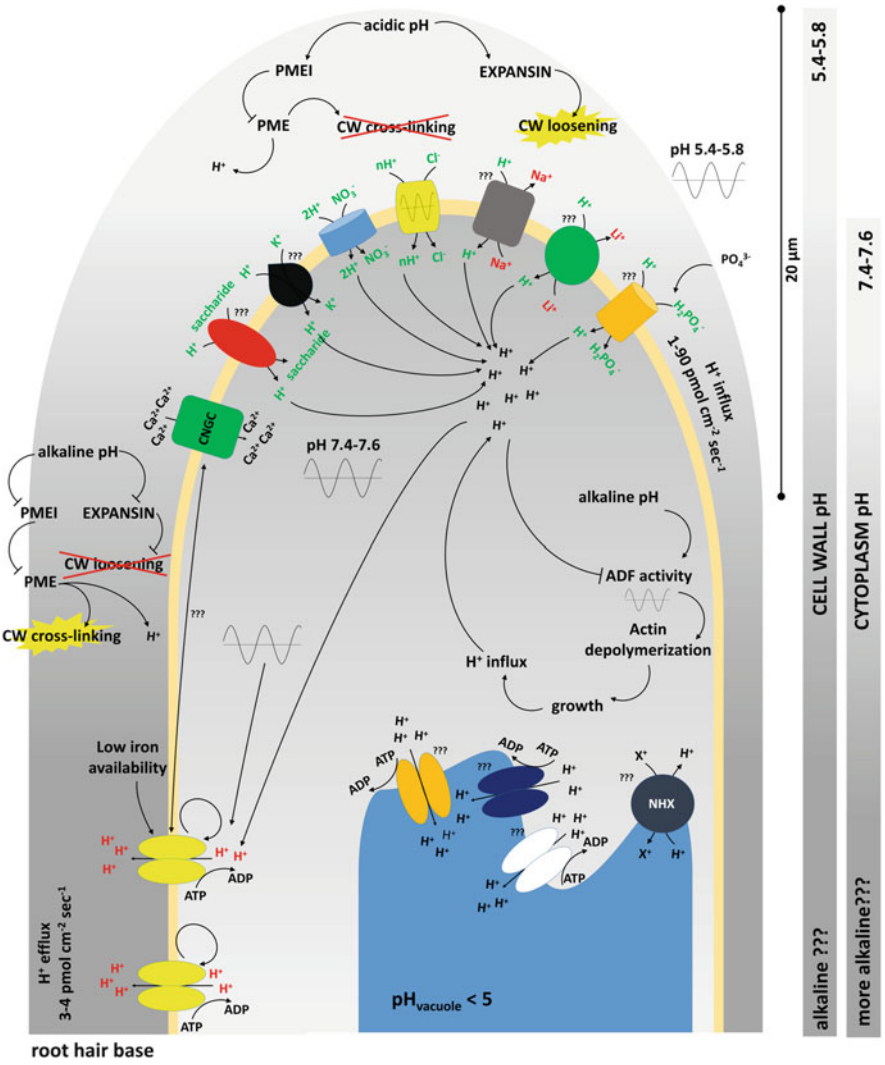

Fig. 9.6 Graphical representation of proton transport and sink systems governing root hair tip growth. *Question marks* refer to hypothetically involved components and mechanisms. The upper 20 µm represents the clear zone. *Sigmoidal curves* represent oscillatory behaviour. *Outer grey area* = cell wall, *yellow outline* = plasma membrane, *inner grey area* = cytoplasm. *Grey gradient* represents pH differences (magnitude depicted on the *right*). All abbreviations are referred to in Sect. 9.4.2

at the pollen grain's surface. During PT growth, efflux of H^+ is observed along the subapical region of the PT. Subsequently, callose plugs are formed in spatially defined regions where no net flux is detected. Upon formation of the primary callose

plug, the efflux ranging from the plug to the grain gradually converts to an influx which finally causes this part of the PT to die.

Compared to PTs, current knowledge in RHs suggests that the spatial distribution of H^+ fluxes in RHs is far less complex. In contrast with PTs, no subapical alkaline band was observed in growing RHs. However, H^+ efflux was found to be the highest at the base of the growing RH (0.03–0.04 amol μm^{-2} s^{-1}; Jones et al. 1995).

Like for the tip-localized Ca^{2+} gradient, the apical pH gradient found in RHs and PTs also occurs in an oscillatory manner (Lovy-Wheeler et al. 2006; Monshausen et al. 2007; Michard et al. 2008). During RH tip growth, the pH_{cyt} oscillates between 7.4 and 7.6. These oscillations have the same frequency as the growth rate oscillations (2–4 min^{-1}), but each growth pulse is preceded by an acidification with a lead of 7 s. Similarly, the apical pH_{cyt} in tobacco PTs was found to oscillate with a magnitude of at least 0.3 pH units and a period of 1–4 min (Michard et al. 2008). In *Lilium* PTs a cytosolic alkalinization preceded a growth pulse by approx. 11 s, and a subsequent acidification followed growth by approx. 8 s (Lovy-Wheeler et al. 2006). These transient acidifications are the result of H^+ influx at the extreme apex of growing PTs and RHs. As such H^+ are (at least partly) cycled through the growing tip. Hence, they are imported at the tip and subsequently exported back to the apoplast either at the base of the clear zone (PTs) or at the base of the shank (RHs).

9.4.2.3 Oscillating H^+ Influx from the Cell Wall

Each cytosolic acidification event is the result of active H^+ transport across the PM at the tip. As such, transmembrane H^+ fluxes also oscillate. Vibrating microelectrode analysis indeed revealed a H^+ influx at the very tip of growing RHs and PTs. However, the measured fluxes strongly differ between studies. Monshausen et al. (2007) reported inward H^+ fluxes up to 0.9 amol μm^{-2} s^{-1} at the tip of growing RHs. Jones et al. (1995) reported on a H^+ influx at the tips of growing *Limnobium stoloniferum* RHs in the range of 0.01 amol μm^{-2} s^{-1}. Feijó et al. (1999) reported on an oscillating H^+ influx in the range of 0–0.04 amol μm^{-2} s^{-1}, whereas Michard et al. (2008) and Messerli et al. (1999) report on a pulsatile influx of 0.1–0.4 and 4.89 $\pm$ 0.81 amol μm^{-2} s^{-1}, respectively, in growing PT tips.

The buffering capacity of the apoplast is generally considered to be much lower than that of the cytosol (in the range of 4 mM H^+ per pH unit; Oja et al. 1999). Logically, periodic efflux of H^+ from the cell wall to the cytosol thus causes pH_{ext} oscillations. Monshausen et al. (2007) found that each cytosolic acidification/H^+ influx peak was indeed accompanied by an extracellular alkalinization. The pH_{ext} was found to oscillate between 5.8 and 5.4 (double the amplitude of pH_{cyt} oscillations). An extracellular alkalinization coincided with cytosolic acidification and followed each growth pulse by 7 s. The nature of transient extracellular alkalinization/acidification events is considered crucial for regulating cell wall dynamics (see section “Cell Wall Pectin Dynamics”).

9.4.2.4 H^+ Transporter Proteins Regulate H^+ Fluxes During Tip Growth

Apical and subapical H^+ fluxes are generated by specific H^+ pumps and non-specific H^+ symporter or antiporter proteins (Fig. 9.6). Several types of H^+ pumps have been reported, either specifically dedicated to H^+ transport (PM-, tonoplast- or endomembrane-localized H^+-ATPases) or relying on H^+ symport for active translocation of nutrients across the PM. The former ATPases actively generate H^+ electrochemical gradients (Palmgren 2001), which are then exploited for active nutrient transport by H^+/nutrient symport or antiporter proteins. Holdaway-Clarke and Hepler (2003) hypothesized that H^+ influx at the tip of growing PTs is regulated by non-specific cation channels, whereas H^+ efflux relates to the specific activity of H^+-ATPases. At the time of writing, little is known about the exact nature of H^+ transporting proteins that act in RH tip growth. However, a quick survey of public transcriptomics data shows that many putative RH-specific proteins might be involved in generating H^+ fluxes across membranes.

H^+ Influx Proteins at the Tip

Whereas H^+-specific microelectrode experiments revealed inward H^+ fluxes across the apical PM, the exact nature of the H^+ transporting proteins is still largely unknown. H^+ influx is generally facilitated by non-specific H^+ symporter or antiporter proteins, which use the transmembrane electrochemical gradient to co-transport nutrients alongside with H^+. Several putative H^+ symport and antiport proteins were shown to be transcribed in pollen, but functional characterization is generally lacking (Bock et al. 2006). A PM-localized pollen-specific H^+/broad-spectrum monosaccharide symporter SUGAR TRANSPORTER 6 (STP6) was identified in *Arabidopsis* (Scholz-Starke et al. 2003). However, *STP6* loss-of-function pollen exhibited no obvious phenotype. Notably, pollen-specific monosaccharide and sucrose transport proteins were also identified in *Petunia* and tobacco, suggesting that a conserved saccharide-based H^+-symport system exists in pollen (Ylstra et al. 1998; Lemoine et al. 1999). More so, in vitro RH and PT tip growth rely on the presence of sucrose. The *Arabidopsis* genome contains 9 sucrose and 14 monosaccharide transport proteins (Scholz-Starke et al. 2003).

At the tip of growing PTs, a tip-focused K^+ influx (6.88 ± 1.44 amol μm^{-2} s^{-1}) coexists alongside the H^+ influx (Messerli et al. 1999). H^+ and K^+ fluxes oscillate in phase relative to each other, but H^+/K^+ peaks lag growth pulses by ± 11 s. Might this suggest the existence of a H^+/K^+ symport system? Interestingly, both in RHs and PTs, several K^+ influx transporter proteins have been identified and characterized, but none of them exhibit H^+ symport activity (Rigas et al. 2001; Ivashikina et al. 2001; Reintanz et al. 2002; Mouline et al. 2002; Desbrosses et al. 2003; Ahn et al. 2004; Lu et al. 2011). Most likely, the coupling of H^+ and K^+ fluxes is due to the strong pH sensitivity of K^+ uptake channels, rather than the existence of a H^+/K^+ symport system (Mouline et al. 2002; Griessner and Obermeyer 2003; Lu et al. 2011). However, the latter option cannot be ruled out.

The current knowledge on H^+ symport proteins in RHs is also quite limited. Patch-clamp analysis revealed the existence of an H^+/NO_3^- symport system in the PM of *Maize* roots (Ruiz-Cristin and Briskin 1991). Similar NO_3^- transport was observed at the PM of *Arabidopsis* RHs (Meharg and Blatt 1995). The latter NO_3^- transport occurred in a pH-dependent manner and involved the co-transport of two protons per single NO_3^- molecule, providing strong evidence for a dedicated H^+/NO_3^- RH-located symport system. Ion-sensitive microelectrode measurements also revealed a H^+/Cl^- symporter current in RHs of *Sinapis alba* (Felle 1994). This current has not been assigned to a specific locus; however, the evidence strongly suggests symport-based coupling of H^+ and Cl^- transport. Upon lowering the pH_{ext}, the $[Cl^-]_{cyt}$ increases and the cytoplasm acidifies. Complementary, the same response was observed when increasing the $[Cl^-]_{ext}$. More so, the Cl^- fluxes were also shown in PTs to oscillate in phase with growth pulses (Zonia et al. 2001).

Hamam and colleagues described a Na^+/H^+ exchange protein at the PM (Hamam et al. 2016), and it is expressed specifically in RH cell files. Interestingly, a Li^+/H^+ exchange protein was characterized at the PM, which is also expressed in RHs (An et al. 2007; Brady et al. 2007).

Finally, a $H^+/H_2PO_4^-$ symporter protein named LePT1 was identified in tomato (Daram et al. 1998). The gene is strongly expressed in RHs and upregulated under phosphate deficiency.

To our knowledge, no other H^+ influx proteins have been characterized at the PM of growing PTs and RHs until now. Thus, a major knowledge gap exists regarding the mechanisms controlling H^+ influx in tip-growing cells.

Subapical H^+ Efflux Proteins

H^+-ATPases constitute the main family of H^+ export proteins. They perform active H^+ transport across the PM through the hydrolysis of ATP. H^+-ATPases are thought to lie at the basis of plant nutrient uptake. Hence, the H^+-ATPase-mediated generation of a PM-localized electrochemical gradient is known to activate nutrient-specific secondary active transporters and membrane channels (Gilroy and Jones 2000; Ashcroft et al. 2009). The *Arabidopsis* genome codes for 12 H^+-ATPase genes (*AHA1-12*). Seven P-type H^+ pumps were found to be expressed during pollen germination and PT growth (Song et al. 2009), and several H^+-ATPases were found to be expressed in RHs (Moriau et al. 1999; Palmgren 2001; Santi and Schmidt 2009; Młodzińska et al. 2015). Indeed, public transcriptomics data confirms that the transcription of several *AHA* genes is restricted to RHs, PTs or both (Table 9.3).

A role for H^+-ATPases in controlling tip growth was first confirmed through a series of pharmacological experiments. PTs grew faster in the presence of fusicoccin (a H^+-ATPase agonist) but seized to grow when supplemented with the H^+-ATPase antagonists vanadate or N-ethylmaleimide (Rodriguez-Rosales et al. 1989; Feijó et al. 1992; Fricker et al. 1997; Pertl et al. 2001; Sun et al. 2009; Lang et al. 2014).

Table 9.3 Transcription of H^+-ATPases in RHs and PTs

AHA gene ID	Transcription in RHs (Brady et al. 2007)	Transcription in PTs (Qin et al. 2009)
1	Yes, highly specific	Yes, low
2	Yes, highly specific	No
3	No	No
4	Yes, highly specific	No
5	Yes, low	No
6	No	Yes
7	Yes, highly specific	Yes
8	No	Yes, highly specific
9	Yes, low	Yes
10	Yes, low	Yes
11	No	No
12	No	No

More so, treatment of tobacco PTs with orthovanadate inhibited the subapical H^+ efflux by 80%, strongly suggesting that H^+-ATPases maintain the latter (Certal et al. 2008). Few studies have focused on the functional annotation of H^+-ATPases in tip growth. Loss of function of *AHA7* was shown to cause a decrease in the number of RHs (Santi and Schmidt 2009). A tobacco pollen-specific AHA with homology to *Arabidopsis* AHA9, AHA8 and AHA6 was found at the subapical PM of PTs (Certal et al. 2008). The fact that it was excluded from the PT tip is consistent with the subapical H^+ effluxes observed in tip-growing cells. Overexpression of *NtAHA* led to the formation of abnormal callose plugs, aberrant H^+ fluxes and overall shorter PTs. Protein-reporter constructs showed that AHA2 is expressed in RHs (Fuglsang et al. 2007; Młodzińska et al. 2015). Santi and Schmidt (2009) found that AHA2 could be involved in controlling iron mobilization through rhizosphere acidification in response to iron deficiency. Most interestingly, both AHA2 and AHA1 were found to interact with CNGC17 (Ladwig et al. 2015). CNGC channels are putative Ca^{2+} channels with the potential of regulating Ca^{2+} dynamics in tip-growing cells (see Sect. 9.4.1.3). *CNGC17* is also specifically expressed in RH cells. As such this raises the question: 'might there be a direct link between the H^+ efflux machinery and the regulation of Ca^{2+} import?'.

A recent study by Veshaguri and colleagues added another layer of complexity to the regulation and dynamics of H^+-ATPase-mediated H^+ fluxes (Veshaguri et al. 2016). Based on single-molecule characterization, they showed that AHA2 switches between three discrete functional states: pumping, inactive and leaky. The intrinsic properties of pH gradients directly determine in which state the pump resides. As such, it is easy to image that also in RHs and PTs, the tip-focused pH signatures regulate the functioning of subapical H^+-ATPases. In addition, all P-type H^+-ATPases contain an autoregulatory domain, which is subject to phosphorylation, thus adding another layer of control.

The Vacuole as H^+ Source/Sink

The role of the vacuole is often neglected when it comes to allocation of H^+. However, (1) several H^+ pumps are thought to localize preferentially to the tonoplast, (2) the vacuolar pH is strongly acidic in comparison to the pH_{cyt} and (3) the vacuole extents until right beneath the clear zone. Several families of putative vacuolar H^+-ATPases exist in the *Arabidopsis* genome, and despite the fact that none of these pumps have been investigated towards their possible involvement in tip growth, transcriptome data leaves no doubt that they might have a pivotal role in controlling RH development. At least 20 vacuolar H^+-ATPase subunits are specifically transcribed in RH cell files (Table 9.4; Brady et al. 2007).

More so, the Na^+/H^+ EXCHANGER 1-4 (NHX1-4) H^+ efflux proteins also localize to the tonoplast (Reguera et al. 2015). Transcriptome analysis shows that *NHX1* is strongly and preferentially expressed in RH cells. Albeit low, *NHX4* is preferentially transcribed in trichoblasts.

9.4.2.5 Signalling Downstream of pH Oscillations

Actin Remodelling

The actin cytoskeleton is a highly spatially organized and dynamic component in tip-growing cells. Experimental evidence derived from studying PTs strongly supports pH-dependent regulation of actin turnover during tip growth. This is based on the facts that (1) an actin fringe of cortical microfilaments co-localizes with the alkaline band in growing PTs, (2) cytosolic acidification disturbs or abolishes this actin fringe and (3) pH-regulated actin depolymerization factor (ADF) proteins co-localize with the actin fringe (Lovy-Wheeler et al. 2006). Lovy-Wheeler and colleagues carefully studied the pH-dependent dynamics of the actin cytoskeleton in growing *Lilium* PTs. They found that artificially lowering the tip-focused pH caused the actin fringe to move closer to the growing tip and resulted in 80% growth reduction (10 mM sodium acetate) or caused the fringe to dissipate completely and growth to stop entirely (100 mM sodium acetate). In addition, treatment of PTs with latrunculin B (LatB; destabilizes actin filaments) causes the dissipation of the acidic domain at the tip and the allocation of the alkaline band towards the apex (Cárdenas et al. 2008). These findings support a role for a feedback loop between tip pH_{cyt} and apical actin dynamics (Fig. 9.6). Furthermore, they showed that ADF co-localizes strongly with the actin fringe and allocates similarly in response to acidification. ADF proteins are thought to promote actin-polymerization in an alkaline environment (Gungabissoon et al. 1998; Allwood et al. 2002; Chen et al. 2002). Contrary to PTs, and consistent with the RH base-positioned H^+ efflux, a subapical alkaline band has not been observed in growing RHs. However, whereas in PTs the apical pH gradient is slightly acidic (located above the alkaline band), in RHs the apical pH gradient is slightly alkaline (Monshausen et al. 2007; Bai et al.

Table 9.4 RH-specific transcription of vacuolar ATPase subunits

V-ATPase domain	V-ATPase subunit	Gene name	Locus ID	Family
V_1	A	AtVHA-A	At1g78920	Vacuolar H^+-pyrophosphatase family
	B2	AtVHA-B2	AT4g38510	V-type ATPase family
	C	AtVHA-C	At1g12840	V-type ATPase family
	D	AtVHA-D	AT3g58730	V-type ATPase family
	F	AtVHA-F	AT4g02620	V-type ATPase family
	H	AtVHA-H	AT3g42050	V-type ATPase family
V_0	a1	AtVHA-a1	At2g28520	V-type ATPase family
	a2	AtVHA-a2	At2g21410	Putative vacuolar H^+-ATPase subunit 1 family
	a3	AtVHA-a3	AT4g39080	Putative vacuolar H^+-ATPase subunit 1 family
	c1	AtVHA-c1	AT4g34720	Vacuolar H^+-ATPase 16 kDa proteolipid family
	c2	AtVHA-c2	At1g19910	Vacuolar H^+-ATPase 16 kDa proteolipid family
	c3	AtVHA-c3	AT4g38920	Vacuolar H^+-ATPase 16 kDa proteolipid family
	c4	AtVHA-c4	At1g75630	Vacuolar H^+-ATPase 16 kDa proteolipid family
	c5	AtVHA-c5	At2g16510	Vacuolar H^+-ATPase 16 kDa proteolipid family
	c″1	AtVHA-c″1	AT4g32530	Vacuolar H^+-ATPase 16 kDa proteolipid family
	c″2	AtVHA-c″2	At2g25610	Vacuolar H^+-ATPase 16 kDa proteolipid family
	d1	AtVHA-d1	At3g28710	V-type ATPase family
	d2	AtVHA-d2	AT3g28715	V-type ATPase family
	e1	AtVHA-e1	AT5g55290	V-type ATPase family
	e2	AtVHA-e2	AT4g26710	V-type ATPase family

2014a). As such, we suggest that ADF activity could also regulate actin dynamics in RHs. More so, both *ADF8* and *ADF11* were shown to be transcribed specifically in RH cell files (Brady et al. 2007; Kandasamy et al. 2007; Ruzicka et al. 2007) and were previously found to be part of the transcriptional core RH machinery (Bruex et al. 2012).

Lovy-Wheeler et al. (2006) suggested a self-sustained cycle controlling pH-mediated actin-remodelling at the tip. An alkaline pH would promote ADF-activity, which in turn favours increased actin depolymerization. The latter would result in faster growth and an increase of the H^+ influx at the tip, leading to cytoplasmic acidification. Subsequent inactivation of ADF would then lead to a decrease of actin polymerization, growth and H^+ influx, allowing for the pH to gradually rise again. Logically, such an autoregulatory loop would result in actin polymerization/depolymerization oscillations at the tip (see Sect. 9.5.1.2).

Cell Wall Pectin Dynamics

The cell wall of tip-growing cells is a highly dynamic compartment, strongly relying on pH oscillations to fine-tune the spatial and temporal balance of cell wall rigidification/loosening (Fig. 9.6). This process is now thought to largely rely on the presence of several cell wall-modifying proteins whose action is pH dependent. In general, RH and PT elongation seem to be consistent with the theory of acid growth, where a more acidic environment promotes cell wall loosening (Rayle and Cleland 1992). More H^+ would generally increase the activity of expansins while inhibiting the action of pectin methylesterases (Cosgrove 2000; Michelli 2001; see Sects. 9.6.2 and 9.6.3).

9.5 The Cytoskeleton

9.5.1 Actin Dynamics Control Apical Cell Growth in RHs

9.5.1.1 Introduction

RH and PT tip growth rely on highly polarized delivery of secretory vesicles to the growing apex and strict subcellular localization of specific organelles. In RHs, directional vesicle and organelle transport is facilitated by the presence of a dynamic actin cytoskeleton (Fig. 9.7) similar to that observed in growing PTs (see Chap. 3).

When RH growth seizes, thick actin bundles protrude the entire cytoplasm, including the apex (Ketelaar et al. 2003; He et al. 2006). It has been shown that treatment with the actin polymerization inhibitor CytD terminates RH growth (Miller et al. 1999). Moreover, like in PTs, depolymerization of F-actin with LatB inhibits RH growth (Gibbon et al. 1999; Baluška et al. 2000). In *Arabidopsis*, there are at least eight isovariants of actin. Ectopic expression of individual isoforms

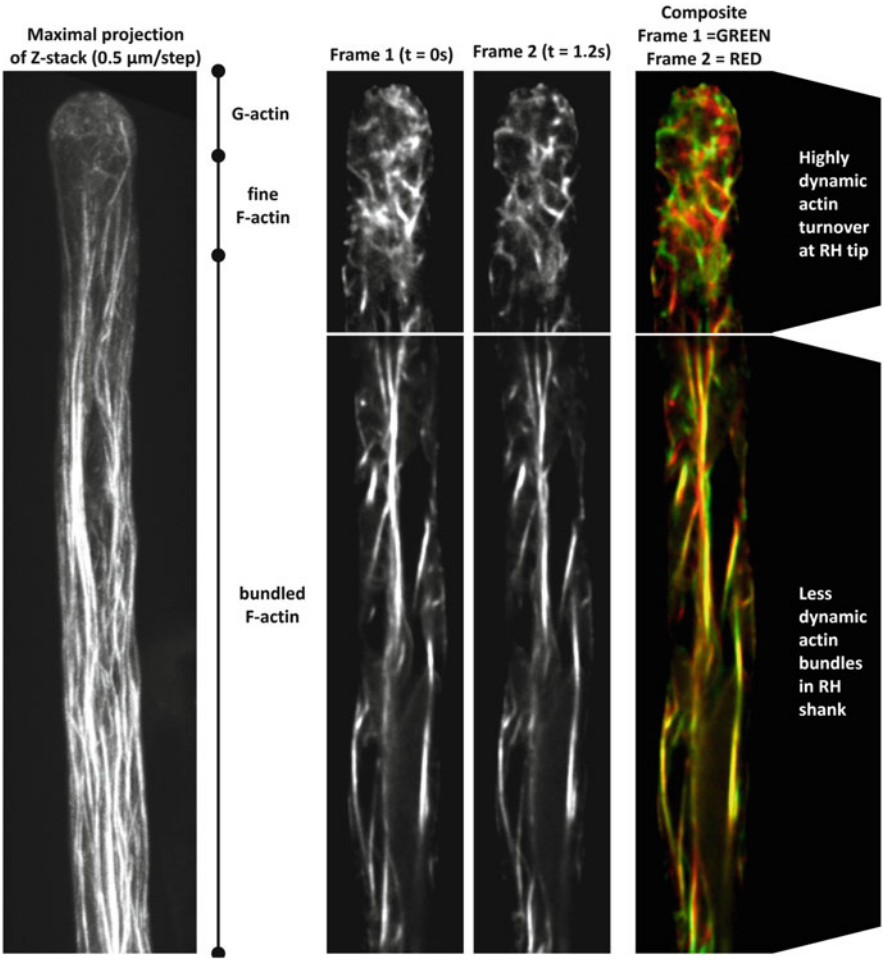

Fig. 9.7 Spinning disc confocal images of the actin cytoskeleton in growing *Arabidopsis* root hairs stably expressing Lifeact-Venus (first employed by Riedl et al. 2008). (*Left*) Maximal projection of a Z-stack (0.5 µm step size) of the actin cytoskeleton. (*Right*) Consecutive frames (taken at 1.2 s interval) showing actin dynamics in the RH tip and shank

resulted in disruption of the actin cytoskeleton and severe developmental defects (An et al. 1996a, b; Huang et al. 1997; Kandasamy et al. 2002, 2007). As such, different actin isoforms seem to relate to different functions. The latter might also imply that they are each associated with different means of regulation. However, at the time of writing, evidence for such isoform-specific control in both RHs and PTs is rather limited. Kandasamy and colleagues did reveal that ectopic expression of *ACT1* resulted in strong developmental defects, likely associated with incorrect interaction with endogenous vegetative actin-binding proteins, since coexpression with the native ACT1-specific ABPs partially rescued the phenotypes.

ACT2, *ACT7* and *ACT8* are expressed in vegetative tissues including RHs. ACT2 and ACT8 are essential for proper tip growth since *act2-1* (aka *enl2* or *der1*) and *act8-2* mutants exhibit stunted RHs, while the *act2-1/act8-2* double mutant is even hairless (Gilliland et al. 2002; Ringli et al. 2002; Nishimura et al. 2003; Kandasamy et al. 2009). Even partial loss of ACT2 functionality results in drastic RH swelling and premature growth arrest (Diet et al. 2004). *ACT7* transcription is auxin inducible and found throughout the root epidermis rather than being restricted to trichoblast cell files (McDowell et al. 1996a; An et al. 1996b; Kandasamy et al. 2001). Its role, however, seems to be restricted to early RH establishment, given that the RH density is decreased in *act7* knockout plants. Together with ACT2, ACT7 regulates planar polarity establishment in early RH development (reviewed in Balcerowicz et al. 2015; Kiefer et al. 2015).

In PTs, several more actin isoforms are expressed besides *ACT2* and *ACT8* (Wang et al. 2008). The remaining isoforms are referred to as the class of reproductive actins (*ACT1*, *ACT3*, *ACT4*, *ACT11* and *ACT12*; McDowell et al. 1996b; McKinney and Meagher 1998), and surprisingly, not much is known about their individual functions. ACT11 was shown to regulate actin turnover and subsequent vesicle turnover rates in *Arabidopsis* PTs (Chang and Huang 2015).

A continuous effort towards the characterization of the actin cytoskeleton and its key regulators has led us to acknowledge its role in PT (see Chap. 3) and RH development. Future research will greatly benefit from novel in vivo labelling and imaging techniques and the availability of a vast number of T-DNA insertional mutants. Here we describe the current state of knowledge regarding the molecular players controlling actin dynamics in growing RHs.

9.5.1.2 Do Actin Polymerization/Depolymerization Oscillations Exist in RHs?

The regulation of polymerization/depolymerization of AFs has been shown to depend on Ca^{2+}, pH, phosphoinositide and ROP-GTPase dynamics, most of which oscillate during tip growth (see Sects. 9.2 and 9.4). As such, it is easily conceivable that actin turnover at the tip could also exhibit an oscillatory pattern. In RHs, initial data revealed clear fluctuations in labelling intensity of tip-localized F-actin + ends (Vazquez et al. 2014). Quantitative analysis of these fluctuations on a broader timescale might reveal the temporal and spatial dynamics of actin oscillations in growing RH. For now, however, the hypothesis that oscillatory actin turnover might exist during RH development is solely based on what has been observed in PTs. Treatment of growing PTs with low [LatB] (2 nM) resulted in reversible inhibition of growth rate and $[Ca^{2+}]_{cyt}$ oscillations (Cárdenas et al. 2008). Importantly, however, PTs did not seize to grow but instead continued to elongate at a basal growth rate. This finding illustrates that actin polymerization is needed to sustain oscillatory but not linear growth, strongly suggesting that actin turnover itself exhibits oscillatory changes. Indeed, through the development of a G-actin-specific ratiometric dye, the authors were able to show that tip-focused G-actin oscillates with the same period

as growth oscillations, but with a phase lag of ±10 s. As such, a low level of G-actin/an increase in F-actin polymerization anticipated an increase in growth rate. Ca^{2+} oscillations lag growth oscillations by approx. 5 s (Messerli et al. 2000) and thus anticipate G-actin oscillations. It is therefore tempting to propose that Ca^{2+} dynamics could regulate oscillatory actin turnover through direct control of ABPs (Pei et al. 2012). Moreover, the ABPs VILLINS and ADFs have been shown to regulate RH growth in a Ca^{2+}-dependent manner. For instance, ZmADF3's actin-severing activity is regulated by a Ca^{2+}-dependent protein kinase (Smertenko et al. 1998; Allwood et al. 2001). VILLIN4 promotes capping and severing of AFs at high (5 μM) but not at low (0.5 μM) [Ca^{2+}] (Zhang et al. 2011a). Considering that these concentrations lie within the physiological range associated with the tip-focused Ca^{2+} gradient, it is easy to imagine that VILLIN activity oscillates together with Ca^{2+} oscillations.

In addition to Ca^{2+}-dependent regulation of actin oscillations, pH oscillations might also play an important role. Based on pharmacological and localization studies, there seems to be a feedback loop controlling actin and pH dynamics at the apex of tip-growing cells (Lovy-Wheeler et al. 2006). Moreover, research has shown that certain ADF proteins are preferentially functional in an alkaline environment, consistent with the base of the clear zone.

9.5.1.3 Actin-Binding Proteins Regulate RH Tip Growth

Emerging evidence shows that the control of actin dynamics by several ABPs is tightly embedded with the core tip growth machinery. This is illustrated by the existence of actin-regulating factors with a specific sensitivity towards Ca^{2+}, pH and phospholipid signalling (Fig. 9.8). Here, we provide an overview of the ABPs shown to be involved in regulating RH tip growth.

Control of Actin Nucleation

Formins are proteins that directly bind to the elongating barbed end of AFs, where they enhance filament elongation while protecting the growing end from capping proteins (Kovar and Pollard 2004; Romero et al. 2004). Their function largely depends on their ability to interact with a variety of proteins, thereby forming large multiprotein complexes generally including profilins (discussed later). Notably, several formin isoforms contain ROP-GTPase-binding domains (Watanabe et al. 1997; Petersen et al. 1998) and were shown to associate with MTs (Deeks et al. 2010; Li et al. 2010; Wang et al. 2013). Formins have a pivotal role in mammals, fungi and plants and were shown to be important for RH and PT development (Deeks et al. 2005; Yi et al. 2005; Ye et al. 2009; Cheung et al. 2010; Huang et al. 2013b). Twenty-one formin-related sequences were identified in the *Arabidopsis* genome alone (Deeks et al. 2002; Cvrckova et al. 2004). Several formins seem to be preferentially transcribed in RH cells (relatively high transcription, *AtFH5*,

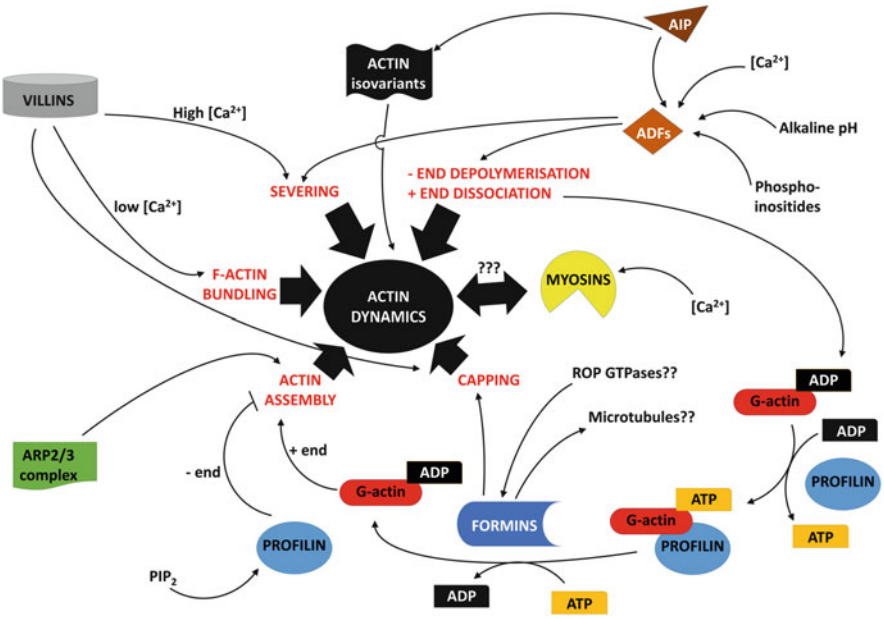

Fig. 9.8 Simplified scheme illustrating the mechanisms controlling actin dynamics in growing root hairs. *Question marks* refer to hypothetically involved components and mechanisms. All abbreviations are referred to in Sect. 9.5.1

AtFH8 and *AtFH1*; moderate transcription, *AtFH10*, *AtFH13*, *AtFH16*, *AtFH19*, *AtFH20*), and overexpression of *AtFH8* lacking its FH2 (actin-binding) domain led to aberrant RH development, with RHs being shorter, seizing growth at the bulge stage or being completely absent from the epidermis (Deeks et al. 2005). The latter suggest that AtFH8 could be involved in both bulge formation (reviewed in Balcerowicz et al. 2015) and tip growth. The authors suggest that in vivo the truncated protein would compete with the interacting proteins of the functional AtFH8, thereby preventing the latter from nucleating the actin cytoskeleton. An additional study revealed that constitutive *AtFH8* overexpression resulted in short, waved, bulged and/or branched RHs with an over-accumulation of AFs (Yi et al. 2005). FH8 was shown to nucleate actin barbed ends in vitro and, curiously, to induce actin severing instead of polymerization. Yet it enhances polymerization in the presence of profilin. The latter has not been shown in any other formin isoform.

AtFH5 is also strongly and specifically expressed in RH cell files (Brady et al. 2007). Corresponding functional characterization in RHs is however still lacking. In PTs, AtFH3 and AtFH5 both regulate apical expansion, but they seem to do so in different ways (Ye et al. 2009; Cheung et al. 2010). Interestingly, present data clearly supports a role for FH5 in subapical vesicle targeting.

OsFH1 (*FH1 Oryza sativa* homologue) knockout plants display very short RHs when no structural support is present (liquid media instead of solid; Huang et al.

2013a, b). When grown on a solid surface, RHs appeared normal. Besides OsFH1 and AtFH8, no other formins have been attributed to regulating RH growth.

Just like formins, the ACTIN-RELATED PROTEIN 2/3 (ARP2/3) complex mediates barbed end actin assembly and thus promotes actin nucleation (Hussey et al. 2006). The seven-protein complex binds to existing AFs and stimulates the formation of new side branches. The complex consists of seven subunits (Arp2, Arp3, ArpC1/p41, ArpC2/p34, ArpC3/p20, ArpC4/p20, ArpC5/p16), which are all expressed in plants (Mathur et al. 2003a). The function of the ARP2/3 complex is considered to be highly conserved between different biological systems, given that *Arabidopsis* subunits can complement their yeast homologues and mammalian homologues can complement mutations in plant subunits (Le et al. 2003; Mathur et al. 2003b). The currently described plant-specific mutations associated with loss of function of *Arp2/3* subunits all resulted in morphogenesis-related phenotypes (Mathur et al. 1999, 2003a; Li et al. 2003) or related to regulation of stomatal aperture (Li et al. 2013, 2014) or salt stress-induced Ca^{2+} signalling (Zhao et al. 2013b). The evidence regarding involvement in RH and PT tip growth is very scarce. It was shown that *AtARP2* is strongly transcribed in pollen grains (Klahre and Chua 1999), and Mathur et al. (2003b) revealed that RHs of the *crooked* mutants, harbouring a loss-of-function mutation in the *ARPC5* subunit, were either short and stunted or curvy and up to double as wide as wild-type RHs. In addition, the occasional presence of two bulges on a single trichoblast cell suggests that the ARP2/3 complex might regulate both RH initiation and elongation. The actin cytoskeleton in *crooked* RHs consisted of alternating regions with thick actin patches (associated with growth restriction) and fine F-actin bundles (associated with growth). The *wurm* (*ARP2* knockout) and *distorted1* (*ARP3* knockout) mutations exhibit a similar short and wavy RH phenotype, as a result of F-actin aggregation (Mathur et al. 2003a). Given the fact that loss of function of each of three *ARP2/ARP3* single subunits causes the same phenotype, it is conceivable that the observed defect is due to loss of function of the entire complex. As such, it can be hypothesized that the ARP2/3 complex regulates the formation of fine filamentous actin cables in RHs, thereby controlling RH polarity and final RH length.

Profilins are G-actin-binding nucleotide exchange factors that catalyse the exchange of ADP to ATP, thereby returning actin monomers to the profiling-actin-ATP pool (Valenta et al. 1993; Gibbon et al. 1998; Pollard and Borisy 2003). They can constitute a large part of the total protein pool (up to 127 μM), largely equimolar with the concentration of G-actin, as observed in *Maize* pollen (Gibbon et al. 1999). In the presence of profilins, plant formins can enhance filament elongation rates by a factor of 10–42 (Zhang et al. 2016). However, profilins can either inhibit spontaneous nucleation of AFs in the absence of formins (at the minus-ends) or promote AF elongation through interaction formation of profilin-G-actin-formin complexes at plus-ends (Kovar et al. 2003; Michelot et al. 2005; Vidali et al. 2009; Zhang et al. 2011b; van Gisbergen and Bezanilla 2013; Ketelaar 2013). Minus-end inhibition of polymerization might be actin isoform specific (Kijima et al. 2016). Five profilin isoforms are transcribed in *Arabidopsis* (Huang et al. 1996) yet only

one is trichoblast specific (*PRF1*; Brady et al. 2007), whereas at least three profilins are expressed in in vivo grown PTs (*PRF1*, *PRF4* and *PRF5*). In RHs, profilin specifically localized to the apex of emerging RH bulges and growing RHs (Braun et al. 1999). This pattern is altered when RH growth stops or upon treatment with the microfilament disruptor CytD and the phosphatidylinositol 4,5-biphosphate [$PI(4,5)P_2$] signalling disruptor mastoparan, strongly suggesting that profilin is involved in controlling tip growth in a $PI(4,5)P_2$-dependent manner. *Prf1* knockout plants exhibit a variety of RH phenotypes related to increased cell elongation (McKinney et al. 2001). The authors suggest that the absence of PRF1 protein could result in more free G-actin, which could enhance filament polymerization. However, the actual mechanism by which PRF1 controls RH elongation has not been characterized. Surprisingly, Vidali and Hepler (1997) found that in PTs profilin localization differs from that observed in RHs. In PTs, profilins were found to localize to the peripheral cytoplasm of the cell and accumulate mostly in the central region of the shank.

Control of Actin Depolymerization

Actin-depolymerizing factors show affinity for both filamentous actin and actin monomers. They enable severing of ADP-actin filaments and promote ADP-actin filament dissociation at the minus-end (Maciver and Hussey 2002; Pollard and Borisy 2003). The latter means that ADF proteins enhance actin turnover by replenishing the monomer pool, and hence, released minus-end monomers can be recycled for plus-end growth. In addition, ADFs might also promote AF bundling and nucleation (Dong et al. 2001a; Andrianantoandro and Pollard 2006; Tholl et al. 2011). Their action is regulated by cytosolic Ca^{2+} (through phosphorylation by CDPKs), pH dynamics and phosphoinositide signalling (Gungabissoon et al. 1998; Smertenko et al. 1998; Allwood et al. 2001, 2002; Tholl et al. 2011; Dong and Hong 2013). As such, it is easy to imagine that ADFs perceive related cytosolic signatures and relay them towards fine-tuning of actin dynamics. The *Arabidopsis* genome encodes at least 11 ADF proteins (Ruzicka et al. 2007). *ADF8* and *ADF11* are specifically transcribed in trichoblast cell files (Brady et al. 2007; Ruzicka et al. 2007; Bruex et al. 2012). However, both genes have not been functionally described in relation to RH tip growth. Overexpression of *AtADF1*, which is expressed in the root epidermis but not specifically in trichoblasts, caused shorter and wider RHs with irregular actin distribution, whereas *adf1* knockout RHs were longer and contained more actin cables (Dong et al. 2001b). These observations are consistent with the function of ADF proteins and indicate that ADF-mediated actin depolymerization is important for RH development. Not *ADF8* and *ADF11* but *ADF7* and *ADF10* are predominantly expressed in growing PTs, and both proteins are associated with the actin fringe and thick actin cables in the PT shank (Ruzicka et al. 2007; Wang et al. 2008; Qin et al. 2009; Bou Daher et al. 2011). ADF7 was shown to regulate AF severing and depolymerization rates in vivo (Zheng et al. 2013).

The actin-interacting protein1 (AIP1) enhances ADF depolymerization activity and has a crucial role in directing RH tip growth (Allwood et al. 2002; Ketelaar et al. 2007, 2004). It caps barbed ends and disassembles ADF-bound filaments (Ono et al. 2004; Okada et al. 2006). Ethanol-induced RNA interference of *AIP1* transcript resulted in drastically shorter RHs and a dramatic decrease of the RH growth rate (Ketelaar et al. 2004). Moreover, consistent with the presumable lack of ADF activity and subsequent absence of actin depolymerization, thick actin bundles reached all the way into the RH apex. Surprising though, the *AIP1* loss-of-function phenotype (short RHs) differs considerably from that observed in *ADF* knockout plants (long RHs; Dong et al. 2001b). This difference might imply an additional role of AIP1 besides stimulation of ADF activity. Ethanol-induced *AIP1* overexpression on the other hand also inhibited RH growth, resulting in short and swollen RHs and thick actin cables (Ketelaar et al. 2007). Interestingly, AIP1 and ADF seem to function in a feedback loop, where AIP1 functioning also depends on ADF presence (Augustine et al. 2011). A recent study showed that AIP1 interacts directly with ACT7 (Kiefer et al. 2015), and together they regulate RH initiation through ROP-mediated planar polarity establishment (reviewed in Balcerowicz et al. 2015), downstream of *WEREWOLF* (*WER*) in an ethylene-dependent manner.

Control of Actin Bundling

The *Arabidopsis* genome encodes 5 *villin-type proteins* (Huang et al. 2005; Khurana et al. 2010), shown to be involved in regulating actin bundling, but also severing, nucleation and plus-end capping (e.g. Yokota et al. 2005; Zhang et al. 2010). As such, villins are considered to be important in regulating actin turnover, and some do so in a Ca^{2+}-dependent manner (Yokota et al. 2005; Zhang et al. 2011a), whereas others are Ca^{2+} insensitive (Khurana et al. 2010). The villin-specific Ca^{2+}-dependency coincides with the presence of at least three Ca^{2+}-binding sites (Huang et al. 2005), and their function regulates tip growth, both in RHs and PTs.

In RHs, double loss of function of *VLN2* and *VLN3* did not result in a RH phenotype (van der Honing et al. 2012). However, GFP-VLN3 was shown to decorate shank-localized F-actin bundles. Loss of function of the RH-specific Ca^{2+}-sensitive *VLN4* that regulates severing and capping, resulted in slowing of RH growth, shorter RHs and alterations of cytoplasmic streaming, due to reduction of actin bundles (Zhang et al. 2011a). Given their apparent redundancy, it would be very interesting to see how RH growth is affected in a triple *vln2 vln3 vln5* knockout mutant.

In PTs VLN2 and VLN5 are thought to act redundantly in regulating actin turnover and construction of actin collars at the PT tip (Zhang et al. 2010; Qu et al. 2013). The two *Lilium* villin homologues P-115-ABP and P-135-ABP regulate Ca^{2+}-dependent AF bundling (Yokota et al. 2005), and P-135-ABP can promote actin turnover. Together, these results identify villins as important regulators of PT and RH growth.

Control of Actin-Mediated Trafficking

Myosins are motor proteins that, by means of ATP hydrolysis, transport vesicles and organelles along AFs (e.g. Reisen and Hanson 2007; Sweeney and Houdusse 2010). Seventeen myosin-class genes are transcribed in *Arabidopsis*, of which 13 belong to the XI-myosin class with an established role in plant development (Hashimoto et al. 2005). Functional characterization of myosin proteins has been challenging due to a high degree of redundancy (Peremyslov et al. 2008, 2010). However, recent advances have provided increasing evidence for their involvement in RH tip growth. The first evidence came from Hashimoto and colleagues, showing that MYOSIN 2 (MYA2) was localized to RHs, appeared as punctate dots (Hashimoto et al. 2005), co-sedimented with F-actin and potentially transported peroxisomes. In addition, MYA2 was suggested to also transport vesicles (Reisen and Hanson 2007; Walter and Holweg 2008). The RH length of *mya2* knockout plants was over 50% decreased compared to wild-type, in parallel with a two times reduction in Golgi, peroxisome and mitochondrial trafficking speeds in RHs (Peremyslov et al. 2008; Avisar et al. 2009). *XI-K* loss of function resulted in the same RH and trafficking defects (Ojangu et al. 2007; Peremyslov et al. 2008; Avisar et al. 2012). *Xi-k* RHs grew slower and seized to grow earlier (Park and Nebenführ 2013). Together with myosin-binding proteins 1 and 2 (MyoB1/MyoB2), XI-K co-localized with yet uncharacterized non-ER-derived endomembrane vesicles along F-actin cables (Peremyslov et al. 2012). Fluorescently tagged XI-K and MyoB1/2 protein exhibited a highly polarized localization in growing RH, which disappeared when growth seized (Peremyslov et al. 2012, 2013). Together, these findings support a role for myosins in regulating fast RH tip growth.

However, at least six myosins are transcribed specifically in RHs, implying that besides MYA2 and XI-K, XI-B/XI-C/XI-D and XI-H might also regulate RH development (Brady et al. 2007). Peremyslov et al. (2010) generated a number of triple and quadruple knockout mutants of XI-family myosins and illustrated that simultaneous loss of function could result in branched, thick or mislocalized RH morphology and a decrease in RH length of up to a factor 10. The fact that RH morphology strongly differed in different knockout combinations suggests that individual myosins serve distinct roles in RH development. Moreover, their results indicate that myosins might also be involved in planar polarity establishment during RH initiation (Peremyslov et al. 2010; reviewed in Balcerowicz et al. 2015).

Also in PTs, at least six XI myosins are expressed (Madison et al. 2015), and PTs of *xi-c xi-e* double-knockout plants grew much slower, resulting in a severely decreased fertilization efficiency. Peroxisome movement and AF organization were disturbed in mutant PTs. Interestingly, a similar myosin-dependent effect on F-actin organization was observed in RHs (Peremyslov et al. 2010; Park and Nebenführ 2013). As such, a feedback loop seems to exist in both RHs and PTs, with F-actin organization regulating myosin functioning, and *vice versa*. The mechanism that controls myosin to actin signalling has not yet been identified.

Finally, cytoplasmic Ca^{2+} might be involved in regulating myosin activity. Myosin XI motility and actin association strongly depend on the Ca^{2+} concentration (Tominaga et al. 2012). At concentrations higher than 1 μM Ca^{2+}, the motility and ATPase activity of a myosin isolated from *Lilium* PTs were strongly decreased (Yokota et al. 1999). Crucially, calmodulin-dependent reversible regulation of myosin motility was observed in the range of 1–10 μM Ca^{2+}, consistent with the $[Ca^{2+}]_{cyt}$ fluctuations observed at the apex of tip-growing cells (Pierson et al. 1996; Wymer et al. 1997; Felle and Hepler 1997). As such, it is tempting to imagine that in tip-growing cells, myosin trafficking could be regulated by the Ca^{2+} signatures present in the apex.

9.5.2 The Microtubule Cytoskeleton

9.5.2.1 Organization of Microtubules in Root Hairs

In RHs, two distinct populations of MTs have been identified. Cortical MTs (CMTs) are present throughout all stages of RH development and, similar to what has been observed in PTs (see Chap. 3), are oriented longitudinally along the shank (Van Bruaene et al. 2004). Studies from several plant species suggest that CMTs, like F-actin, are absent from RH apices (Sieberer et al. 2002; Weerasinghe et al. 2003; Van Bruaene et al. 2004; Vassileva et al. 2005). Endoplasmic MTs (EMTs), which exhibit a more irregular arrangement in comparison to CMTs, form when tip growth begins and accumulate in the subapical region and around the nucleus. EMTs are extremely dynamic and display higher sensitivity to depolymerizing drugs than CMTs (Sieberer et al. 2002; Van Bruaene et al. 2004). As for PTs, the role of MTs in regulating RH development remains poorly understood. It is suggested that EMTs might be involved in positioning of AFs and transport of nucleation complexes from the nucleus; however, their exact role remains unclear (Van Bruaene et al. 2004). Pharmacological analysis revealed that EMTs are required for nuclear positioning within RHs of *Medicago* (Sieberer et al. 2002) but not of *Arabidopsis* (Ketelaar et al. 2002).

9.5.2.2 Disruption of Microtubules Affects Root Hair Morphology

Both pharmacological and genetic studies have shown that MTs play a role in RH development by maintaining the direction of tip growth. Treatment of *Arabidopsis* roots with the MT stabilizing drug taxol and the MT depolymerizing drug oryzalin results in wavy RH growth and occasionally causes formation of multiple tips (Bibikova et al. 1999; Ketelaar et al. 2002). In addition, manipulations of the Ca^{2+} gradient and application of a touch stimulus trigger formation of new growth points in taxol-treated RHs, whereas in the control RHs, this only leads to the transient reorientation of growth (Bibikova et al. 1999). Consistent with the effects

of MT disturbing drugs, crooked and/or branched RH have also been reported for mutants in α-tubulin (Bao et al. 2001), *MICROTUBULE ORGANIZATION 1* (*MOR1*; Whittington et al. 2001) and *ARMADILLO REPEAT-CONTAINING KINESIN 1/MORPHOGENESIS OF ROOT HAIR 2* (*ARK1/MRH2*; Jones et al. 2006; Sakai et al. 2008). *MOR1* encodes a MT-associated protein (MAP) with several HEAT repeats that might serve as MT-binding sites. The point mutation in the N-terminal HEAT motif of *MOR1* leads to temperature-dependent disruption of CMTs indicating that MOR1 plays a role in stabilizing MTs (Whittington et al. 2001). In contrast, ARK1/MRH2 has recently been shown to promote MT disassembly through a yet unknown mechanism. Low concentrations of oryzalin were able to partially restore normal RH growth in *ark1-1* mutants, whereas taxol caused even more severe RH defects. As such, the role of ARK1/MRH2 in RH tip growth seems to be maintaining the pool of free tubulin which is required for rapid MT polymerization (Eng and Wasteneys 2014). However, it is important to note that a previous study suggested that ARK1/MRH2 acts as a MT-stabilizing factor (Yang et al. 2007). Interestingly, in contrast to RHs, taxol and oryzalin do not have a significant effect on PT growth indicating possible differences in the role of the MT cytoskeleton in these two cell types (Gossot and Geitmann 2007; Poulter et al. 2008; Bou Daher and Geitmann 2011; Zhou et al. 2015b).

9.5.2.3 The Microtubule/Actin Interface

There is growing evidence that MTs contribute to tip growth through interaction with the actin cytoskeleton. It has been demonstrated that administration of the actin-disrupting agent cytochalasin B (CytB) causes an inhibition of reverse fountain streaming in *Hydrocharis* RHs and that the effect of the drug is fully reversible (Shimmen et al. 1995). However, simultaneous treatment with CytB and the MT-destabilizing herbicide propyzamide followed by removal of CytB does not lead to recovery of long actin cables and cytoplasmic streaming (Tominaga et al. 1997). It therefore appears that the presence of MTs is essential for re-establishment of AFs. On the other hand, in *Medicago*, longer treatment with the actin-depolymerizing drug LatB leads to formation of MT bundles in the (sub)apical region of RH cells (Timmers et al. 2007). Consistently, in tobacco PTs, long MTs organized as bundles replace the short and randomly oriented ones in the presence of a low dose of LatB (Idilli et al. 2012). Thus, the actin cytoskeleton has an influence on MT dynamics too. In *Arabidopsis* RHs, the cross-talk between MTs and AFs might involve the action of ARK1/MRH2 since an ARM domain-containing fragment of this kinesis has the ability to bind polymerized actin in vitro and *mrh2–mrh3* mutant displays increased sensitivity to LatB (Yang et al. 2007). Moreover, a mutation in *ARK1* enhances the phenotype of CA-ROP2 (Yang et al. 2007), and ROP2 regulates actin organization in RHs (Jones et al. 2002).

9.6 The Cell Wall as a Dynamic System

Much like in PTs, the protoplasm of RHs is surrounded by a dynamic cell wall that consists of cellulose, hemicellulose, pectin and several types of proteins (Somerville et al. 2004). In addition, the basic mechanisms controlling cell wall deposition in RHs are similar to those found in PTs, as well as the resulting biomechanical properties along the RH axis. More so, several cell wall-related genes are transcribed in both RH and PT cells (Becker et al. 2014). Nevertheless, despite these commonalities, the detailed molecular composition of the RH cell wall seems to differ considerably from that of PTs. Here we review the current knowledge on the RH cell wall.

9.6.1 Cellulose Deposition in Root Hair Cell Walls

The main load-bearing network of plant cell walls, cellulose microfibrils tethered by hemicelluloses such as xyloglucan, is partly responsible for the restraining feature in the RH's shank (Cosgrove 2005). Electron microscope experiments revealed that most terrestrial plants have a random and short cellulose microfibril organization in the primary wall deposited at the RH tip (Newcomb 1965; Emons and van Maaren 1987; Akkerman et al. 2012; Rounds and Bezanilla 2013). In the shank of the RH, a second layer of more ordered cellulose microfibrils is deposited at the inside of the random wall, helicoidal or parallel to the RH axis (Akkerman et al. 2012). Addition of the cellulose synthase inhibitor, 2,6-dichlorobenzene (DCB) or cellulose-specific endo-(1→4)-β-glucanase (cellulase) to RHs resulted in rapid cessation of growth and even induced cell rupture at the tip, suggesting that deposition of cellulose or cellulose-like polysaccharides is a necessity for controlled tip growth of RHs (Park et al. 2011).

While most of the cell wall components like pectin, xyloglucan and other hemicelluloses are delivered by secretory vesicles, cellulose is synthesized at the PM by cellulose synthase (CESA) complexes (CSCs; Cosgrove 2005). From the 10 *CESA* genes present in *Arabidopsis* (Doblin et al. 2002), only *CESA1, CESA3* and *CESA6* are involved in primary cellulose synthesis (Arioli et al. 1998; Fagard et al. 2000; Scheible and Pauly 2004). Nevertheless, several lines of evidence suggest that other proteins than these three CESAs are involved in cellulose synthesis at the hair's tip. When the temperature-sensitive mutant *radial swollen 1* (*rsw1*), mutated in the *CESA1* gene, was grown at restrictive temperature, RH morphology was altered, but the tip growth process was not completely abolished (Baskin et al. 1992; Arioli et al. 1998; Williamson et al. 2001), which suggests that at least CESA1 is not essential for tip growth in RHs. Fluorescent fusion proteins of the two other CESAs involved in cellulose synthesis in primary cell walls, CESA3 and CESA6, do not localize to the apical PM of growing RHs, nor to the vesicle-rich zone in the hair apex, which are the sites one expects when these proteins would play

an essential role in cellulose deposition at the tip (Park et al. 2011). In addition, isoxaben, interfering with CESA3 and CESA6, was not able to alter RH elongation. All data so far suggest that CESA1, CESA3 and CESA6 are not required for RH tip growth, but it cannot be ruled out that other CESAs take part in cellulose deposition at the tip. Interestingly, mutants of two members of the cellulose-like super family, *CSLD2* and *CSLD3* (*KOJAK*), produce RHs with a range of abnormalities, with many RHs rupturing late in development (Bernal et al. 2008), or contain RHs that burst prematurely (Favery et al. 2001; Wang et al. 2001), respectively. Additionally, both YFP-tagged proteins are localized to the PM and in the vesicle-rich zone in the expanding tip. The *CSLD* family is the closest one of the *CSLs* to the *CESA* genes, with 35% identity at the amino acid level (Richmond and Somerville 2001), and even though the (1→4)-β-glucan synthase activity of CESA6 can functionally replace the catalytic activity of the CSLD3 domain, it is not clear at the moment whether the products of CSLD3, (1→4)-β-glucan polysaccharides, assemble into cellulose-like microfibrils (Galway et al. 2011; Park et al. 2011).

Most importantly, unlike in RHs where cellulose is the predominant cell wall component, PTs have a lower amount of cellulose but a very high amount of callose in their cell walls (see Chap. 3). In contrast to RHs, PT cellulose synthesis does seem to rely on CESA6 (Chebli et al. 2012). Yet here also the CSLD family plays a role since mutants of *CSLD1* and *CSLD4* show reduced pollen germination ability in vitro (Bernal et al. 2008), although it is not sure whether this is a pre- or post-germination effect.

9.6.2 Xyloglucan

Xyloglucan (XyG), the most abundant form of hemicellulose in dicots, interacts with cellulose microfibrils forming the load-bearing cellulose-xyloglucan network (Carpita and Gibeaut 1993). As it tethers adjacent cellulose microfibrils (Cosgrove 2005), it is expected that alterations in XyG might result in changes in mechanical properties of the cell walls and hence potential growth defects. Pena et al. (2012) reported that a unique acidic XyG is exclusively present in the *Arabidopsis* RH cell wall and that a loss-of-function mutation in *ROOT HAIR SPECIFIC8/XYLOGLUCAN-SPECIFIC GALACTURONOSYLTRANSFERASE1* (*RHS8/XUT1*), catalysing the synthesis of this galacturonic acid-containing XyG, causes a short RH phenotype, indicating the importance of this form of XyG in polarized expansion. Furthermore, Cavalier et al. (2008) have shown that double mutants in xylosyltransferases (*XXTs*), *xxt1/xxt2*, that catalyse the addition of xylosyl to the glucan backbone of XyG lack detectable amounts of XyG and produce short RHs with swollen bases. The single mutant *xxt5* and the triple mutant *xxt1 xxt2 xxt5* have short and swollen RHs as well (Zabotina et al. 2008, 2012), but do not show rupturing tips. Selective degradation of XyG in growing RHs using xyloglucanase leads to the same conclusion that XyG absence results in reduced

RH elongation, but not to rupture as was the case when cellulose was targeted (Park et al. 2011).

Besides altered synthesis, modifications of XyG can also affect cell wall mechanical properties. Xyloglucan endotransglucosylases/hydrolases (XTHs) are cell wall enzymes that cut XyG chains and reform bonds with water (hydrolase activity, XEH) and other xyloglucan acceptor substrates (endotransglycosylase activity, XET), potentially altering the distance between adjacent cellulose microfibrils and hence loosening cell walls (Nishitani and Vissenberg 2007; Van Sandt et al. 2007). High XET activity was indeed found in elongating epidermal cells and RHs at all stages of growth (Vissenberg et al. 2000, 2001, 2003), yet *xth* mutants with a detectable RH phenotype are still absent. Surprisingly, addition of recombinant XTH proteins leads to RH swelling, reduction and even cessation of RH growth (Maris et al. 2009), although one should take care since the XTH proteins are experimentally added from the outside of the wall, while under normal circumstances, they are secreted at the inside and younger part of the cell wall. Expansins are another class of proteins that interact with XyG and break their hydrogen bonds with cellulose at more acidic pH, thereby weakening the wall (Cosgrove 2005). A reduction of *expansin A7* (*EXPA7*) expression resulted in shorter RHs (Lin et al. 2011a), confirming that expansin A7 likely performs active cell wall modification activities during the elongation of the hair tip.

In PTs, fucosylated XyG epitopes were uniform all along the cell wall of straight growing PT, with a slightly lower abundance at the tip compared with the distal region (Chebli et al. 2012). At the moment, no effects of XyG absence are described on PT and pollen development.

Taken together, XyG plays a role in the growth of RHs, but its absence does not lead to cell rupture, suggesting that cellulose is not only connected to XyG. Indeed, Dick-Pérez et al. (2011) demonstrated interactions between pectin and cellulose microfibrils, and both pectic polysaccharides and xylans seem to have an enhanced role in the walls from the *xxt1 xxt2* double mutant that lacks detectable XyG (Park and Cosgrove 2012).

9.6.3 The Pectin Matrix

The above findings demonstrate that next to the cellulose/XyG load-bearing network, other cell wall components such as pectin, forming a highly hydrated matrix for the cellulose-xyloglucan framework, greatly contribute to RH growth. Enzymatic degradation of pectin using pectate lyase indeed inhibited RH elongation, but did not induce RH rupture (Park et al. 2011). In *Arabidopsis*, mutations in *UDP-4-KETO-6-DEOXY-D-GLUCOSE-3,5-EPIMERASE-4-REDUCTASE 1* (*UER1*) and *UDP-D-GLUCURONATE 4-EPIMERASE 6* (*GAE6*), both enzymes involved in the synthesis of pectic substrates, cause a short RH phenotype that can be rescued by applying exogenous precursors to the growth media (Pang et al. 2010). Furthermore, plants lacking LRR-extensin1 (LRX1) are defective in RH cell wall formation and

produce short, collapsed and branched RHs. A suppressor screen has revealed that the *lrx1* phenotype can be reversed by mutation in *RHAMNOSE BIOSYNTHESIS1* (*RHM1*). Since rhamnose is a major component of pectin and the *rhm1* mutation changes the expression of other cell wall-related genes, the suppression of the *lrx1* phenotype is likely due to changes in cell wall composition (Diet et al. 2006). In addition, the pectin cell wall dynamics in tip-growing cells has been analysed using propidium iodide. In RHs and PT the fluorescence intensity of apical propidium iodide shows an oscillatory pattern in which each peak precedes growth rate oscillations. However, the distribution of fluorescence in both cell types is distinct, with RHs exhibiting predominant labelling along the shanks, whereas in PT this is clearly seen at the apex (Rounds et al. 2011). These results are consistent with previous findings that the tip region of growing PTs is mainly composed of pectins, especially homogalacturonan (HG). HGs are deposited in a highly methylesterified form at the apex through exocytosis and they become demethylated by pectin methylesterase (PME) activity (see Sect. 9.3.5). Spatial and temporal regulation of PME activity depends strongly on PMEIs, the Ca^{2+} concentration in the cell wall and local pH differences. Inactivation of a pollen-specific PME, PPME1, resulted in curved, irregularly shaped PTs that were dramatically stunted because of reduced elongation rates (Tian et al. 2006). Similar unstable and retarded PT growth with occasional tube bursting was seen in mutants of *VANGUARD1* (*VGD1*), another pollen-expressed PME (Jiang et al. 2005). Surprisingly, at the moment, evidence for the involvement of PMEs in regulating RH growth is largely lacking.

9.6.4 Control by Second Messengers and Cell Wall Sensing/Modifying Proteins

The presence and correct assembly of extensins (EXTs), cell wall structural proteins, is crucial in the development of RHs. As mentioned before, LRX1 needs to interact with the RH cell wall, and this depends on the oxidative cross-linking of tyrosine (Tyr) residues in its EXT domain (Baumberger et al. 2001; Ringli 2010). Absence of this interaction results in impaired RH development. It is known that during plant defence reactions, EXT cross-linking can be mediated by peroxidases, and they have also been predicted to form the required cross-links with EXTs during RH formation (Almagro et al. 2009). Furthermore, cell wall self-assembly of EXTs requires correct O-glycosylation, and this is needed for normal RH elongation (Velasquez et al. 2011; Boron et al. 2014). Localization and interference with degrading enzymes indicate that EXTs are present in the PT wall and that they are required for normal PT elongation as well (García-Hernández and Cassab López 2005; Zhang et al. 2014).

The abundance and arrangements of apoplastic components determine the cell wall mechanical properties at the RH and PT tip, yet this can be changed by the activity of several other actors. Ca^{2+} can interact with demethylesterified pectin to

stiffen the wall, and local changes in apoplastic pH can change the activity of cell wall remodelling enzymes and proteins (Cosgrove 2005; Maris et al. 2009, 2011). Furthermore, ROS seem to play dual roles, as hydroxyl radicals can cleave cell wall polymers, thereby loosening the wall, whereas hydrogen peroxide can cause the opposite by helping peroxidases to cross-link certain cell wall components. On top of that, the complexity rises as certain cell wall defects in RHs and PT might not be the direct cause of reduced or ceased elongation, since certain RLK family members can serve as cell wall integrity sensors (Guo et al. 2009; Wolf et al. 2012), conferring information from the cell wall to the inside of the cell, regulating, for example, ROS production through ROPs (Duan et al. 2010), which affects the elongation of tip-growing cells.

9.7 Conclusion and Perspective

The number of identified players in the molecular regulation of RH tip growth is increasing rapidly, but their specific interactions and the mechanisms that integrate internal and environmental signals to fine-tune RH growth remain far from clear. Comparing the more comprehensive knowledge on PTs with that of RH tip growth, we identified many possible targets for future RH research. However, many challenges remain to be addressed in order to better understand the vast complexity and integrated nature that lie at the basis of RH tip growth and tip growth in general. A problem of particular concern regarding the study of RH development lies within the fact that RH cells are an integral part of the total root system. Unlike for PTs, it is currently very challenging to acquire a pure or undiluted RH cell extract without employing harsh techniques such as protoplast isolation. Consecutively, high throughput -omics techniques which cannot be performed on single cells have been left unexploited, despite the fact that they could provide enormous insight and identify several new targets in studying RH tip growth. Similarly, the cell wall has proven to be a very complex, dynamic and difficult structure to probe. Most importantly, however, the outcome of intracellular-extracellular signalling pathways is the targeted secretion, synthesis and modification of new cell wall material. Despite its importance, very little is known about tip-related cell wall dynamics. Plants have been shown to contain a diverse set of putative cell wall sensing proteins (CrRLK1Ls), which likely perceive specific information on cell wall composition and subsequently relay the latter towards the cell wall-controlling tip growth machinery. How do these proteins sense cell wall composition, and is RH growth disturbance/cessation in cell wall mutants a direct consequence of aberrant cell wall structure or is it the result of CrRLK1L-mediated signal transduction?

In addition, we now know that cell wall dynamics largely depend on extracellular changes in ion and ROS concentrations. However, it is most likely that highly localized ion transport and ROS generation lead to the existence of micro domains in which cell wall modification strongly differs from the surrounding environment, much like the regulatory micro domains observed at the surface of membranes. The

fluxes leading to these extra- as well as intracellular ion accumulation/depletion dynamics have been experimentally observed in several independent experiments. Based on their characteristics, they are thought to relate to a vast array of ion-transporting transmembrane proteins localized either to the tip or the shank of growing RHs. The identification of the loci corresponding to plasma membrane and endomembrane-localized transporters is however still largely lacking. More so, whereas different ion oscillations have been shown to influence one another, the way in which they are interconnected is poorly understood. For instance, it is unclear how Ca^{2+}, ROS and pH dynamics interact to control RH elongation.

Furthermore, the signals encoded by oscillatory ion signatures are mostly unknown. Why do ion concentrations oscillate, and how do these oscillations translate into oscillatory growth rates? How is the oscillatory nature of ion transport regulated? Recent research efforts are gradually providing insight into these processes. However, the possible involvement of Ca^{2+} and pH-sensitive proteins, which interpret and relay the ion-specific signal towards downstream targets, has been largely neglected. Feedback loops involving several of these proteins are likely embedded throughout the signalling cascade leading up to successful RH and PT growth. Importantly, ROP GTPases are now thought to function as signalling hubs, 'sensing' and integrating the key molecular RH growth machinery mentioned above. But what are the direct and downstream targets of these proteins, and how is their signal relayed to govern tip growth?

ROP GTPases have been shown to regulate cytoskeleton dynamics, but despite the well-established role of the actin cytoskeleton, the role of microtubules in both RH and PT elongation is still dubious. Finally, the cytoskeleton has a pivotal role in the establishment of a highly organized subcellular organization in tip-growing cells. However, the role of individual subcellular compartments and the way in which they might interact to regulate RH elongation are poorly understood.

It is clear that many challenges lie ahead to better understand RH development. Here we have identified many interesting targets for future research. Studying the molecular pathways governing tip growth and integrating them into a network that leads up to successful RH elongation will have to go hand in hand with the development of new techniques which will allow us to probe the complexity of RH cells and their building blocks. Many questions remain open, yet combined efforts on PTs and RHs will lead to exciting discoveries and a better understanding of the tip growth process.

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Part V
Tipomics: Omic Approaches in Tip Growth

Chapter 10
When Simple Meets Complex: Pollen and the -Omics

Jan Fíla, Lenka Záveská Drábková, Antónia Gibalová, and David Honys

Abstract Pollen, an extremely reduced bi-cellular or tri-cellular male reproductive structure of flowering plants, serves as a model for numerous studies covering a wide range of developmental and physiological processes. The pollen development and subsequent progamic phase represent two fragile and vital phases of plant ontogenesis, and pollen was among the first singular plant tissues thoroughly characterised at the transcriptomic level. Here we present an overview of high-throughput tools applied in pollen research on numerous plant species. Transcriptomics, being the first experimental approach used, has provided and continues providing valuable information about global and specific gene expression and its dynamics. However, the proteome does not fully reflect the transcriptome, namely, because post-transcriptional regulatory levels, especially translation, mRNA storage and protein modifications, are active during male gametophyte development and during progamic phase. Transcriptomics therefore should be complemented by other -omic tools to get more realistic insight, most importantly proteomics and other specialised approaches mapping the involvement of regulatory RNAs and protein post-translational modifications as well as experiments designed to identify the subsets of total -omes like translatome, secretome or allergome.

Keywords Pollen development • Gene expression • Regulation • -Omics • Transcriptome • Proteome

Abbreviations

2-D DIGE	two dimensional fluorescence difference gel electrophoresis
2-DE	two dimensional gel electrophoresis
bHLH	basic helix-loop-helix transcription factor

Jan Fíla and Lenka Záveská Drábková contributed equally to this work.

J. Fíla • L. Záveská Drábková • A. Gibalová • D. Honys (✉)
Laboratory of Pollen Biology, Institute of Experimental Botany, Academy of Sciences of the Czech Republic, Rozvojová 263, 165 02 Praha 6, Czech Republic
e-mail: david@ueb.cas.cz

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bZIP TF	basic leucine zipper transcription factor
cAMP	cyclic adenosine monophosphate
CAGE	cap analysis of gene expression
cGMP	cyclic guanosine monophosphate
DEFL protein	defensin-like family protein
EAR motif	ethylene-responsive element binding factor-associated amphiphilic repression motif
EPP	EDTA/puromycin-resistant particle
GO	gene ontology
IMAC	immobilized metal affinity chromatography
LC–MS/MS	liquid chromatography–tandem mass spectrometry
MADS-box TF	family of transcription factors containing conserved MADS DNA-binding domain
MALDI–TOF/TOF	matrix-assisted laser desorption/ionization–time-of-flight tandem mass spectrometry
MIKC* type proteins	subfamily of MADS-box proteins with conserved domain structure, where the MADS (M) domain is followed by Intervening (I), Keratin-like (K) and C-terminal domains
MOAC	metal oxide/hydroxide affinity chromatography
MPSS	massively parallel signature sequencing
mRNP	messenger ribonucleoprotein particle
MYB family proteins	transcription factor protein family characterised by the presence of MYB (myeloblastosis) DNA-binding domain
PKA	cAMP-dependent protein kinase
PKC	protein kinase C
PKG	cGMP-dependent protein kinase
R2R3-MYB	MYB-protein subfamily characterised by the R2R3-type MYB domain
RNAseq	RNA deep sequencing technologies
RPM	reads per million
SAGE	serial analysis of gene expression
SIMAC	sequential elution from IMAC
TCTP	translationally controlled tumour protein
TF	transcription factor

10.1 Introduction

Reproduction is one of the most important processes performed by living organisms on Earth, which enables the survival of species. Plant reproduction has several important aspects, some of which differ from animal reproduction. In plants (Archaeplastida), there are two altering generations that differ in their ploidy. The diploid sporophyte produces haploid spores by meiosis, and the spores give rise to haploid gametophyte, in which gametes are produced by mitosis (unlike animals,

gametes of which are formed by meiosis). Two haploid gametes fuse together, forming a diploid zygote, from which a new sporophyte generation develops.

The ancestors of higher plants, Streptophyta, spent a vast majority of their lives as haploid gametophytes (reviewed in Qiu et al. 2012). Their zygote that was formed by two fusing gametes immediately underwent meiosis and gave rise to four haploid spores. In their descendants, meiosis was delayed, and a multicellular diploid generation was formed by several rounds of zygote mitosis. During subsequent evolution, one of the generations tended to be reduced. In bryophytes (Bryophyta *sensu lato*)—i.e. mosses, hornworts and liverworts—gametophyte dominates, and sporophyte is dependent on it. On the other hand, sporophyte is dominant in ferns (Monilophyta) and lycophytes (Lycopodiophyta), but they still form gametophytes as an independent generation. Later, in gymnosperms (Gymnospermae) and angiosperms (Angiospermae), gametophytes were notably reduced. In gymnosperms, the male gametophyte contains 4–40 cells, whilst the female gametophyte comprises several thousands of cells. Angiosperm gametophytes are even more reduced, having male gametophyte composed of 2–3 cells, whereas female gametophyte comprises typically 7 cells with 8 nuclei (although there exist several other alternative arrangements with different number of cells in the embryo sac; Reiser and Fischer 1993).

The mature angiosperm plants belong to diploid sporophyte generation. Angiosperms produce spores, gametophytes and gametes of separate sexes—male and female. The male gametophyte is formed inside the anthers of a flower; two initials differentiate from the sporophytic tissue in the anther—tapetal initial (which gives rise to tapetum) and pollen mother cell (microsporocyte; see McCormick 1993). A pollen mother cell divides into four microsporocytes by meiosis. The microspore tetrad is first connected by callose, which is later digested by the activity of callases (enzymes digesting callose) released by the tapetum. The freed microspores increase their size and vacuolise, and their nuclei migrate to the periphery (McCormick 1993; Borg and Twell 2010). The microspores then undergo highly asymmetric pollen mitosis I (PMI) leading to the production of a large vegetative cell and a small generative cell. The generative cell, the origin of male germline, is then engulfed by the vegetative cell and migrates into its cytoplasm (Berger and Twell 2011). The asymmetry of PMI is of key importance, which was proven by the serious phenotypic defects of *Arabidopsis thaliana* mutants *gemini pollen 1* (Park et al. 1998) or *two-in-one* (Oh et al. 2005). The generative cell undergoes one more round of cell division, pollen mitosis II (PMII), which occurs either before mature pollen is discharged or afterwards. Two sperm cells are formed from a generative cell by PMII. Consequently, the mature pollen can be shed as bi-cellular or tri-cellular (Brewbaker 1967).

Initially, the ancestral state of angiosperm pollen grains was inferred as bi-cellular, because all ancient woody Magnoliales shed this pollen type. Moreover, this bi-cellular pollen is phylogenetically widespread, and it was believed that tri-cellular pollen was restricted to aquatics, grasses and some herbs (Elfving 1879; Strasburger 1884). More recent investigations revealed that pollen tri-cellularity is not irreversible and that tri-cellular lineages diversify slowly and sometimes

reverse to bi-cellular lineages. This reflects a linkage between the evolution of sporophyte lifestyle and developmental lability of male gametophyte (Williams et al. 2014a). The ancient aquatic plant *Ceratophyllum* and several monocot lineages, such as Araceae, Alismataceae and Nymphaeaceae, support the tri-cellular ancestry of pollen, which in this case represents a selective advantage over bi-cellular pollen. Williams et al. (2014a) proposed that bi-cellular pollen evolved secondarily from tri-cellular ancestors during shifts away from rapid life cycle or from limited reproduction. In total, thirteen orders of angiosperms shed prevalently tri-cellular pollen (Fig. 10.1). In the orders with bi-cellular pollen, about 2–44% species produce also tri-cellular pollen.

The simultaneous presence of both pollen types in one species is very uncommon. For instance, the coexistence of bi- and tri-cellular pollen grains at the same time is in early-divergent angiosperm *Annona cherimola* (Magnoliales, Annonaceae). There, the production of the actual pollen type depends on environmental factors such as temperature and humidity during the pollen maturation (Lora et al. 2009).

Upon reaching the stylar papillary cells, pollen grain rehydration and activation occurs (Vogler et al. 2015). Later on, pollen tube growth through the female connective tissues starts processes, which are accompanied by reciprocal communication of pollen tube and pistil tissues (Hafidh et al. 2014, 2016b; Higashiyama 2015, see also Chap. 8). The pollen tube delivers two sperm cells (male haploid gametes) to the embryo sac. One sperm cell fuses with the egg cell (female haploid gamete) giving rise to a diploid zygote, which represents a start of a novel diploid sporophyte generation. The zygote subsequently undergoes several rounds of mitotic divisions giving rise to embryo and a new plant. The second sperm cell fuses with the central nucleus of the embryo sac, giving rise to the triploid endosperm. The double fertilisation is typical in angiosperms and was reviewed in more detail by Raghavan (2003).

In this chapter, we will discuss how various -omic techniques notably broadened the wealth of information about male gametophyte development. There were over hundred -omic studies performed on male gametophyte that were published so far (Table 10.1). Of these studies, transcriptomics represented the dominant experimental approach with 51% of all -omics experiments followed by proteomics (26%). The remaining 23% of experiments were shared by the identification and analyses of translatome, miRNAome, methylome, phosphoproteome, allergome, secretome and metabolome. Phylogenetically, most main orders (altogether 15) representing all major groups of seed plants are covered but to a different extent according to the distribution of model species. Therefore, the majority of information (75% experiments) was gathered on four well-distributed orders, Poales (Monocots, Commelinids, key models *Oryza sativa* and *Zea mays*), Brassicales (Rosids, key model species *Arabidopsis thaliana*), Solanales (Asterids, key models *Nicotiana tabacum* and *Solanum lycopersicum*) and Liliales (Monocots, key model *Lilium longiflorum*). Moreover, these model species were subjected to a combination of several -omic approaches, which enabled the mutual comparison of various -omic datasets of different origins.

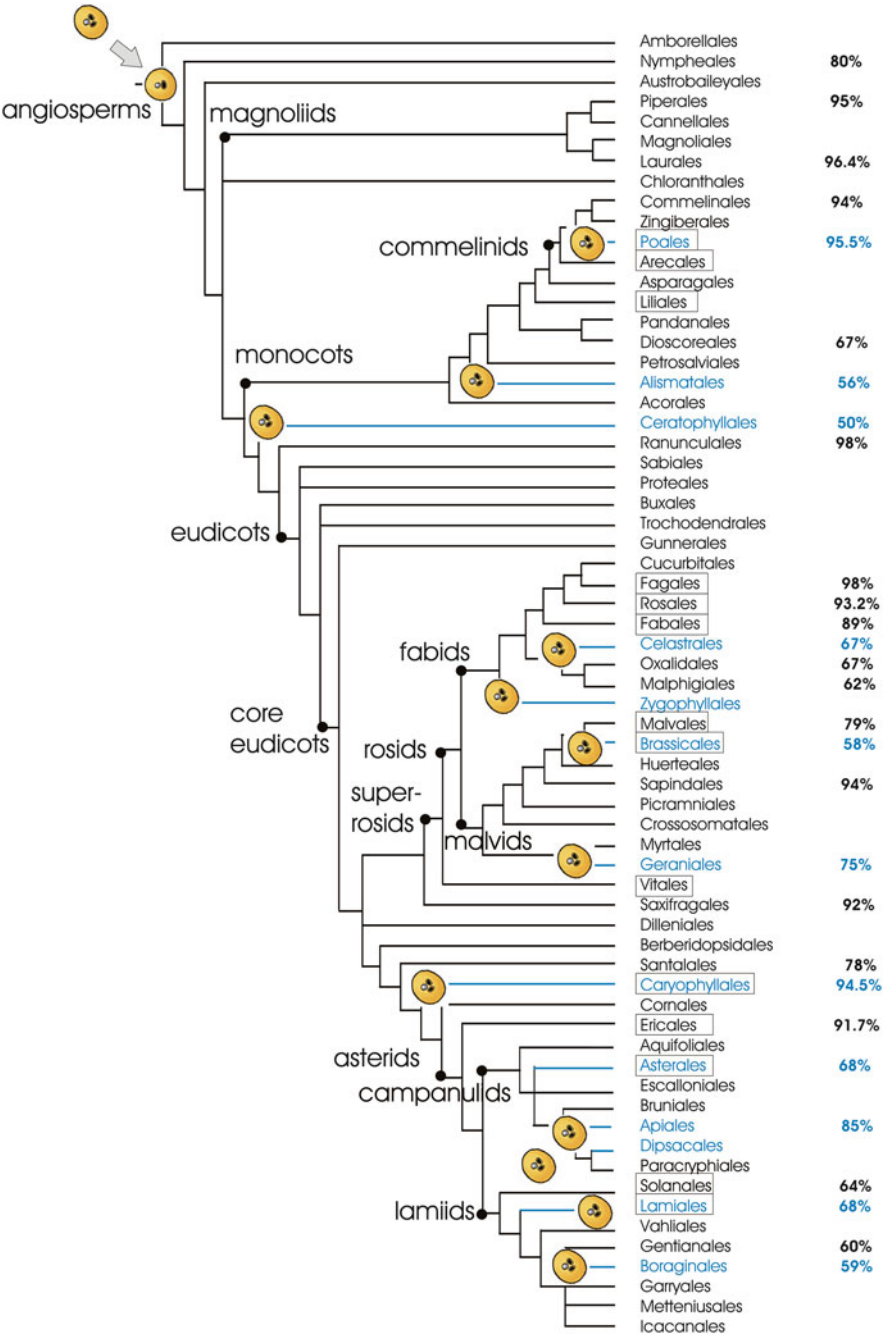

Fig. 10.1 Distribution of tri-cellular and bi-cellular pollen within angiosperms. Interrelationships are based on APG IV (APG IV, 2016). Percentages summarise all taxa with tree species matches from Williams et al. (2014a). Tri-cellular pollen is dominant in *blue-coloured* orders. *Boxes* indicate that some of the omics is already done for the order

Table 10.1 Summary of published male gametophyte -omics studies

Order	Experiments	T	TL	miR	MT	P	PP	A	S	M
Pinales	8	2		1		4	1			
Liliales	10	5				4				1
Arecales	1							1		
Poales	33	18		3		6	1	5		
Vitales	1	1								
Fabales	1	1								
Rosales	3	1				1		1		
Fagales	2					2				
Malvales	1	1								
Brassicales	34	20	1	3	2	7	1			
Funariales	1	1								
Ericales	2	1				1				
Lamiales	4	2						2		
Solanales	16	7				6	2		1	
Asterales	7	3				1		3		
Summary	124	63	1	7	2	32	5	12	1	1

The numbers show the number of performed experiments in the respective category. The plant species are divided into gymnosperms (Pinales) and angiosperms (all other orders). *T* transcriptome; *TL* translatome; *miR* miRNAome; *MT* methylome; *P* proteome; *PP* phosphoproteome; *A* allergome; *S* secretome; *M* metabolome

10.2 Transcriptomics

Transcriptomics was the first -omics technique applied to the male gametophyte (Becker et al. 2003; Honys and Twell 2003; Lee and Lee 2003) and so far transcriptomic profiles of at least one male gametophyte developmental stage were published for 22 seed plant species, 21 of which were angiosperms (Table 10.2).

Transcriptomic studies underwent development from sequencing of cDNA or EST libraries through serial analysis of gene expression (SAGE) and microarray-based studies to the most recent deep sequencing technologies (RNAseq). Sanger sequencing of cDNA or EST libraries is relatively low-throughput method, expensive and generally not quantitative. To overcome these limitations, tag-based methods were developed, including SAGE, cap analysis of gene expression (CAGE) and massively parallel signature sequencing (MPSS). These approaches are high throughput and measure precise gene expression levels. However, most of them are based on Sanger sequencing, and a significant portion of the short tags cannot be uniquely mapped to the reference genome. Moreover, only a portion of the transcripts can be analysed, and gene isoforms are generally indistinguishable from each other. These disadvantages limited the application of traditional sequencing technology in annotating the structure of transcriptomes (Wang et al. 2009). DNA microarrays started to appear during the late 1990s; however the first article was

Table 10.2 Overview of published male gametophyte -omics studies. Analysed species are put in the appropriate orders

Order	Species	-celled	no. exp.	Technique	Developmental stage												Fraction						References
					MEI	TET	UNM	BCP	TCP/lBCP	MPG	GP	PT	SIV-PT	IVPT	GC	SC	VC	TOT	NUC	APO	MEM	EPP	CGR	
Transcriptome																							
Pinales	*Cryptomeria japonica*	–	2	EST-seq/ microarray						X								X						Futamura et al. (2006), Tsubomura et al. (2016)
Liliales	*Lilium longiflorum*	2	5	EST-seq/ microarray/ RNAseq			X			X	X	X		X	X			X						Huang et al. (2011), Lang et al. (2015), Obermeyer et al. (2013), Okada et al. (2006, 2007)
Poales	*Oryza sativa*	3	8	Microarray/ RNAseq	X	X	X	X	X	X	X					X	X	X						Anderson et al. (2013), Aya et al. (2011), Hirano et al. (2008), Hobo et al. (2008), Luo et al. (2011), Peng et al. (2012), Russell et al. (2008, 2012), Shen et al. (2011), Suwabe et al. (2008), Tang et al. (2010), Wei et al. (2010), Wu et al. (2014)

(continued)

Table 10.2 (continued)

Order	Species	-celled	no. exp.	Technique	Developmental stage													Fraction						References
					MEI	TET	UNM	BCP	TCP/IBCP	MPG	GP	PT	SIV-PT	IVPT	GC	SC	VC	TOT	NUC	APO	MEM	EPP	CGR	
	Zea mays	3	7	EST-seq/ microarray/ RNAseq	X					X						X		X						Chettoor et al. (2014), Davidson et al. (2011), Dukowic-Schulze et al. (2014), Engel et al. (2003), Ma et al. (2008), Zhang et al. (2014)
	Triticale	3	1	Microarray						X								X						Tran et al. (2013)
	Sorghum halepense	3	1	RNAseq						X								X						Campbell et al. (2015)
	Phleum pratense	3	1	RNAseq						X								X						Schulten et al. (2013)
Vitales	*Vitis vinifera*	2	1	Microarray						X								X						Fasoli et al. (2012)
Fabales	*Glycine max*	2	1	Microarray						X								X						Haerizadeh et al. (2009)
Rosales	*Fragaria vesca*	3	1	RNAseq			X			X								X						Hollender et al. (2014)
Malvales	*Pyrus bretschneideri*	2	1	RNAseq						X	X	X						X						Zhou et al. (2016)

Brassicales	*Arabidopsis thaliana*	3	19	SAGE/microarray/ RNAseq	X		X	X	X	X	X	X	X			X		X					Adamczyk and Fernandez (2009), Becker et al. (2003, 2014), Borges et al. (2008), Chen et al. (2010), Costa et al. (2013), Gibalová et al. (2009), Honys and Twell (2003, 2004), Lee and Lee (2003), Lin et al. (2014), Loraine et al. (2013), Miyazaki et al. (2009), Ohr et al. (2007), Pina et al. (2005), Qin et al. (2009), Schmid et al. (2005), Verelst et al. (2007a, b), Wang et al. (2008)
	Brassica napus	3	1	EST-seq			X			X								X					Whittle et al. (2010)
Caryo-phyllales	*Plumbago zeylanica*	3	1	Microarray												X	X	X					Gou et al. (2009)
Ericales	*Camellia sinensis*	2	1	RNAseq								X						X					Wang et al. (2016)
Solanales	*Nicotiana tabacum*	2	2	EST-seq/ microarray			X	X	X	X		X				X		X					Bokvaj et al. (2015), Hafidh et al. (2012a, b), Xin et al. (2011)
	Solanum lycoper-sicum	2	2	Microarray/ RNAseq						X								X					Frank et al. (2009), Loraine et al. (2015)

(continued)

Table 10.2 (continued)

Order	Species	-celled	no. exp.	Technique	Developmental stage													Fraction						References
					MEI	TET	UNM	BCP	TCP/lBCP	MPG	GP	PT	SIV-PT	IVPT	GC	SC	VC	TOT	NUC	APO	MEM	EPP	CGR	
	Solanum tuberosum	2	1	RNAseq						X								X						Sanetomo and Hosaka (2013)
	Solanum demissum	?	1	RNAseq						X								X						Sanetomo and Hosaka (2013)
	Petunia inflata	2	1	RNAseq						X								X						Williams et al. (2014b)
Lamiales	*Olea europaea*	2	2	RNAseq						X		X		X				X						Carmona et al. (2015), Iaria et al. (2016)
Asterales	*Ambrosia artemisiifolia*	3	3	RNAseq						X								X						Bordas-Le Floch et al. (2015), El Kelish et al. (2014), Kanter et al. (2013)
Translatome																								
Brassicales	*Arabidopsis thaliana*	3	1	Microarray						X		X		X				X						Lin et al. (2014)
microRNAome																								
Pinales	*Pinus taeda*	–	1	Microarray						X	X							X						Quinn et al. (2014)
Poales	*Oryza sativa*	3	2	RNAseq			X	X	X									X						Peng et al. (2012), Wei et al. (2011)
	Zea mays	3	1	RNAseq						X	X							X						Li et al. (2013)
Brassicales	*Arabidopsis thaliana*	3	3	RNAseq						X						X		X						Chambers and Shuai (2009), Grant-Downton et al. (2009b), Slotkin et al. (2009), Borges et al. (2011)
Methylome																								
Brassicales	*Arabidopsis thaliana*	3	2	RNAseq			X	X		X														Calarco et al. (2012), Ibarra et al. (2012)

Proteome																							
Pinales	*Pinus strobus*	–	1	Gel based						X		X						X						Fernando (2005)
	Pinus occidentalis	–	1	Gel based						X								X						Rodriguez de Francisco et al. (2016)
	Picea meyeri	–	1	Gel based								X						X						Chen et al. (2009)
	Picea wilsonii	–	1	Gel-free						X								X						Chen et al. (2012)
Liliales	*Lilium longiflorum*	2	1	Gel-free						X		X						X			X			Pertl et al. (2009)
	Lilium davidii	2	3	Gel based/gel-free						X		X			X	X	X	X	X		X			Han et al. (2010), Yang et al. (2016), Zhao et al. (2013)
Poales	*Oryza sativa*	3	3	Gel based/gel-free						X	X					X		X		X				Abiko et al. (2013), Dai et al. (2006, 2007)
	Zea mays	3	2	Gel-free						X								X						Chao et al. (2016), Zhang et al. (2012)
	Sorghum halepense	3	1	Gel based						X								X						Campbell et al. (2015)
Rosales	*Parietaria judaica*	2	1	Gel based						X								X						Barranca et al. (2010)
Fagales	*Betula pendula*	3	1	Gel-free						X								X						Bryce et al. (2010)
	Quercus ilex	2	1	Gel based						X								X						Valero Galvan et al. (2012)
Brassicales	*Arabidopsis thaliana*	3	6	Gel based/gel-free						X	X	X						X		X				Ge et al. (2011), Grobei et al. (2009), Holmes-Davis et al. (2005), Noir et al. (2005), Sheoran et al. (2006), Zou et al. (2009)
	Brassica napus	3	1	Gel based						X	X							X						Sheoran et al. (2009a)

(continued)

Table 10.2 (continued)

Order	Species	-celled	no. exp.	Technique	Developmental stage													Fraction						References
					MEI	TET	UNM	BCP	TCP/IBCP	MPG	GP	PT	SIV-PT	IVPT	GC	SC	VC	TOT	NUC	APO	MEM	EPP	CGR	
Ericales	*Camellia sinensis*	2	1	Gel based						X								X						Li et al. (2008)
Solanales	*Nicotiana tabacum*	2	2	Gel based/gel-free	X	X	X	X		X		X						X				X		Honys et al. (2009), Ischebeck et al. (2014)
	Solanum lycopersicum	2	4	Gel based/gel-free	X	X	X			X								X			X			Chaturvedi et al. (2013), Paul et al. (2016), Sheoran et al. (2007, 2009b)
Asterales	*Helianthus annuus*	3	1	Gel-free						X								X						Ghosh et al. (2015)
Phosphoproteome																								
Pinales	*Picea wilsonii*	–	1	TiO_2–gel-free						X								X						Chen et al. (2012)
Poales	*Zea mays*	3	1	IMAC–gel-free						X								X						Chao et al. (2016)
Brassicales	*Arabidopsis thaliana*	3	1	IMAC/TiO_2-gel-free						X								X						Mayank et al. (2012)
Solanales	*Nicotiana tabacum*	2	2	TiO_2–gel-free						X	X							X						Fíla et al. (2012, 2016)
Allergome																								
Arecales	*Cocos nucifera*	2	1	Gel based						X								X						Saha et al. (2015)
Poales	*Sorghum halepense*	3	1	Gel based						X								X						Campbell et al. (2015)
	Phleum pratense	3	3	Gel based						X								X					X	Abou Chakra et al. (2012), Schmidt et al. (2010), Schulten et al. (2013)

	Cynodon dactylon	3	1	Gel based						X								X						Kao et al. (2005)
Rosales	*Parietaria judaica*	2	1	Gel based						X								X						Barranca et al. (2010)
Lamiales	*Ligustrum lucidum*	2	1	Gel based						X								X						Mani et al. (2015)
	Plantago lanceolata	3	1	Gel based						X								X						Sousa et al. (2014)
Asterales	*Ambrosia artemisiifolia*	3	1	Gel based						X								X						Zhao et al. (2016)
	Ambrosia psilostachya	3	1	Gel-free						X								X						Bordas-Le Floch et al. (2015)
	Helianthus annuus	3	1	Gel based						X								X						Ghosh et al. (2015)
Secretome																								
Solanales	*Nicotiana tabacum*	2	1	Gel-free									X	X				X						Hafidh et al. (2016b)
Metabolome																								
Liliales	*Lilium longiflorum*	2	1	GC-MS						X	X	X						X						Obermeyer et al. (2013)

The 'Exp' column shows the number of performed experiments in the respective category. Tissue/developmental stage: *MEI* meiocytes; *TET* tetrads; *UNM* uninucleate microspores; *BCP* bi-cellular pollen; *lBCP* late bi-cellular pollen; *TCP* tri-cellular pollen; *MPG* mature pollen grain; *GP* germinating pollen; *PT* pollen tubes; *SIV-PT* semi-in vivo-cultivated pollen tubes; *IVPT* in vivo grown pollen tubes; *GC* generative cell; *SC* spermatic cells; *VC* vegetative cell. *F* fraction specification: *TOT* total RNA/proteins/metabolites; *NUC* nuclear proteome; *APO* apoplastic proteins; *MEM* membrane proteins; *EPP* EDTA/puromycin-resistant particles; *CGR* cytoplasmic granules

published by Schena et al. (1995). Gene chip technique brought the quantum leap in gene expression studies and became standard because of its well-established sample preparation and data analysis protocols, rapid turnaround time, wealth of archived data and data-mining methodologies. However, the use of expression microarrays is limited, as they require fabrication, and alongside their rigidity, they also depend on prior knowledge of genes and gene sequences (Loraine et al. 2013). Recently, the development of novel DNA deep sequencing technologies such as RNAseq has started to be a dominant technique, mainly because it requires only a little a priori knowledge of the genome, and therefore it enables transcriptome studies in non-model plant species. It allows both mapping and quantifying transcriptomes.

The majority of male gametophytic transcriptomics studies are still based on microarray analyses representing 33 experiments in 11 species (Table 10.2). Affymetrix has been the most commonly used platform, but a significant share of experiments used alternative Agilent (*Zea mays*, *Nicotiana tabacum*) and Roche NimbleGen (*Cryptomeria japonica*, *Arabidopsis thaliana*, *Vitis vinifera*) platforms. However, the number of model systems investigated by RNAseq is boosting; it currently reached 23 experiments in 15 species. Since all transcriptomic studies employing RNAseq were published in last few years, they are responsible for the recent massive increase in plant species with analysed pollen transcriptomes, especially among models, genomes of which have not been sequenced and annotated yet. For example, Rutley and Twell (2015) mentioned in their review only 10 angiosperm species. There is a limited overlap of species with male gametophyte being analysed on both platforms—microarrays and RNAseq. There were only five key models—*Arabidopsis thaliana*, *Oryza sativa*, *Zea mays*, *Solanum lycopersicum* and *Lilium longiflorum* (Table 10.2). Of them, four provided sufficient wealth of information, because the microarray transcriptome profiling of *L. longiflorum* pollen was achieved on a custom cDNA microarray. However, such overlap was sufficient for the comparisons of both platforms. Not surprisingly, RNAseq enabled the identification of larger transcriptome fraction mainly due to the absence of probes for numerous genes on microarrays and as a result of continuous refined genome annotations leading to further reduction of reliable gene models. However, such increase of the number of identified genes was not dramatic. The number of genes expressed in *Arabidopsis* mature pollen was calculated 6044 (Rutley and Twell 2015) as an average value from the range of 3954–7235 genes published in original mature pollen microarray-based datasets (Borges et al. 2008; Honys and Twell 2004; Pina et al. 2005; Qin et al. 2009; Schmid et al. 2005; Wang et al. 2008). On the contrary, of the 5525 annotated protein-coding loci that have no corresponding probe set on the Affymetrix ATH1 microarray, 451 genes were identified as expressed in pollen by RNAseq with normalised expression values of 5 reads per million (RPM) or greater (Loraine et al. 2013). Similarly, RNAseq transcriptomes of *Zea mays* mature pollen comprised 13,418 (Davidson et al. 2011) or 14,591 genes (Chettoor et al. 2014) in comparison to 10,539 genes previously identified using Agilent 44K maize microarray (Ma et al. 2008). Higher sensitivity is not the only advantage of RNAseq, the sequencing of non-exonic transcripts allowed for the first time the broad view on the alternative splicing in *Arabidopsis* pollen

including the discovery of novel pollen-specific splicing patterns (Loraine et al. 2013). For the same reason, the over-representation of transposable element-related transcripts was observed in maize pollen, although to a lesser extent than in embryo sac transcriptomes sequenced in parallel (Chettoor et al. 2014). Similar pattern was detected for transcripts encoding small signalling peptides of DEFENSIN/LURE (DEFL) family since probes for small peptide genes were often omitted from earlier microarray studies (Chettoor et al. 2014).

Not surprisingly, 49 out of 63 datasets (77%) related to mature pollen (Table 10.2). Only a fraction of experiments included also pollen developmental stages. Full pollen development including at least four developmental stages is available for three species—*Arabidopsis thaliana*, *Oryza sativa* and *Nicotiana tabacum* (Bokvaj et al. 2015; Honys and Twell 2004; Wei et al. 2010). Considering less developmental stages sufficient for the evaluation of transcriptome dynamics throughout pollen development would lead to the addition of four more species—*Zea mays* (meiocytes and pollen; Chettoor et al. 2014; Dukowic-Schulze et al. 2014; Xu et al. 2012), *Lilium longiflorum* (microspores and mature pollen; Okada et al. 2007), *Brassica napus* (microspores and mature pollen; Whittle et al. 2010) and *Fragaria vesca* (microspores and mature pollen; Hollender et al. 2014). The inclusion of progamic phase changed the list of analysed species. More than one time point of pollen germination and in vitro pollen tube growth was analysed in four species—*Arabidopsis thaliana* (germinating pollen and pollen tubes; Wang et al. 2008), *Lilium longiflorum* (hydrated pollen, germinating pollen and pollen tubes; Lang et al. 2015; Obermeyer et al. 2013), *Nicotiana tabacum* (4h and 24h pollen tubes; Hafidh et al. 2012a, b) and *Pyrus bretschneideri* (hydrated pollen and pollen tubes; Zhou et al. 2016). Only one time point of progamic phase (germinating pollen) was analysed in *Oryza sativa* (Wei et al. 2010). Finally, only in vitro pollen tube transcriptome without the reference mature pollen sample is available for *Camellia sinensis* (Wang et al. 2016). The quantification of transcriptome dynamics showed similar expression pattern throughout pollen development and progamic phase in all species analysed. In general, the complexity of male gametophyte transcriptome was lower than that of any sporophytic tissue analysed. It reached its maximum in early developmental stages and was subsequently reduced until mature pollen reaching only 61% in *A. thaliana* and *N. tabacum* and even 46% in *O. sativa* of the maximum value in the male gametophyte. (Honys and Twell 2004; Wei et al. 2010; Peng et al. 2012; Bokvaj et al. 2015; Rutley and Twell 2015). During progamic phase, the size of pollen tube transcriptomes remained relatively stable, similar to that of mature pollen or slightly larger increasing usually only by 0.1–3% in comparison to mature pollen in *Pyrus bretschneideri*, *Arabidopsis thaliana*, *Oryza sativa* and *Nicotiana tabacum* (Qin et al. 2009; Wei et al. 2010; Hafidh et al. 2012a, b; Zhou et al. 2016). Therefore, there was no apparent difference between fast growing tri-cellular pollen tubes and less advanced but metabolically more active bi-cellular pollen tubes. The only exception was another *Arabidopsis* study, in which the transcriptome complexity in 4h pollen tubes increased by 24% (Wang et al. 2008).

Pollen development is tightly regulated primarily at the level of transcription; it is under the control of at least two successive global gene expression programmes, early and late. The switch point between both developmental programmes occurs after pollen mitosis I in both tri-cellular (*A. thaliana*, Twell et al. 2006) and bi-cellular (*N. tabacum*, Honys et al., unpublished data) pollen. The initiation of the late programme therefore more likely reflects the progress of pollen maturation rather than the timing of pollen mitosis II (Hafidh et al. 2012a; Rutley and Twell 2015), supporting the uniqueness of the late male gametophytic transcriptome (Honys and Twell 2004) as shown also by principal component analyses in several species (Tang et al. 2010; Russell et al. 2012; Bokvaj et al. 2015; Rutley and Twell 2015). Generally, genes involved in cell cycle control and transcription regulation were expressed in both early and late male gametophyte transcriptomes, however, with variable expression of individual transcription factor (TF) genes and gene families, like MYB/MYB related, AP2-EREBP, C2H2, bHLH, MADS, bZIP, WRKY and TCP. On the contrary, the gene ontology (GO) category of protein synthesis/translation was over-represented in early developmental stages, whereas cell wall synthesis, cytoskeleton, signalling, protein turnover and localisation were upregulated closer to pollen maturation and in growing pollen tubes (Twell et al. 2006; Wei et al. 2010; Hafidh et al. 2012a; Costa et al. 2013, Zhou et al. 2016).

Comparative and developmental transcriptomic studies served as an information background for follow-up research including reverse genetic screens for male gametophytic transcription factors (Reňák et al. 2012), F-box proteins (Ikram et al. 2014), signalling proteins (Chen et al. 2014) and numerous functional studies. Unlike them, transcriptomic studies comparing wild-type and mutant pollen were rare, and besides the search for genetic interactions in pollen tubes deficient in two arabinogalactan protein-coding genes, *agp6* and *agp11* (Costa et al. 2013), they aimed to identify the transcriptional networks that regulate cell differentiation and define cell-specific functions during pollen development (Verelst et al. 2007b; Gibalová et al. 2009).

The comparison of wild-type and *agp6/agp11* double-mutant pollen tubes revealed 1022 differentially expressed genes (14.7% of the pollen tube transcriptome), almost equally distributed among upregulated and downregulated sets. GO categorisation of these genes was similar as in other late pollen transcriptomes; however, the over-representation of several protein groups (F-box proteins, receptor-like protein kinases, protein chaperones and proteins involved in calcium signalling) highlighted the interactions of AGP6 and AGP11 with members of the pollen tube endosome machinery enabling the recycling of AGPs to perform their signalling role (Costa et al. 2013).

MADS-domain transcription factors play key roles in the development of higher eukaryotes functioning as higher-order complexes. Five members of the MIKC* subgroup of the MADS-box family (AGL30, AGL65, AGL66, AGL94 and AGL104) were strongly upregulated in late stages of *Arabidopsis* pollen development (Pina et al. 2005) and were shown to form several heterodimeric complexes preferentially binding MEF2-type CArG-box sequence motifs (consensus $CTA(A/T)_4TAG$) also over-represented in promoters of late pollen-expressed genes

(Verelst et al. 2007a). Transcription profiling of double, triple (Verelst et al. 2007b) and even quadruple (Adamczyk and Fernandez 2009) mutants deficient in several combinations of MIKC* genes revealed the intriguing complexity of MADS-box TF network directing cellular differentiation during pollen maturation, a process that is essential for male reproductive fitness in flowering plants (Verelst et al. 2007b; Adamczyk and Fernandez 2009). Interestingly, the importance of MIKC* MADS-box TFs ZmMADS2 for maize pollen development was demonstrated even earlier on (Schreiber et al. 2004). The functional conservation of MIKC* MADS-box complexes in *Arabidopsis* and rice indicated that the function of heterodimeric MIKC* protein complexes in pollen development has been conserved since the divergence of monocots and eudicots, roughly 150 million years ago (Liu et al. 2013).

Basic leucine zipper (bZIP) transcription factors act as homo- or heterodimers and these effector-type TFs control many aspects of plant development including reproduction. Pollen-expressed TF AtbZIP34 is active during late stages of male reproductive development with a complex sporophytic and gametophytic mode of action. Transcription profiling of *atbzip34* mutant pollen led to the finding that AtbZIP34 regulon comprises membrane-associated transporters and proteins involved in lipid metabolism and cell wall synthesis (Gibalová et al. 2009).

Transcription profiling was used also to unravel the regulon of male germline-specific R2R3-MYB transcription factor, DUO POLLEN1 (DUO1, Durbarry et al. 2005; Rotman et al. 2005), playing an important role in sperm cell differentiation. However, Borg et al. (2011) adopted different strategies as they analysed the transcriptomes of seedlings with ectopically expressed DUO1 in an estradiol-inducible manner and identified 63 candidate targets. Moreover, DUO1 was shown to directly regulate its target promoters through binding to the canonical MYB sites (Borg et al. 2011). The role of two DUO1 target genes *DAZ1* and *DAZ2* has been characterised; they both encode EAR[1] motif-containing C2H2-type zinc finger proteins that are important for both generative cell division and DUO1-dependent germ cell differentiation (Borg et al. 2014).

In connection with bi- and tri-cellular pollen types in angiosperms, DUO1 may have been involved in the control of the timing of generative-cell division during the evolution of the pollen type (Hafidh et al. 2012b; Rotman et al. 2005). In order to illustrate complicated processes and interrelationships in plant protein families, we adopted the *DUO1* gene as an example for the demonstration of frequently used genomic tools for studying the roles of specific proteins in the cell and to briefly summarise current knowledge and line out the distribution of different orthologs within angiosperms. Up to 2015, the lack of knowledge has hindered the comprehension of the origin and evolutionary history of MYB gene family

[1]EAR motif is short *E*thylene-responsive element binding factor-associated *A*mphiphilic *R*epression motif present in plant transcriptional repressors that mediates transcriptional repression by the association with corepressors responsible for chromatin modification (Kagale and Rozwadowski 2011).

across plants. It was reported that the intron patterns of R2R3-MYB transcription factors were greatly conserved in model higher plants (Matus et al. 2008; Du et al. 2012). However, the prevalence of R2R3-MYBs was quite different indicating that their introns were established in the common ancestor of land plants. Moreover, intron patterns in algae were variable and different from land plants. These findings suggested that algae and land plant lineages used different splicing patterns. Du et al. (2015) confirmed that R2R3-MYBs were older than 3R-MYBs which may be evolutionarily derived from R2R3-MYBs via intragenic domain duplication. The interesting feature is that intron patterns of land plant R2R3-MYBs were exclusively conserved within each subfamily. Phylogenetic relationships of *DUO1* and related MYB-family transcription factors expressed in pollen from selected plant species including basal angiosperms are shown in Fig. 10.2. Members of different orders tend to cluster together within a given clade indicating that clades could have been expanded after divergence from their common ancestor (Du et al. 2015). This finding suggests the common origin of family members. All dicots clustered in their own clades and are separated from monocots and basal angiosperms, which exhibit lineage-specific expansion. The resolution within main orders is species dependent and possibly suffers from missing data (i.e. representatives from many groups across the phylogenetic tree that are not available yet) and long-branch attraction artefacts. Alternatively, the species-specific R2R3-MYBs may represent genomic relics that evolved independently as Du et al. (2015) adumbrated. These results show that there may be more lineage-specific subfamilies in *DUO1* gene, and their evolution history could be solved in a future by using a more representative set of species and by combination with the expression -omics studies.

It has been established that pollen tube growth in vitro and in vivo differ and that the directional pollen tube growth is greatly influenced by pistil tissues (Palanivelu and Preuss 2006; Palanivelu and Tsukamoto 2012). Therefore, pollen tubes growing in vitro and in vivo were compared in *Lilium longiflorum* (Huang et al. 2011). Similar analysis including also mature pollen was performed in *Olea europaea* (Carmona et al. 2015; Iaria et al. 2016). To characterise the changes in gene expression of *A. thaliana* pollen tubes growing in vivo and pistil-activated pollen tubes, the semi-in vivo approach was applied (Qin et al. 2009). It was shown that the pistil activation induced the expression of 1254 genes (18% of the overall transcriptome), many of which were pollen specific. None of these genes were active in in vitro growing pollen tubes. Similar analysis was performed on *A. thaliana* semi-in vivo pollen tubes growing under the influence of isolated ovules that induced the expression of 719 genes (Chen et al. 2014). In both studies, genes with potential function in signalling, pollen tube growth/cell extension and transcription were over-represented among the pistil-activated genes, including three MYB transcription factors (MYB97, MYB101, MYB120, Qin et al. 2009) that were later shown to play a crucial role during pollen tube differentiation required for sperm release (Leydon et al. 2013; Liang et al. 2013). Eighteen candidate genes for

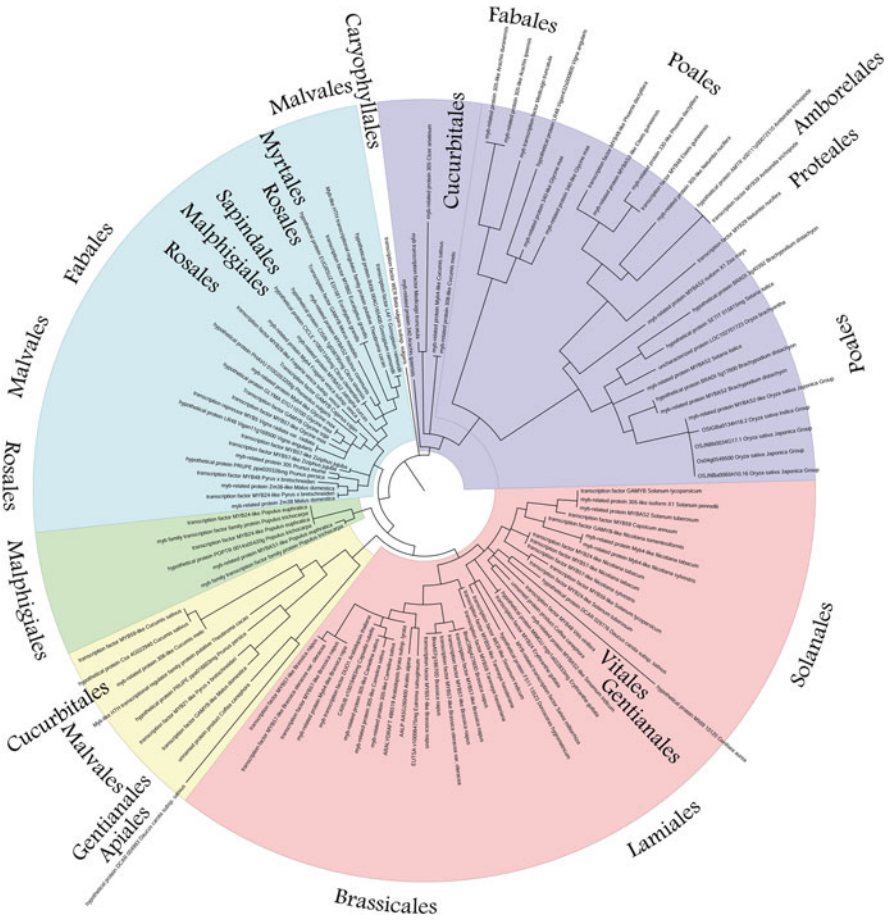

Fig. 10.2 Phylogenetic relationships of pollen-specific DUO POLLEN 1 gene. The evolutionary history was inferred by using the maximum likelihood method based on the JTT matrix-based model. The tree with the highest log likelihood (−2637.8067) is shown. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The tree was divided into five main phylogenetic subgroups with bootstrap support >80% shown in different colours

pollen tube guidance including TIR-NBS-LRR[2] proteins, DEFL proteins, protein kinases and receptor-like protein kinases were selected for reverse genetic screen, however, only with limited success, due to the functional redundancy in these large gene families (Chen et al. 2014).

[2]TIR-NBS-LRR is a large receptor subfamily, a part of the 'R' gene superfamily implicated in pathogen recognition. TIR-NBS-LRR proteins contain N-terminal domain with toll/interleukin-1 receptor homology (TIR), nucleotide binding site (NBS) and leucine-rich repeat (LRR) domains (Meyers et al. 2003).

Both cell types forming mature pollen–'somatic' vegetative cell and generative cell/sperm cells as the male germline–have very different cell fates that are reflected in their gene expression. Therefore, several experiments compared the transcriptomes of isolated male gametes with either whole mature pollen or even with isolated vegetative nuclei. In plants dispersing tri-cellular pollen, mature pollen grains were used. The first, although limited sperm cell transcriptome obtained by EST library sequencing, was published for *Zea mays* (Engel et al. 2003). In *Plumbago zeylanica*, producing dimorphic sperm cells, their individual transcriptomes were compared to that of vegetative nuclei (Gou et al. 2009), whereas in *Arabidopsis thaliana* (Borges et al. 2008) and *Oryza sativa* (Russell et al. 2012; Anderson et al. 2013), sperm cell transcriptomes were compared to intact mature pollen grains. Similar analysis was performed in *Lilium longiflorum* that releases bi-cellular pollen and therefore the transcriptome of generative cell was studied (Okada et al. 2006, 2007). To isolate sperm cells of plants producing bi-cellular pollen, *Nicotiana tabacum* pollen was germinated semi-in vivo, sperm cells were collected from pollen tubes emerging from cut pistils and their transcriptome was obtained by conventional EST sequencing (Xin et al. 2011). Seven studies of sperm cells from six plant species covered both dicots and monocots as well as plants producing bi- and tri-cellular pollen, providing us with an ample material for comparisons. The identified fractions of individual sperm cell transcriptomes varied according to the method used, from around 600 (*Plumbago zeylanica*, EST sequencing, Gou et al. 2009) to tens of thousands genes (*Oryza sativa*, RNAseq, Anderson et al. 2013). Such numbers clearly prove that sperm cells were definitely not transcriptionally and metabolically inactive entities. In all species, sperm cell transcriptomes were different not only from sporophytic control datasets but also from the transcriptomes of whole pollen/vegetative nuclei (i.e. Russell et al. 2012) as visualised also by PCA analyses (Borges et al. 2008; Russell et al. 2012). Moreover, there were striking differences between the transcriptomes of both types of dimorphic *Plumbago zeylanica* sperm cells, destined to fuse with egg cell and central cell, respectively (Gou et al. 2009). Interesting similarities were found in the presumed function of sperm cell-expressed proteins including the significant fraction of proteins of unknown function. Sperm cell transcriptomes were enriched in GO categories of cell cycle proteins, membrane-associated proteins, proteins involved in signal transduction, protein destination, ubiquitin-mediated proteolysis and epigenetic modifications (Engel et al. 2003; Okada et al. 2006, 2007; Borges et al. 2008; Xin et al. 2011; Russell et al. 2012; Anderson et al. 2013). On the contrary, genes for RNAi machinery were downregulated (Russell et al. 2012; Anderson et al. 2013). The fact that sperm cell transcriptomes of *A. thaliana*, *N. tabacum* and *Z. mays* shared only 0.3% genes (7.6% of genes were shared by at least two of three datasets, Xin et al. 2011) pointed out either the possibility that still only limited transcriptome fractions were identified and/or that there were more significant differences between sperm cells of dicots and monocots as well as those formed in bi- and tri-cellular pollen than originally expected. In any case, this fact is stimulating for further exciting research.

Finally, transcriptome profiles of gametophyte generation are available also for a few species of bryophytes and ferns—*Physcomitrella patens* (O'Donoghue et al. 2013; Ortiz-Ramirez et al. 2016; Xiao et al. 2011), *Tortula ruralis* (Oliver et al. 2004), *Marchantia polymorpha* (Higo et al. 2016) and *Pteridium aquilinum* (Der et al. 2011). However, since we concentrate solely on seed plants, gymnosperms and angiosperms, these datasets are not present in Table 10.2.

10.3 Proteomics

Transcriptomic analyses have provided and continue providing valuable information about global and specific gene expression and its dynamics. Transcriptomic data, however, do not provide complete information about gene expression since the proteome does not fully reflect the transcriptome (de Groot et al. 2007). This is especially true for systems with high level of translational regulation, such as the male gametophyte. The principal reason is that it is not technically feasible for transcriptomics to take into account the possible contribution of post-transcriptional regulatory levels of gene expression (Keene 2007). The effect of splicing (Collins 2011; Lorkovic and Barta 2004; Reddy et al. 2012) including the identification of potentially alternatively spliced transcripts (Kazan 2003; Sanchez et al. 2011; Xing and Li 2011) can be studied with the complete tilling gene chip (Whole-Genome ChIP Tilling Array ATH6, Roche-NimbleGen Systems, Inc.) or, ideally, by RNAseq (Loraine et al. 2013). However, further post-transcriptional regulatory levels, especially translation and mRNA storage, are active during male gametophyte development (Hafidh et al. 2011, 2016a). For all above reasons, it remains necessary to complement transcriptomics with proteomic data to get more realistic insight.

Soon after the first pollen transcriptomic analyses appeared in the last decade, the initial studies of pollen proteome were published. Surprisingly, the first plant species with published pollen proteome, though very incomplete, was a gymnosperm, *Pinus strobus* (Fernando 2005). Since then, numerous angiosperm species followed, for example, *Arabidopsis thaliana* (Grobei et al. 2009; Holmes-Davis et al. 2005; Noir et al. 2005; Sheoran et al. 2006; Zou et al. 2009), *Oryza sativa* (Dai et al. 2006, 2007), *Solanum lycopersicum* (Lopez-Casado et al. 2012; Sheoran et al. 2007), *Lilium longiflorum* (Pertl et al. 2009), *Brassica napus* (Sheoran et al. 2009a), *Quercus ilex* (Valero Galvan et al. 2012), and *Helianthus annuus* (Ghosh et al. 2015).

The pioneering proteomic studies were based on the excision of intact proteins in the isolated spots acquired from gels after 2-D gel electrophoresis (2-DE), which were protease-treated and analysed by mass spectrometry. Therefore, it resulted in only a very limited fraction of the total proteome, comprising usually several hundred proteins, almost invariably fewer than 1000. Therefore, 2-D gel-based proteomics has much lower coverage than transcriptomics and a little overlap between individual experiments. For example, three first published *Arabidopsis* pollen proteomic datasets identified 135 (Holmes-Davis et al. 2005), 121 (Noir et al.

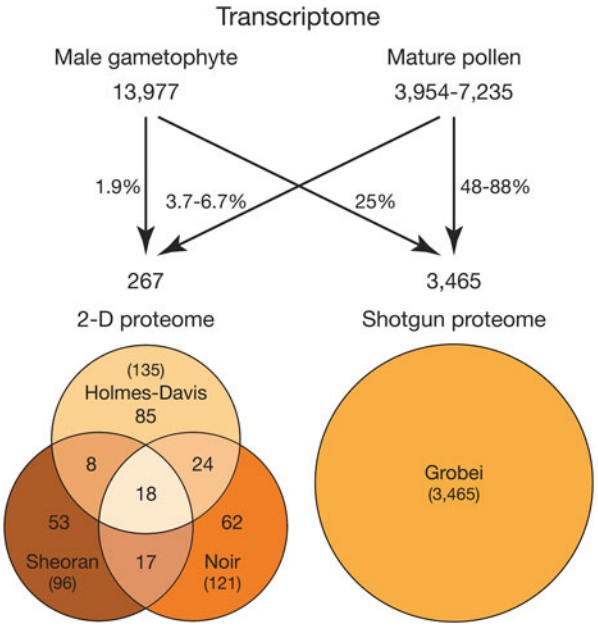

Fig. 10.3 Quantification of *Arabidopsis thaliana* transcriptomic and proteomic studies

2005) and 96 (Sheoran et al. 2006) proteins, respectively (Fig. 10.3). Considering overlaps between these datasets, 2-DE proteomics enabled the identification of 267 mature pollen proteins. Affymetrix ATH1 gene chip harboured probes for 237 of these proteins representing only very limited fraction of 13,977 genes active during pollen development (Honys and Twell 2004), of which 6044 were identified in mature pollen (Rutley and Twell 2015). Of identified 267 mature pollen proteins, 200 (75%) were found only in a single study, whilst only 18 proteins (7%) were identified by all three groups. This very small coverage of the pollen proteome is not surprising. One can assume that proteins found by more authors were encoded by most strongly expressed genes. Indeed, all 18 genes encoding these proteins were among the most abundant in pollen transcriptome. They also belonged to the functional categories containing usually only a limited number of typically very highly expressed genes: energy metabolism (8), stress response (4), synthesis and metabolism of the cell walls (2), cytoskeleton (2), protein synthesis (1), and metabolism (1). The result was also influenced by protein extraction protocol used by different groups that affects the composition of purified proteome fraction drastically, as independently demonstrated in tobacco pollen (Fíla et al. 2011).

Characterisation of although a limited part of the pollen proteome enabled the functional categorisation and comparison of pollen transcriptome and proteome. Again, there is significantly higher proportion of functional categories grouping abundant proteins into proteomic datasets. On the contrary, the obvious variability between individual 2-DE-analysed pollen proteomes is not as high as might be

expected from their variability. As an exception, stress-related proteins and proteins of unknown function differ significantly. However, especially here, the possible influence of the downstream processing of the biological material cannot be excluded. In comparison to transcriptome, there is higher proportion of proteins involved in massive processes of general and energy metabolism and of protein synthesis and metabolism. Similar trends can be seen in the reference datasets characterising *Oryza sativa* pollen proteome (Dai et al. 2006, 2007), another model species with tri-cellular pollen. In this respect, the original *Pinus strobus* pollen proteome (Fernando 2005) is significantly different, not only because pine is a gymnosperm, in which male gametophyte structure and development differ from the angiosperms, but at least partly due to the significantly smaller size of identified proteome fraction and the lack of known genomic sequence of any gymnosperm at the time of publication. Therefore, there was particularly high fraction of unknown proteins in *Pinus strobus* proteome.

A fundamental breakthrough not only in pollen research was the introduction of gel-free proteomic techniques, which increased the efficiency of peptide and protein identification by one order. Gel-free techniques also enabled more accurate quantification of protein abundance. However, only a limited number of plant species were used for gel-free pollen proteome characterisation (Table 10.2). The first shotgun proteomic study identified 3465 proteins in *Arabidopsis* pollen (Grobei et al. 2009) that represented almost 13-times enlargement of a known fraction of the mature pollen proteome including the vast majority of proteins previously identified by 2-DE techniques. Such extension was also reflected in the functional categories, to which the identified proteins belonged, which were closer to the transcriptomic studies including the significant representation of the stable structural proteins (Fig. 10.3). Comparison of proteomic and transcriptomic datasets showed that the vast majority of 2928 (85% of described proteome) genes identified in pollen by proteomic and transcriptomic approaches was encoded by transcripts present already in early stages of male gametophyte development (Grobei et al. 2009; Honys and Twell 2004), thus representing an independent confirmation of the extent of translational regulation of gene expression in pollen (Honys et al. 2000, 2009; Honys and Twell 2004). Finally, 537 pollen proteins (15% of pollen proteome) had not been described in any transcriptomic study known at that time which made proteomics an attractive method for gene expression studies complementary to transcriptomics.

Similarly to transcriptomics, proteomic studies also aimed at the characterisation of the proteome dynamics during the pollen development and pollen tube growth. The list of species is shorter containing only *Solanum lycopersicum* (Chaturvedi et al. 2013), *Nicotiana tabacum* (Ischebeck et al. 2014) representing angiosperms and *Picea wilsonii* (Chen et al. 2012) as a representative of gymnosperms. Chen et al. (2012) investigated the influence of the limited nutrient supply on pollen tube growth, namely, the deficiency of sucrose, calcium and boron. In total, 166 proteins and 42 phosphoproteins (see also next section) were identified by LC-MS/MS as differentially regulated. Such number, low for gel-free approach, was mainly caused by the lack of conifer genome sequence data at that time. The identified proteins were involved in a variety of signalling pathways, providing new insights into the

multifaceted mechanism of nutrient function including indicated nutrient-specific effects (Chen et al. 2012). Remaining two studies provided the most comprehensive male gametophytic proteomic datasets at the moment; they covered numerous stages of pollen development of related *Solanaceae* species providing evidence for developmentally controlled processes that might help to prepare the cells for specific developmental programmes and environmental stresses. In *Solanum lycopersicum*, five pollen developmental stages were compared—microsporocytes, tetrads, microspores, polarised microspores and mature pollen (Chaturvedi et al. 2013). In *Nicotiana tabacum*, the covered period was even broader—diploid microsporocytes, meiosis, tetrads, microspores, polarised microspores, bi-cellular pollen, mature pollen and pollen tubes, altogether eight stages (Ischebeck et al. 2014). In tomato, 1821 proteins were identified (Chaturvedi et al. 2013), whereas the tobacco analysis led to the identification of 3817 protein groups (Ischebeck et al. 2014). In both species, principal component analyses (Chaturvedi et al. 2013; Ischebeck et al. 2014) provided a similar picture as those resulted from transcriptomic studies (Bokvaj et al. 2015; Rutley and Twell 2015) and demonstrated that pollen development is highly controlled sequential process also at the proteome level. From the predicted functions, energy-related proteins are upregulated during the later stages of tomato pollen development. It indicates that pollen germination depends upon presynthesised proteins in mature pollen. In contrast, heat stress-related proteins are highly abundant in very early developmental stages, suggesting a dominant role in stress protection (Chaturvedi et al. 2013). Similar observations were made in tobacco, where the early developmental stages were enriched also with ribosomal and other translation-related proteins (Ischebeck et al. 2014).

In several cases, the individual studies targeted specific cell types, namely the generative or sperm cells in *Lilium davidii* (Zhao et al. 2013), and *Oryza sativa* (Abiko et al. 2013). In rice, sperm cell gel-free proteome was compared to the whole mature pollen grain. Of 2179 sperm cell-expressed proteins, 77 were preferentially present in the male gametes (Abiko et al. 2013). The comprehensive study by Zhao et al. (2013) employed 2-D DIGE followed by MALDI-TOF/TOF mass spectrometry to identify 101 proteins differentially expressed in lily generative and sperm cells. These proteins are involved in diverse cellular and metabolic processes, with preferential involvement in the metabolism, cell cycle, signalling, the ubiquitin/proteasome pathway, and chromatin remodelling, i.e. similar categories as revealed by transcriptomics. Impressively, almost all proteins in ubiquitin-mediated proteolysis and the cell cycle were upregulated in sperm cells, whereas those in chromatin remodelling and stress response were downregulated (Zhao et al. 2013).

Other studies were devoted to specialised cellular compartments, including membranes (*Lilium longiflorum*, Pertl et al. (2009); *Lilium davidii*, Han et al. (2010); and *Solanum lycopersicum*; Paul et al. (2016)), nuclei (Yang et al. 2016) and messenger ribonucleoprotein (mRNP) complexes (Honys et al. 2009). Other studies focused on specific protein groups (e.g. allergens) or proteins characterised by specific post-translational modifications, notably phosphorylation (Fíla et al. 2012, 2016; Mayank et al. 2012, see below). In all species analysed, membrane proteomes confirmed the presence of expected membrane-associated proteins on/in plasma membrane as well

as endomembranes (Pertl et al. 2009; Han et al. 2010; Paul et al. 2016). In *Lilium longiflorum*, the differences in abundance of various protein types were observed in both membrane fractions in mature pollen and in several time points of pollen tube growth. For example, increase in the abundance of proteins involved in cytoskeleton, carbohydrate, energy metabolism, as well as ion transport was observed before pollen germination (10–30 min), whereas proteins involved in membrane/protein trafficking, signal transduction, stress response and protein biosynthesis decreased in abundance during this time (Pertl et al. 2009). Similar proteins were identified in membrane proteomes of two tomato cultivars, and the presence of proteins corresponding to energy-related pathways (glycolysis and Krebs cycle) enabled to present a hypothetical model of energy reservoir of the male gametophyte (Paul et al. 2016). *Lilium davidii* pollen and pollen tubes plasma membrane proteome fraction comprised also proteins of translational apparatus and DNA/RNA-binding proteins with preferential occurrence of ribosomal proteins. The identification of these proteins probably resulted from the presence of cytoskeleton-binding polysomes anchored to the plasma membrane via actin filaments or targeted to lipid rafts (Han et al. 2010). The association of translation apparatus and RNA-storage particles with the actin cytoskeleton was observed also in tobacco pollen and pollen tubes (Honys et al. 2009) where the protein composition of large ribonucleoprotein particles (EPPs) was studied. EPP complexes are formed in immature pollen where they contain translationally silent mRNAs. Although massively activated at the early progamic phase, they also serve as a long-term storage of mRNA transported along with the translational machinery to the tip region. Since EPPs contain ribosomal subunits, rRNAs and a set of mRNAs, they were hypothesised to represent well-organised machinery devoted to mRNA storage, transport and subsequent controlled activation resulting in protein synthesis, processing and localisation, extremely useful in fast tip-growing pollen tube. Expression of vast majority of the closest orthologues of EPP proteins also in *Arabidopsis* male gametophyte further extended this concept from tobacco to *Arabidopsis*, the model species with advanced tri-cellular pollen (Honys et al. 2009). The last cellular compartments analysed for the proteomic perspective were the vegetative, generative and sperm cell nuclei of *Lilium davidii* (Yang et al. 2016). The profiling of histone variants of all five histone families in all three cell types revealed 92 identities representing 32 histone variants. Generative and sperm cells had almost identical histone profiles and similar histone H3 modification patterns, significantly different from those of vegetative nuclei. These results suggested that differential histone programmes, important for the identity establishment and differentiation of the male germline, may be established following the asymmetric division (Yang et al. 2016).

To summarise, it is obvious that proteomics studied a broader spectrum of species (including plants not representing the classical models) compared to microarray transcriptomics since proteomics is not limited by sequenced genomic DNA of the particular species. The EST sequences or protein sequences from related species can be used instead. It also highlighted post-transcriptional levels of gene expression that could not be addressed by transcriptomics.

10.4 Phosphoproteomics

Pollen rehydration and activation is accompanied by two main regulatory mechanisms of gene expression. The first one is represented by translation regulation. A notable part of stored, translationally regulated transcripts are localised in EDTA/puromycin-resistant particles (EPPs) in tobacco (*Nicotiana tabacum*, Honys et al. 2000, 2009). The transcripts inside these complexes are stored in a translationally silent form in mature pollen, whereas upon pollen rehydration, the mRNAs are being de-repressed and translated. Since most growing processes inside the pollen tube are localised to the tip, EPP complexes are transported towards the pollen tube tip (Honys et al. 2009). The second mechanism of gene expression regulation is phosphorylation, which is one of the most dynamic post-translational modifications of proteins. Protein phosphorylation in reaction to rehydration was revealed not only in *Nicotiana tabacum* male gametophyte (Fíla et al. 2016) but also in rehydrated plants of the xerophyte *Craterostigma plantagineum* (Röhrig et al. 2008) and in the cells in *Zea mays* leaf growing zone (Bonhomme et al. 2012). Large-scale studies of protein phosphorylation usually employ various enrichment protocols to enable the identification of phosphorylated peptides in the total complex protein crude extract. The enrichment can be either carried out at the level of intact proteins or alternatively from the peptide mixture acquired after cleavage of the complex total protein crude extract by a specific protease (Fíla and Honys 2012). Both these phosphoproteomic approaches showed their advantages as well as limitations.

The first phosphoproteomic study performed on male gametophyte was that of *Arabidopsis thaliana* mature pollen (Mayank et al. 2012, Table 10.3). This study applied a combination of three phosphopeptide-enriching protocols: immobilised metal affinity chromatography (IMAC), metal oxide/hydroxide affinity chromatography (MOAC) and sequential elution from IMAC (SIMAC). The study presented collectively 962 phosphopeptides carrying 609 phosphorylation sites that belonged to 598 phosphoproteins. From the functional point of view, most identified phosphoproteins took part in the regulation of protein metabolism and function, metabolism, protein fate, protein binding, signal transduction and cellular transport. Several kinases were also among the identified phosphoproteins, particularly AGC[3] protein kinases, Ca^{2+}-dependent protein kinases and sucrose non-fermenting protein kinases 1.

The next male gametophyte of angiosperms, which was subjected to phosphoproteomic techniques, was tobacco (*Nicotiana tabacum;* Fíla et al. 2012, 2016, Table 10.3). The former study identified 139 phosphoprotein candidates from mature pollen and pollen grains activated in vitro for 30 min. In order to improve the number of unambiguously positioned phosphorylation sites, titanium

[3]AGC kinases represent the subgroup of serine/threonine protein kinases named after three representative families, the cyclic guanosine monophosphate (cAMP)-dependent protein kinase (PKA), the cyclic guanosine monophosphate (cGMP)-dependent protein kinase (PKG) and the protein kinase C (PKC) families (Pearce et al. 2010).

Table 10.3 Comparison of published angiosperm male gametophyte phosphoproteome studies in particular from *Arabidopsis thaliana*, tobacco and maize

Parameter	*Arabidopsis* pollen phosphoproteome (Mayank et al. 2012)	Tobacco pollen phosphoproteome I (Fíla et al. 2012)	Tobacco pollen phosphoproteome II (Fíla et al. 2016)	Maize pollen phosphoproteome (Chao et al. 2016)
Mature pollen	✓	✓	✓	✓
Pollen activated in vitro 5 min			✓	
Pollen activated in vitro 30 min		✓	✓	
Phosphoprotein enrichment method	–	$Al(OH)_3$–MOAC	–	–
Phosphopeptide enrichment method	IMAC, TiO_2–MOAC and SIMAC	TiO_2–MOAC[a]	TiO_2–MOAC	IMAC
Number of identified phosphoproteins	598	139	301	2257
Number of identified phosphopeptides	962	52	471	4638
Number of phosphorylation sites	609	52	432	5292
The pS/pT/pY ratio	86:14:0.16	67.3:32.7:0[b]	86.4:13.4:0.2	81.5:14.5:4.0
Number of phosphorylation motifs (pS/pT/pY)	2:0:0	N/A[c]	5:1:0	23:4:0

[a]The TiO_2–MOAC phosphopeptide enrichment in Fíla et al. (2012) was presented only for phosphopeptides already identified after the phosphoprotein enrichment
[b]The pS/pT/pY ratio in case of Fíla et al. (2012) is biased by a too small dataset studied
[c]The phosphorylation motifs were not searched in Fíla et al. (2012)

dioxide phosphopeptide enrichment was performed on trypsin-digested mature pollen crude extract, which led to the identification of 51 more phosphorylation sites localised in the phosphoproteins already identified in mature pollen giving a total of 52 unambiguous phosphorylation sites. In order to understand the processes during pollen grain activation and the start of pollen tube growth, pollen grains activated in vitro for 5 min were also taken into consideration in the subsequent study (Fíla et al. 2016). To increase the probability of phosphoprotein identification, phosphopeptide-enriching MOAC with titanium dioxide matrix was applied exclusively. In the mentioned three stages of tobacco male gametophyte, 471 phosphopeptides were identified, which carried 432 unambiguously determined phosphorylation sites. These phosphorylated peptides were assigned to 301

phosphoproteins. The phosphopeptide enrichment of the three stages increased notably the number of identified phosphorylation sites. The dominant functions were transcription, protein synthesis, protein destination and storage and signal transduction. It is also worth mentioning that almost one fifth of identified phosphopeptides was put into categories with unknown function or unclear classification. These results are in agreement with tobacco male gametophyte proteome, where approx. fifteen percent of male gametophyte-specific proteins were of unknown classification. The unknown phosphoproteins represent likely male gametophyte-specific or male gametophyte-enriched proteins, function of which will be probably important for regulation of pollen activation and pollen tube growth. A notable part of the identified phosphopeptides showed a significant regulatory trend in the progamic phase of male gametophyte. Most of the regulated peptides were shown to be exclusive for mature pollen grains. The only alternative study that considered other stages than mature pollen was performed with pollen tubes from *Picea wilsonii* (Chen et al. 2012). However, it differed in two ways from the above studies: (1) a gymnosperm was studied instead of an angiosperm, and (2) the proteome and phosphoproteome of *Picea wilsonii* pollen tubes were studied not from the developmental point of view but as a reaction to growth media lacking sucrose or Ca^{2+} ions (which serve as important nutrients for pollen tube growth). The *Picea wilsonii* study thus revealed 166 proteins and 42 phosphoproteins playing their roles in signalling of media deficiency.

Most recently, *Zea mays* became the first monocot with a published mature pollen phosphoproteome (Chao et al. 2016), but again, no other gametophyte stages were studied (Table 10.3). Despite this, the maize pollen phosphoproteomic dataset became the largest one since it presented 4638 phosphopeptides, which belonged to 2257 phosphoproteins. These phosphorylated peptides led to the identification of 5291 phosphorylation sites with many multiply phosphorylated phosphopeptides and also carrying more than one phosphorylation site. The dominant molecular functions were ion binding, kinase activity, transmembrane transporter activity, oxidoreductase activity, and DNA binding, whilst the enriched biological processes were represented by protein posttranslational modification, cell organisation, signalling G-proteins, calcium signalling, abiotic stress, protein targeting, and RNA–RNA binding.

The functional categories of the identified phosphopeptides were quite similar in male gametophytes of all studied species. Since pollen tube tip growth requires several cellular mechanisms, such as small GTPase signalling, ion gradient formation, cytoskeleton organisation and transport of secretory vesicles (Palanivelu and Preuss 2000; Šamaj et al. 2006), dominant phosphoproteins common to all datasets belonged to at least some of these categories. Moreover, in tobacco, the protein synthesis category included proteins likely responsible for translation regulation, including EPP particles (Honys et al. 2009). The highest number of proteins responsible for the actual regulatory processes was identified in the maize phosphoproteome, including male sterility-associated proteins together with proteins influencing maize productivity (Chao et al. 2016). The number of proteins identified in tobacco could be influenced by the fact that the identifications of the

second tobacco male gametophyte phosphoproteome relied on expressed sequence tags (EST sequences) that were acquired mainly from sporophyte tissues and thus could lack gametophyte-specific proteins.

The phosphoproteomic datasets are usually analysed whether they contain any over-represented sequence context surrounding the phosphorylation site. However, such a motif over-representation compared to the background dataset remains speculative, and the rare sequence motifs could remain undetectable. Moreover, the link between a particular kinase and target protein still remains to be experimentally proven. In *Arabidopsis thaliana* pollen phosphoproteome (Mayank et al. 2012), only serine-phosphorylated peptides were subjected to motif search, and only two motifs were identified: xxxxxxS*Pxxxxx and xxxRxxS*xxxxxx (phosphorylated amino acid is indicated by an asterisk behind the one-letter code). The prolyl-directed phosphorylation is usually mediated by mitogen-activated protein kinases and/or cyclin-dependent protein kinases, whereas the latter basic motif is recognised by Ca^{2+}-dependent protein kinases (Lee et al. 2011). Later, the motif search in the second tobacco male gametophyte phosphoproteome (Fíla et al. 2016) enabled the identification of five motifs with central phosphoserine (particularly xxxxxxS*Pxxxxx, xxxRxxS*xxxxxx, xxxKxxS*xxxxxx, xxxxxxS*DxExxx, and xxxxxxS*xDDxxx) but also one with a phosphothreonine in the middle (xxxxxxT*Pxxxxx). The prolyl-directed phosphorylation (regardless whether a serine or a threonine occupied the middle position of the motif) was mediated by mitogen-activated protein kinases and/or cyclin-dependent protein kinases (Lee et al. 2011). Then, there were two alkaline and two acidic motifs. The former motifs were represented by an arginine or a lysine on the third position before the actual phosphorylated serine, which were recognised by Ca^{2+}-dependent protein kinases and Ca^{2+}-dependent protein kinases–sucrose-non-fermenting protein kinases (CDPK–SnRK) (Lee et al. 2011). The latter, acidic motifs can be in principle merged to one phosphorylation motif, xxxxxxS*(D/E)(D/E)(D/E)xxx, which was reported to be targeted by casein kinase 2 (CK2) (Lee et al. 2011). The broadest spectrum of kinase motifs was identified in maize mature pollen phosphoproteome (Chao et al. 2016), but several motifs were in principle shared with tobacco and *Arabidopsis.* However, these shared motifs were more specified in maize by other amino acid positions around the phosphorylation site so one phosphorylation site was actually split into more similar motifs differing in the specified position(s). In spite of more identified phosphorylation motifs in the most recent study, it still had common phosphorylation motifs with tobacco and *Arabidopsis* pollen phosphoproteomes. It is likely that pollen activation will bear similarities across various angiosperm species. Chao and colleagues thus identified 23 phosphoserine motifs and 4 phosphothreonine motifs, which were further sorted into 4 groups: 8 phosphorylation motifs were considered as prolyl-directed phosphorylation, 5 motifs were alkaline and 4 were acidic. The remaining ten phosphorylation motifs were collected in the group ‘others’. Several phosphorylation motifs (mainly from the group ‘others’) were considered as novel, and these motifs can represent the male gametophyte-specific regulatory pathways. Finally, it should be noted that all mentioned phosphorylation motifs were acquired by *in*

silico data search, and it will be required to perform additional experiments in order to link a particular protein kinase with its target protein(s).

10.5 Specialised Pollen -Omics

To make the list of pollen -omics complete, we cannot leave out more specialised studies covering generally only limited number of model species. Of them, translatomics, methylomics and miRNAomics are based on transcriptomic approaches since they characterised specialised RNA populations functionally related to mRNA fate in the cytoplasm and translation. Likewise, pollen allergome and secretome were identified by proteomic techniques, whereas metabolomics employs a different set of techniques which will be discussed elsewhere in this book (see Chap. 12).

The study of Lin et al. (2014) represents the first and so far the only attempt to identify and characterise the subset of actively translated transcripts in in vivo-growing pollen tubes of *Arabidopsis thaliana*. The authors adopted elegant solution for the isolation of polysome-RNA complexes from pollen tubes growing through tiny *Arabidopsis* pistils; they were affinity purified via HIS6-FLAG dual-epitope tagged ribosomal protein RPL18 expressed under pollen vegetative cell-specific promoter LAT52. The comparison with in vitro-cultivated pollen tubes revealed over 500 transcripts specifically enriched in in vivo-elongating pollen tubes including transcripts encoding proteins involved in micropylar guidance, pollen tube burst and repulsion of multiple pollen tubes in embryo sac (Lin et al. 2014). Although the similar functional categorisation of genes upregulated in in vivo translatome (Lin et al. 2014), semi-in vivo transcriptome (Qin et al. 2009) and pollen–pistil interaction-induced transcriptome (Boavida et al. 2011), there was only very little overlap at the level of individual genes. However, it is difficult to conclude, whether such differences reflected a different nature of *de novo* transcription during in vivo and semi-in vivo pollen tube growth or only a subset of induced transcripts is being actively translated.

MicroRNAs (miRNAs) represent only a small portion of transcriptome, but they play an important role in post-transcriptional regulation of gene expression, mRNA cleavage, mRNA destabilisation through poly(A) tail shortening and translation inhibition (Brodersen et al. 2008; Carthew and Sontheimer 2009). Therefore, the identification of pollen miRNAs and their targets and especially the dynamics of miRNAome is of key importance for understanding the fine modulation of gene expression in the male gametophyte and in the process of the male germline differentiation. Of the seven studies published so far, one was devoted to gymnosperm *Pinus taeda* (Quinn et al. 2014) and the remaining six to three angiosperm model plants *Arabidopsis thaliana* (Chambers and Shuai 2009; Grant-Downton et al. 2009b; Borges et al. 2011), *Oryza sativa* (Peng et al. 2012; Wei et al. 2011), and *Zea mays* (Li et al. 2013). In these studies, 24-nt miRNAs represented the most abundant class. Alongside the identification of known and novel miRNA families,

these studies also identified their putative mRNA targets, and in few cases, they even demonstrated the regulatory function of the respective miRNAs.

Chambers and Shuai (2009) profiled the expression of 70 known miRNAs in *Arabidopsis* mature pollen using miRCURY microarray and the comparison of their expression with transcriptomic profiles of their putative targets indicated the activity of several candidate miRNAs in pollen. The first large-scale study employing *de novo* sequencing was, as usual, conducted on *Arabidopsis* mature pollen and pollen miRNAome was compared to that of leaves (Grant-Downton et al. 2009b). Out of 33 miRNA families identified in pollen, expression of 17 was validated by RT-PCR, and most of them were found to be enriched in the male gametophyte with three (miR157, ath-MIR2939, and miR845) being putatively pollen specific. Moreover, the study reported, for the first time, the presence of *trans*-acting siRNAs in pollen (Grant-Downton et al. 2009b). Borges et al. (2011) analysed miRNA populations sequenced by Slotkin et al. (2009) with a special interest in miRNAs active in the male germline. They confirmed most of the previously identified pollen-expressed miRNAs (with the exception of miR776, Grant-Downton et al. 2009b) and found even higher representation of miRNA families both in mature pollen (75 families) and in sperm cells (83) including 25 potentially novel miRNAs processed in sperm cells and pollen. Of them, miR159 was particularly interesting, since it was highly enriched in sperm cells and was predicted to be involved in the regulation of *DUO1* (Palatnik et al. 2007, Grant-Downton et al. 2009a). miR156 and miR158 represented other candidates for the role in the male germline as they were enriched in sperm cells and likely to associate with sperm cell-enriched ARGONAUTE 5 (see Borges et al. 2011). Rice studies (Peng et al. 2012; Wei et al. 2011) described the miRNAome dynamics during male gametophyte development in stages previously used for the transcriptome analysis (Wei et al. 2010). The authors identified numerous known and novel miRNAs, often pollen-enriched, and showed the correlation of their expression profiles with their potential targets (Wei et al. 2011). In maize, the comparison of miRNA populations isolated from mature pollen, in vitro-cultivated-pollen tubes and non-pollinated as well as pollinated silks identified 56 miRNAs (40 conserved and 16 novel) differentially expressed between pollen and pollen tubes and 38 miRNAs (30 conserved and 8 novel) showing differential expression pattern between mature non-pollinated and pollinated silks. The analyses of these miRNAs and their potential targets (predominantly auxin signal transduction and transcription regulation) also showed the participation of miRNA pathway in the regulation of pollen–pistil interactions (Li et al. 2013). In loblolly pine, miRNA populations were compared between mature and germinating pollen and 47 miRNAs (23% of 208 identified in total) representing 22 families were upregulated and downregulated (14 and 8 families, respectively) in germinated pollen. Together with other similarities and differences with *Arabidopsis* and rice pollen miRNA populations, it highlighted that the microRNA pathway is active also during pollen germination in gymnosperms (Quinn et al. 2014).

The methylome sequencing of haploid cell types during male gametogenesis highlighted the differential methylation patterns in vegetative and sperm cells (Calarco et al. 2012, Ibarra et al. 2012). Plant male germline retains symmetric DNA

methylation, whereas the asymmetric methylation is lost there. On the contrary, asymmetric DNA methylation is restored in vegetative cells and during post-fertilisation embryo development. This *de novo* CHH methylation is a result of the activity of DOMAINS REARRANGED METHYLTRANSFERASE2 (DRM2) and employs 24-nt siRNAs. Differential genome reprogramming in pollen contributes to epigenetic inheritance, imprinting and transposon silencing (Calarco et al. 2012).

Of the two specialised proteomics-based techniques, allergomics was applied to a wider selection of plant species covering five orders of monocots and dicots (Table 10.2). For the obvious relation to pollen allergenicity, the selection lacks the usual models but concentrates mainly on wind-pollinated plants producing large amounts of pollen, predominantly grasses (Abou Chakra et al. 2012; Campbell et al. 2015; Kao et al. 2005; Schmidt et al. 2010; Schulten et al. 2013) and ragweed (*Ambrosia* spp.; Bordas-Le Floch et al. 2015; Zhao et al. 2016). Therefore, all studies were performed on mature pollen and were gel based. The purified proteins were separated by 2-DE, human IgE-binding proteins were identified, excised and analysed by mass spectrometry. Most of the major pollen allergen families were found in both dicot and monocot pollen—profilin, expansin, berberine bridge enzyme, pectate lyase, Ole e 1, cytochrome C and group 5/6 grass allergen (ribonuclease) families followed by enolase, EF hand, polygalacturonase, pathogenesis-related and prolamin families (Abou Chakra et al. 2012; Bordas-Le Floch et al. 2015; Campbell et al. 2015; Kao et al. 2005; Schmidt et al. 2010; Schulten et al. 2013; Zhao et al. 2016). In *Phleum pratense*, the major grass allergens were confirmed also in pollen cytoplasmic granules released from pollen grains that may represent respirable vectors of allergens (Abou Chakra et al. 2012).

Sexual reproduction in plants requires extensive cell–cell communication at many stages and at many levels including pollen–pistil interaction involving female sporophytic and gametophytic tissues that ends by the direct communication between male and female gametes preceding fertilisation. The extreme compactness of flower tissues as well as the need to separate individual cells made research in this area immensely difficult, and so far, most of the information came from the female side (reviewed by Kessler and Grossniklaus 2011, see also Chap. 8). The attempts to characterise the male–female crosstalk at the global scale first represented transcriptomic and proteomic studies of complex female tissues typically before and after pollination. Most of these studies also studied the phenomenon of pollen (in)compatibility. However, before that, it was interesting to analyse the pollination interface. Sang et al. (2012) analysed the differences in the proteomes of wet and dry stigmas in *Nicotiana tabacum* (wet) and *Zea mays* (dry) and compared them with the exudates from wet tobacco stigmas. With 177 identified proteins, tobacco stigmatic exudates were richer in proteins than stigmatic exudates of *Lilium longiflorum* and *Olea europaea* comprising 51 and 57 proteins, respectively (Rejon et al. 2013). Similarly, Nazemof et al. (2014) identified proteins involved in Triticale stigma development. However, these studies had only limited coverage because of the use of gel-based proteomics. Most recently, protein composition of ovular secretes, pollination drops, on female cones of two closely related gymnosperm species—*Cephalotaxus koreana* and *C. sinensis*—was similar to that of other

gymnosperms including gnetophytes and contained mainly defence-related proteins and carbohydrate-modifying enzymes (Pirone-Davies et al. 2016). Therefore, a deeper insight was achieved through transcriptomics when transcriptomes of stigmatic papillary cells in three *Brassicaceae* species, *Arabidopsis thaliana*, *A. halleri* and *Brassica rapa*, showed great degree of similarity. Fifty-eight percent of papilla-expressed genes were shared by all three species (Osaka et al. 2013), and only a minor fraction of expressed genes was species specific. Interestingly, gene expression in *Arabidopsis* papillar cells does not seem to be much influenced by the pollination, since 77% of genes were active in non-pollinated papillar cells as well as in these cells after the pollination with compatible and incompatible pollen (Matsuda et al. 2014).

Another step forward represented the comparison of non-pollinated and pollinated pistils studied at both transcriptomic and proteomic levels. Proteome differences caused by pistil pollination were studied in *Glycine max* (Li et al. 2012), *Oryza sativa* (Li et al. 2016), *Prunus armeniaca* (Feng et al. 2006), *Solanum pennellii* (Chalivendra et al. 2013) and, interestingly, also in basal angiosperm *Liriodendron chinense* (Li et al. 2014). Transcriptomic studies comprised *Arabidopsis thaliana* (Boavida et al. 2011), *Olea europaea* (Carmona et al. 2015; Iaria et al. 2016), *Oryza sativa* (Li et al. 2016), *Citrus clementina* (Caruso et al. 2012) and the direct comparison of self-compatible *Solanum pimpinellifolium* and self-incompatible *Solanum chilense* (Zhao et al. 2015). These studies confirmed that not only pollination but also cross- versus self-pollination induced novel gene expression and protein synthesis.

For the characterisation of proteins directly involved in male–female interactions, it became necessary to analyse proteins secreted from both main players (see also Chap. 8). The comparison of apoplastic proteins isolated from *Arabidopsis thaliana* mature pollen and pollen tubes cultivated in vitro for 6 h enabled the identification of 71 novel proteins expressed after pollen germination. Of them, 50 proteins were secreted; they were involved in cell wall modification and remodelling, protein metabolism and signal transduction (Ge et al. 2011). This study provided the first insight into pollen-secreted proteins functioning in pollen germination and pollen tube growth. However, the use of DIGE limited the number of identified proteins. More importantly, it was already shown that the contact with female tissues significantly changed and enriched pollen tube gene expression (Qin et al. 2009; Lin et al. 2014). Therefore, it became necessary to evaluate the situation in vivo or semi-in vivo. Recently, such secretomes were published for two related species. Proteomic analysis of *Solanum chacoense* ovule exudates isolated by tissue-free gravity-extraction method enabled the identification of 305 ovule-secreted proteins, 58% of which appeared to be ovule specific (Liu et al. 2015). Similarly, gel-free proteomics was used to characterise the secretome of *Nicotiana tabacum* semi-in vivo cultivated pistil-activated pollen tubes (Hafidh et al. 2016b). Here, 801 proteins were identified with high frequency of small proteins <20 kDa. Interestingly, the majority (57%) of pollen tube-secreted proteins lacked signal peptide and were shown to be secreted unconventionally. This study not only highlighted a potential mechanism for unconventional secretion of pollen tube proteins but also indicated

their potential functions in pollen tube guidance towards ovules for sexual reproduction. Hafidh et al. (2016b) demonstrated that the knockdown of unconventionally secreted translationally controlled tumour protein (TCTP) in *Arabidopsis thaliana* pollen tubes caused their poor navigation to the target ovule and low transmission of the mutant allele through the male. Unconventional protein secretion was described also in the ovules (Liu et al. 2015) but to a much smaller extent. The combination of both datasets, although obtained from different species, represents a significant contribution to our current efforts for dissecting possible mechanism for cell–cell communication between the pollen tube and female reproductive cells.

10.6 Conclusion and Perspective

The introduction of -omics techniques brought a very notable insight into the research of male gametophyte development, its dynamics and regulation. The past two decades and recent few years in particular faced the outburst of novel -omics techniques. However, transcriptomics still represents the main source of information and usually the first choice mainly due to its robustness, established infrastructure and data processing pipelines. Both microarrays and RNAseq offer big gene coverage, but the microarrays depend on a *priori* knowledge of sequence information and cannot display, for instance, splicing variants and/or shortened or altered transcripts. Although these issues are circumvented by deep sequencing technologies, the majority of data was achieved by the microarrays. That is why mostly male gametophytes of model plants were studied and only a limited number of alternative species have a known pollen transcriptome. Nevertheless, no transcriptomic technique solved the issue of the correlation of the abundance of transcripts to corresponding proteins. Therefore, proteomics is considered more reflecting gene regulation since it proves the presence of a protein. The original in-gel techniques showed limited gene coverage since they usually identified no more than 1000 proteins with a little overlap between independent experiments. On the other hand, because proteomics does not rely on the knowledge of genomic sequences, male gametophyte proteomes of non-model plants were studied. The versatility of proteomic approaches also enabled specialised studies like specific sets of proteins (membrane proteome, secretome, allergome) as well as functional and regulatory post-translational modifications, usually on limited samples only. Of them, phosphoproteome was studied in male gametophyte, namely, in three angiosperms—*Arabidopsis thaliana*, tobacco and maize. In tobacco, phosphoproteome dynamics during pollen activation was studied. In order to evaluate the regulation of early phases of pollen activation in bi- and tri-cellular species, it would be interesting, although challenging, to perform similar experiments on the tri-cellular species to acquire properly activated pollen, but the novel information brought by these approaches is definitely worth the effort.

In spite of the growing list of proteins with known function, pollen -omics revealed many proteins with unknown function or unclear classification that will

surely deserve a further investigation by the subsequent functional studies. These unknowns likely represent pollen-specific proteins or proteins with a notable function related to male gametophyte. Integration of phylogenetic comparative methods with the analysis of genomic data enables to design experiments and generate new insights concerning the origin and structure of the genomes. The phylogenomic approach based on sequence similarities can identify gene duplications—orthologs vs. paralogs, infer evolutionary rate variation among taxa and separate sequence convergences from shared origins. The prediction of gene function can be improved by incorporating the evolutionary history of the genes themselves and reconstructing their historical sequence and function using a phylogenetic framework. However, the majority of available plant genome sequences originate from crop plants that make deeper phylogenomic analyses still unfeasible. In the future, with more data available, the comparison of gene families among species from a phylogenetically different group of plants will allow a comprehensive study of pollen-specific genes and gene family evolution in plants.

Perhaps the most interesting trend in modern work has been a move towards synthesis. During the past two decades, various high-throughput plant -omic studies have revealed a boom due to technological advancement. Numerous articles focused not only on model plants but also other organisms. However, some of these analyses were carried out with expertise on the bioinformatics field, but with minimal biological relevance. The most interesting part of the research started with integration of data from various resources as well as the combination of these two fields.

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Chapter 11
The Pollen Membrane Proteome

Heidi Pertl-Obermeyer

Abstract The male gametophyte (or pollen) is a highly specialized organ essential for sexual reproduction of higher plants. Their reduced complexity constitutes them as an ideal experimental system for analyses of biological processes maintaining tip growth. Rapid advances in proteomic technologies and a vast choice of metabolic labelling and label-free quantitation protocols as well as the availability of full genome sequences allow comprehensive analyses of various pollen proteomes. Pollen membrane proteome consists of integral and membrane-associated proteins involved in regulation of many cellular functions. In this chapter, novel insights into identification of membrane proteins by proteome analysis and how their dynamic subcellular localization contributes to the initiation of pollen grain germination and maintenance of tube growth are discussed.

Keywords Mass spectrometry • Membrane proteins • Protein-protein interactions • Proteomics

Abbreviations

2D-PAGE	Two-dimensional polyacrylamide gel electrophoresis
2D DIGE	Two-dimensional difference in gel electrophoresis
ACA	Autoinhibited-type Ca^{2+} ATPase
BiFC	Bimolecular fluorescence complementation
CaM	Calmodulin
COP	Coat protein complex
ECA	Endoplasmic reticulum-type Ca^{2+} ATPase
ER	Endoplasmic reticulum
ESI Q-TOF MS/MS	Electrospray ionization quadrupole time-of-flight tandem mass spectrometry
GFP	Green fluorescent protein

H. Pertl-Obermeyer (✉)
Molecular Plant Biophysics and Biochemistry, Department of Molecular Biology, University of Salzburg, Billrothstr. 11, 5020 Salzburg, Austria
e-mail: h.pertl-obermeyer@sbg.ac.at

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IEF	Isoelectric focusing
LC-MS^n	Liquid chromatography coupled with multistage accurate mass spectrometry
MALDI-TOF MS	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometer
mbSUS	Mating-based split-ubiquitin system
p*I*	Isoelectric point
PIP	Plasma membrane intrinsic proteins
PM	Plasma membrane
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment protein receptor
TGN	Trans-Golgi network
VDAC	Voltage-dependent anion channel

11.1 Introduction

Due to its simple morphology and highly specialized function, the growing pollen tube is a well-established model system to study tip growth mechanisms. In general, tip-focused growth of pollen tubes is regulated by a network of cellular processes and components that are mainly localized in the tube apex and subapex region. Many of these processes are related to membranes, e.g. membrane-associated cytoskeleton, a highly active membrane trafficking system for endo- and exocytosis, signal transduction pathways including membrane receptors, cytosolic Ca^{2+} concentration (Ca^{2+}_{cyt}) and pH (pH_{cyt}) as well as localized ion fluxes across the plasma membrane of pollen tubes and grains (Feijó et al. 1995, 2001; Holdaway-Clarke and Hepler 2003; Bibikova et al. 2004; Cheung and Wu 2008; Michard et al. 2009, 2017). Of special note, ion transporters in the plasma membrane are generating the typical current pattern of ion fluxes that possibly functions as a navigation system for the tube's growth rate and growth direction (Holdaway-Clarke and Hepler 2003; Michard et al. 2009, 2017). Fluxes of protons (H^+), potassium (K^+), chloride (Cl^-) and calcium ions (Ca^{2+}) across the plasma membrane contribute to cytosolic ion concentrations but also to the complex electrical current pattern along the pollen tube. Although electrophysiological techniques like the ion-sensitive vibrating probe or patch-clamp analysis give a detailed view of the ion current pattern around growing pollen tubes and characterize single ion transporters, they cannot identify the particular ion transporter protein or protein complex that is generating the current nor give a comprehensive view of all ion transporters involved in current pattern generation. In addition, signal transduction pathways are also involved in the regulatory network that contribute to activate or inactivate cellular processes including membrane receptors and components that modulate ion transporter activities like the cytosolic-free Ca^{2+} concentration, the cytosolic pH, protein phosphorylation and 14-3-3 proteins, G-proteins and phospholipids (Kost et al. 1999; Pertl et al. 2001; Potocky et al. 2003; Monteiro et al. 2005; Michard et al.

2008). All mentioned components were found to be essential for tip growth, and it is assumed that they also interact with each other forming a complex spatial-temporal regulatory network controlling the events necessary for pollen tube growth.

Despite the important role of the polar distribution of plasma membrane proteins, only a few plasma membrane proteins were localized in pollen tubes by immunolocalization or GFP-fusion proteins, including the PM H^+ ATPase (Obermeyer et al. 1992; Certal et al. 2008), a Ca^{2+} ATPase (Schiott et al. 2004), the SLAH3 anionic channel (Gutermuth et al. 2013), members of the cyclic nucleotide-gated channels (CNGC; Frietsch et al. 2007; Tunc-Ozdemir et al. 2013; Gao et al. 2016) and components of signal transduction pathways, e.g. calcium-dependent protein kinase (Myers et al. 2009; Gutermuth et al. 2013), CIPK/CBL pairs (Steinhorst et al. 2015) or inositol phosphate kinases (Ischebeck et al. 2008), among many others. However, an intrinsic property of a regulatory network is that all components are somehow connected and the network as a whole functions in tube growth. Therefore, we need to investigate as many network components as possible under the same experimental conditions to understand the dynamics of the pollen tube growth process entirely. A proteomic approach identifying the membrane proteins and membrane-associated signalling proteins may be a first step to understand the protein interaction network responsible for maintenance of pollen tube elongation.

11.2 Pollen Proteome

11.2.1 Basic Proteomic Approaches for Model and Non-model Organisms

Rapid advances in proteomic technologies are tightly associated with completion of genome sequences of model plants or relevant crop plants like *Arabidopsis* (Arabidopsis Genome Initiative 2000), rice (*Oryza sativa,* Yu et al. 2002; Goff et al. 2002), maize (*Zea mays,* Schnable et al. 2009), barrelclover (*Medicago truncatula,* Young et al. 2011), tobacco (*Nicotiana benthamiana,* Bombarely et al. 2012), tomato (*Solanum lycopersicon*, The Tomato Genome Consortium 2012) and poplar (*Populus trichocarpa,* Tuskan et al. 2006) as well as the availability of comprehensive public sequence databases. Functional composition analysis of the *Arabidopsis* pollen transcriptome revealed that the mRNAs presented in pollen mainly encode proteins involved in cell wall metabolism, vesicle transport, cytoskeleton and signalling (Becker et al. 2003; Honys and Twell 2003, 2004; Pina et al. 2005; Wang et al. 2008; Loraine et al. 2013) which reflects their functional specialization in initiation of pollen germination and maintenance of tube growth. Although the analysis of mRNA expression is useful for general studies, the presence or absence of a transcript does not necessarily reflect the expression of the encoded protein. Thus, in addition to transcriptome analyses, a detailed analysis at the protein level is an essential step towards the further identification of functional

components involved in pollen germination and tube growth. Proteomics, however, provide new insights on the mechanisms of pollen development and germination in higher plants (reviewed in Dai et al. 2007b), but so far, only few detailed proteome studies have been carried out on pollen considering the temporal dynamics of pollen tube growth process.

Recently, a comparative proteome analysis of tobacco pollen showed that pollen development can be divided into three phases. The early phase that ends with release of the microspores is characterized by the so-called sporophytic proteome, which is relatively static. During the intermediate phase, the sporophytic proteome is partially degraded, whereas the late phase presents a 'gametophytic proteome' that contains many proteins required for pollen tube growth and cell wall synthesis (Ischebeck et al. 2014). In addition, a combination of two-dimensional gel electrophoresis (2-DE) and mass spectrometry (MALDI-TOF MS and ESI Q-TOF MS/MS) applied to mature rice pollen identified several new proteins involved in signal transduction, cell wall remodelling and metabolism, protein synthesis, assembly and degradation, as well as carbohydrate and energy metabolism (Dai et al. 2006). Also, new proteins involved in defence mechanisms, energy conversion, hormone metabolism and signalling, and membrane transport were identified in tomato pollen by proteome analysis (Sheoran et al. 2007). Quite recently, the pollen membrane proteome from two tomato cultivars was analysed to obtain a general overview on membrane function in pollen (Paul et al. 2016). In both cultivars, more than 150 proteins were assigned as putative membrane proteins, mainly involved in energy-related processes (Krebs cycle and glycolysis) that emphasize the protein content of mature pollen as energy reservoir for subsequent pollen germination and pollen tube growth. *Arabidopsis thaliana* is excellently suited for large-scale proteome analyses because of its small genome size and well-annotated gene databases. Of the approximately 29,000 genes in the *Arabidopsis* genome (Alonso et al. 2003), approximately 6600 are expressed in pollen, and only 10–20% of these genes are pollen specific (Holmes-Davies et al. 2005). Nearly half of the pollen-specific mRNAs were found to encode for proteins needed for building a cytoskeleton, for cell wall metabolism and for signal transduction that also supports the hypothesis that mature pollen grains contain mRNAs which then encode proteins for rapid germination and maintenance of pollen tube growth. In that context, most identified proteins are necessary for cell and cell wall structure (glycosyl hydrolase family protein, pectin methylesterase, pectin methylesterase inhibitor family protein, actin, actin-depolymerizing factors and tubulin), energy metabolism (ATPase synthase subunits, malate and isocitrate dehydrogenases and malate oxidoreductase) and signal transduction (calnexin 1, GTP-binding protein SAR1B, inositol 1,3,4-triphosphate 5/6 kinase, Holmes-Davies et al. 2005).

Proteome analyses of pollen were mainly performed by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and by comparison between protein spot pattern of wild-type and mutant plants. 2D gel-based proteomics has valuable features, e.g. its reproducibility and robustness, which make it often an attractive choice when a proteomic strategy must be selected (Rabilloud 2014). Nevertheless, 2D-PAGE based on isoelectric focussing (IEF) is technically limited because it is

mainly restricted to soluble and/or high abundant proteins. In addition, 2D-PAGE is subject to restrictions, which include limited dynamic ranges and difficulties in handling hydrophobic proteins and in detecting proteins with extreme molecular weights and isoelectric point (p*I*) values. The use of this technique may account for the relative low number of identified membrane proteins in earlier pollen proteome studies (Dai et al. 2006, 2007b; Sheoran et al. 2006). Therefore, alternative approaches that are based on mass spectrometry techniques for identification of complex peptide or protein mixture and quantification methods have been developed. Mass spectrometry-based protein quantification represents an important enhancement to simple identification proteomics by enabling a comparison of protein expression levels between different samples or treatments. Comparative protein quantification by mass spectrometry typically employs stable isotope incorporation, such as isotope-coded affinity tag (ICAT), stable isotope labelling by amino acids in cell culture (SILAC), $^{15}N/^{14}N$ metabolic labelling and isobaric tags for relative and absolute quantification (iTRAQ). However, certain limitations, including additional sample processing steps, cost of labelling reagents, insufficient labelling, difficulty in analysis of low abundance peptides and limitation of sample number, are associated with the use of these labelling techniques (Zhang et al. 2013). As an alternative approach, comparative quantification of label-free LC-MS^n proteomics has emerged. Quantification of protein expression using a label-free method can be achieved by two methods: (1) spectral counting where the frequency of peptide identification of a particular protein is used as a measure of relative abundance and (2) ion intensity, which uses the mass spectrometric chromatographic signal intensity of peptide peaks belonging to a particular protein. Label-free approaches are applicable to all types of biological samples and are not limited by the source of the sample, the number of samples, or the number of time points to be compared. Spectral counting is based on the rationale that an increase in protein abundance will result in an increase in the number of product ion spectra (spectral count) collected for peptides from that protein (Kota and Goshe 2011). However, spectral counting performs poorly with low abundance proteins and does not take into account posttranslational modifications (PTMs) (Schulze and Usadel 2010). Such a label-free 'shotgun' proteomic approach identified approximately 3500 proteins in mature *Arabidopsis* pollen, and the integration of obtained proteomic data with published transcriptomics data sets resulted in more than 500 proteins that were not previously identified in mature pollen (Grobei et al. 2009). Additionally, this analysis revealed that proteins of the categories transcription, cell cycle and DNA processing are underrepresented in mature *Arabidopsis* pollen supporting the old idea that transcription is not essential for pollen tube growth (see Mascarenhas 1975). As a first comprehensive as well as detailed shotgun pollen proteome analysis in a non-model organism, namely, *Lilium longiflorum*, more than 270 membrane and membrane-associated proteins of different organelle membrane fractions were identified at five physiologically important time points for pollen germination and tube growth (Pertl et al. 2009). Identified proteins were mainly involved in cytoskeleton turnover, carbohydrate and energy metabolism, as well as ion transport, membrane/protein trafficking, signal transduction, stress response

and protein biosynthesis. In an iTRAQ-based comparative quantitative proteomic approach in lily (*Lilium davidii*) focussing on plasma membranes of pollen grains and pollen tubes, more than 220 integral and membrane-associated proteins were identified (Han et al. 2010). Only 14 proteins showed a differential expression pattern in the plasma membrane during the transition from pollen grains to pollen tubes, but these proteins are mainly involved in signalling, transport and membrane trafficking.

In addition to internal compartments, the apoplast of plant tissue, which includes the cell wall matrix and the intercellular space, has some distinct functions such as growth regulation, sustaining skeleton, homeostasis of the internal environment and transportation route (Sakurai 1998). However, detailed information about the functions of apoplastic proteins in pollen germination and during pollen tube growth is scarce. Most knowledge comes from screening of mutants, in which identified proteins were mainly involved in adhesion, hydration and maintenance of pollen tube growth (Cheung and Wu 2008; Mayfield and Preuss 2000; Jiang et al. 2005). To gain a deeper insight into the function of apoplastic proteins, Ge and co-workers (Ge et al. 2011) analysed the apoplastic proteome of mature and germinated *Arabidopsis* pollen grains by 2D DIGE and LC-MS/MS. 103 spots were significantly differentially expressed after pollen germination, and 98 spots (representing 71 proteins) were identified. Of these, 50 proteins were found to be apoplastic and mainly involved in cell wall modification and remodelling, protein metabolism and signal transduction. As the apoplast is the first subcellular component of the pollen to be exposed to signals from the surrounding stigma or pistil tissues, it is hypothesized to have important roles in signal transduction. During pollen tube growth, new cell wall has to be synthesized quickly, and, on the other side, the cell wall of the stigma and the transmitting tract then have to be loosened. Additionally, the rapid growth requires a fast turnover of proteins. Taken together, these results from proteome studies highlight the importance of signal transduction, energy metabolism, transport of ions and nutrients, protein biosynthesis as well as cell wall biosynthesis and endocytosis in maintenance of polarized tip growth.

11.2.2 Pollen Allergomics

Since pollen allergens are mainly proteins of the pollen apoplast and pollen coat or associated with the pollen cell wall, much information on pollen proteins and pollen cell wall proteomes can be found in studies of pollen allergens. However, these data were collected in respect to allergic reactions in humans and are, therefore, only of limited use for plant physiologists but still provide a convenient source of sequence information, especially for non-sequenced pollen species. Because pollen allergy affects about 25% of the population in industrialized countries, and numbers are still increasing, the determination of the allergenic potential of several pollen proteins is of public interest. Allergies are immunological disorders, characterized

by immune responses directed against normally harmless environmental substances, such as airborne grass or tree pollen, as well as weed pollen species. Sensitive people then produce IgE antibodies that lead to allergic symptoms, ranging from rhinitis to bronchial asthma (Petersen et al. 2006).

Plant allergens have various roles: some serve as storage proteins, some are involved in plant defence and lipid transport, and others act as protease inhibitors, actin-binding proteins or structural proteins. Pollen allergens are classified according to their biochemical structure and immunological reactivity. For detection of allergens in plants, proteomic techniques that target allergens, i.e. allergomics, have become powerful tools for comprehensive allergen analysis (Nakamura and Teshima 2013, see also Chap. 10). Compared to conventional methods, e.g. classical isolation methods of single proteins, proteomics has clearly accelerated identification of multiple allergens in pollen. Important issues regarding allergen identification and quantification are the sensitivity and the specificity of the applied method. Mass spectrometry-based approaches are useful to investigate the various biological questions related to pollen allergy and have some advantages over routinely used methods to detect and quantify allergens. For example, ELISAs, where IgG antibodies from immunized animals are used for allergen detection, are only as good as the specificity of the used antibodies. On the one hand, an IgG antibody may not recognize all isoforms of a particular allergen or, on the other hand, may also recognize isoforms of the allergen that do not trigger an immune response (Schenk et al. 2010). Mass spectrometry methods have the advantage that they detect the allergens directly and independently of antibody specificity, namely, unbiased. One limitation of allergomics is that it is impossible to know the allergenic potential of the novel, identified IgE-binding proteins, because not all IgE-binding proteins cause allergic responses (Nakamura and Teshima 2013).

To analyse putative allergenic pollen proteins by mass spectrometry, special care has to be taken about the source of pollen material and the way how the material is prepared. Allergen expression levels have been shown to vary depending on genetic backgrounds and environmental factors during pollen development. For identification of allergenic pollen proteins, pollen grains are often incubated in solutions to release the allergenic proteins. These fractions have to be denoted as ‘pollen exudates’ or ‘pollen diffusates’ to distinguish them from pollen extracts which are prepared by damaging the pollen grains, and therefore, the high amounts of intracellular proteins would cover the less abundant allergens (Hoidn et al. 2005; Pertl-Obermeyer and Obermeyer 2013). Allergens are often members of multigene families, whose genes encode for similar isoforms that differ only by a few amino acids (Schenk et al. 2010). Different isoforms of pollen allergens often vary in their immune reactivity as shown for the major birch allergen Bet v 1 (Ferreira et al. 1996) with hypoallergenic isoforms that can be used as putative valuable tools for immunotherapy. Their allergenic potential is not determined by the total allergen content but by the quantity of the hypoallergenic isoform or the quantity of their specific peptides. Mass spectrometry methods can determine their quantities by the use of stable isotope-labelled internal standards or by label-free approaches, i.e. comparison of signal intensity or spectral counting. Such a label-free MS procedure

was used to screen pollen of different birch species and varieties for identification of hypoallergenic birches (Silva et al. 2005). Nevertheless, the main drawbacks for identification of allergens by mass spectrometry are the still incomplete databases used for peptide search.

11.3 Pollen Membrane Proteome

The ultimate challenge of membrane proteomics is to develop a method that allows extracting, separating and identifying the often low abundant peripheral and integral membrane proteins out of the highly heterogeneous mixture of proteins within a cell. Membrane proteins vary in abundance and show extremely different physicochemical properties, e.g. different hydrophobicity or extreme isoelectric points (p*I*), which makes it difficult to separate them by chromatography techniques. Therefore, an analysis of membrane proteins very often requires the combination of various extraction and fractionation strategies.

In pollen, the tip-focused growth is regulated by a network of cellular processes, including a highly active and dynamic membrane/protein trafficking system including endo- and exocytosis. For example, plasma membrane (PM) proteins move through the endoplasmic reticulum (ER) and the Golgi apparatus before they reach their sites of action and are recycled via endocytosis. Therefore, only a comprehensive view of many membrane compartments reflects the dynamic subcellular distribution of membrane proteins. Although pure membrane fractions can be achieved by aqueous two-phase partitioning systems, it gives only limited insights because only a single membrane compartment can be isolated during one preparation and some subcellular organelle membranes are still difficult to purify (Larson 1983). To overcome these limitations, separation of endomembrane and plasma membrane vesicles by (dis)continuous density centrifugation (Fig. 11.1) allows analysing the dynamics of protein abundance in different cellular compartments (Obermeyer et al. 1996; Pertl et al. 2005, 2009). One of the major advantages in using a (discontinuous) sucrose step gradient is that all organelle fractions from different physiological states during pollen grain germination and tube growth can be separated and enriched in a single preparative step for subsequent mass spectrometry analysis.

To assign the interphases of the step gradient to organelle membrane-enriched fractions, the distribution of typical organelle marker proteins across the gradient has to be analysed (Fig. 11.2). Therefore, well-characterized membrane proteins with known location to different subcellular compartments, such as mitochondria, endoplasmic reticulum, Golgi apparatus, plasma membrane and vacuole, have to be chosen. For example, known residents of the plasma membrane are members of the PM H^+ ATPase family or plasma membrane-localized aquaporins (PIPs). Markers for the endoplasmic reticulum are members of ER-localized Ca^{2+}-ATPases (ECAs) and ER-localized chaperons such as calreticulins (CRTs) and members of the HSP70 family (BIPs, Dunkley et al. 2006; Nikolovski et al. 2012). F-type ATPases

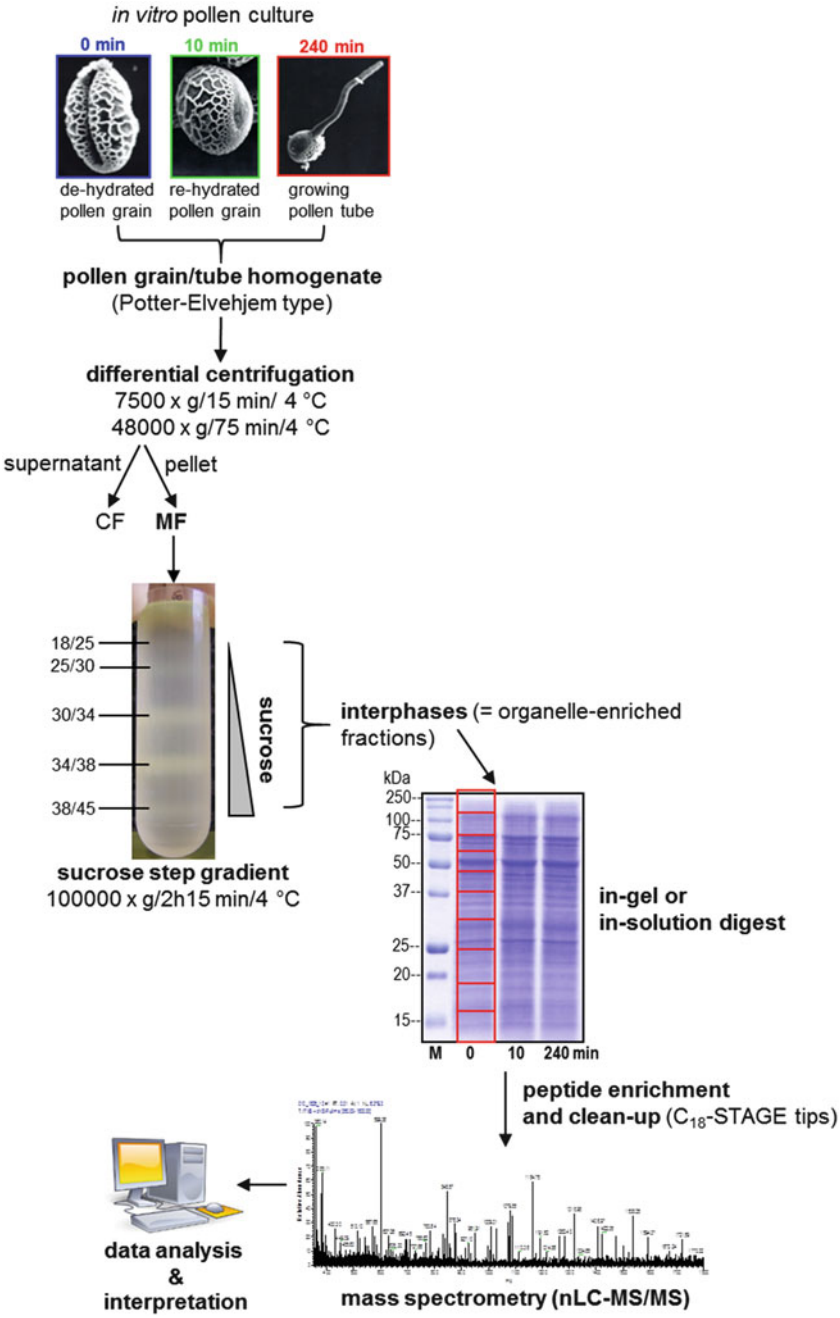

Fig. 11.1 Experimental design of a label-free shotgun proteomic workflow. A microsomal fraction (*MF*) was prepared from lily (*Lilium longiflorum*) pollen grains and tubes incubated for various times in germination medium representing specific states for lily pollen. The MFs obtained from the different time points were further separated by discontinuous sucrose density centrifugation resulting in interphases 18/25%, 25/30%, 30/34%, 34/38% and 38/45%. Proteins of each interphase and each time point were separated by SDS-PAGE or used directly (in-solution digest), and trypsin-digested proteins were analysed by mass spectrometry (nLC-MS/MS)

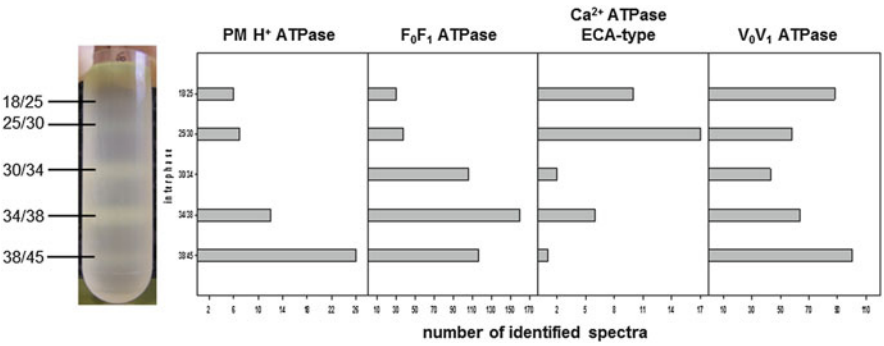

Fig. 11.2 Distribution of typical organelle marker proteins in the discontinuous sucrose gradient. Most spectra identifying the PM H^+ ATPase were found in interphase 38/45; for the F_0F_1 ATP synthase, most spectra were identified in interphase 34/38. For the ER-localized Ca^{2+}-ATPase, highest number of spectra were found in interphase 25/30, and in interphases 18/25 and 38/45, most spectra for the V_0V_1 ATPase were identified (Pertl et al. 2009)

and cytochrome c oxidase II, and ADP-ribosylation factors (ARFs) as well as glycosyl transferases are markers for mitochondria and Golgi-derived membranes, respectively. To assign proteins to vacuole-enriched fractions V-ATPases, the H^+-PPase or tonoplast-localized aquaporins (TIPs) are used as marker proteins (Pertl et al. 2009; Gattolin et al. 2009). A discontinuous sucrose gradient combined with marker analysis was used in a 'shotgun' proteomic approach to identify membrane and membrane-associated proteins from lily pollen grains and pollen tubes (Pertl et al. 2009). Thereby, an increase in abundance of proteins involved in cytoskeleton, carbohydrate and energy metabolism as well as ion transport before pollen grain germination was observed (10–30 min), whereas proteins involved in membrane/protein trafficking, signal transduction, stress response and protein biosynthesis decreased during early stages of pollen grain germination/tube growth (<30 min) followed by an increase just after tube germination (60 min, Fig. 11.3). Time-dependent changes in protein abundance may depict the up- and downregulation of a biological process during characteristic phases of pollen action. The changes in peptide/protein abundance between 10 and 30 min after starting the pollen culture emphasize the importance of this time period for initiating pollen germination. Although no visible signs of germination are observable during the first 30 min, a number of processes were postulated to take place inside the pollen grain: adjustment of ionic concentrations and pH, building up of a membrane potential and osmotic gradient, reorganization of the organelle membranes and the cytoskeleton, respiration and energy supply. These processes are well mirrored by the dynamic changes in protein abundances at this time, for example, the fast increase in the number of identified peptides involved in cytoskeleton organization and turnover or in energy metabolism and in ion transport. Proteins involved in some other biological processes, such as protein biosynthesis and membrane/protein trafficking, became more abundant after 30 min when the pollen grain starts to germinate,

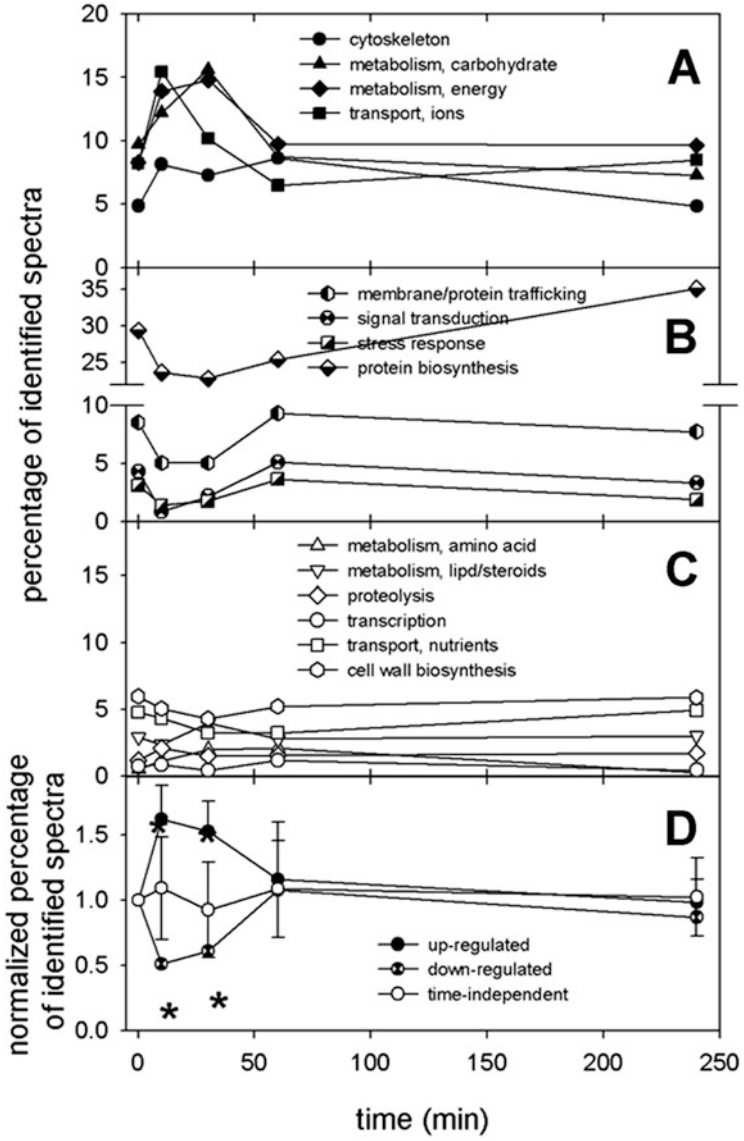

Fig. 11.3 Time dependence of functional classes during pollen grain germination and tube growth. Temporal changes in protein abundance could be observed for some biological processes during pollen grain germination and tube growth. The relative numbers of identified spectra were plotted against time. (**a**) Biological processes that show a higher abundance of peptides (spectra) in the first 30 min are 'cytoskeleton', 'carbohydrate metabolism', 'energy metabolism' and 'ion transport'. (**b**) The amount of peptides/spectra of the functional categories 'membrane/protein trafficking', 'signal transduction', 'stress response' and 'protein biosynthesis, folding and modification' was lower during the first 30 min and increased after rehydration and germination of the pollen grains. (**c**) All other biological relevant processes did not show significant changes during the 240 min of pollen grain in vitro culture. (**d**) The up- and the downregulated processes at time points 10 and 30 min were significantly different from their time point 0 min (Pertl et al. 2009)

thus reflecting the need of a constant supply of new proteins and the synthesis and delivery of membranes to the germination site/growing tube tip.

11.3.1 Membrane Proteome in Signalling and Stress Response

During their development or during the progamic phase, pollen grains can be exposed to stress conditions, e.g. like changes in metabolic activity or in response to desiccation. The pollen response to these stresses is accompanied by the activation of many genes and protein expression involved in stress perception and in transduction of stress signals, which results in further modulation of gene activity or protein expression. In tomato pollen, a member of aldehyde dehydrogenase (ALDH) gene family localized to plastids was identified. ALDHs are expressed during oxidative stress and are known to be critical in detoxification of aldehydes generated as a product of different cellular processes (Paul et al. 2016). In this study, also two plasma membrane-localized glycerophosphodiester phosphodiesterase-like family of proteins (GDPL) were identified, which play pivotal roles in conditions of inorganic phosphate (P_i) starvation. In tomato as well as in lily pollen, members of the dehydration-responsive protein-like family (DRP) localized to different cellular sub-compartments have been identified (Paul et al. 2016; Pertl et al. 2009). LEA (late embryogenesis abundant) proteins provide protection from dehydration and play important roles in pollen function at maturity and during subsequent germination and tube growth. Many LEA proteins are induced by cold or osmotic stress or by exogenous ABA and were found in *Arabidopsis* pollen (Sheoran et al. 2006; Grobei et al. 2009) and in lily pollen (Pertl et al. 2009). As stated, the external stress signals have to be sensed and transduced to the cell interior. In this context, PM-/ER-localized phosphoinositide phosphatases (PIPase) have been found. These proteins modulate the phosphoinositide (PI) level within cell membranes, thereby regulating various signal transduction processes. Moreover, membrane-associated proteins such as 14-3-3 proteins, phospholipase D, calmodulin and heterotrimeric G-protein complexes, all of them well known to play important roles in stress signal transduction, were identified (Dai et al. 2006; Pertl et al. 2009). 14-3-3s are major regulators in plant development and stress physiology, typically via a phosphorylation-dependent interaction with target proteins (Pertl et al. 2011; van Kleeff et al. 2014). Phospholipase D (PLD) hydrolyzes membrane phospholipids for the production of phosphatidic acid (PA) which play important roles in signal transduction via a proposed activation of the PM H^+ ATPase activity (Shen et al. 2011; Potocký et al. 2014). Calmodulin is a multifunctional receptor protein for intracellular Ca^{2+}, and heterotrimeric G-proteins are conserved signal-transducing proteins in the plasma membrane involved in tip growth (Ma et al. 1999). Recently, it was shown that G-protein subunit α participates in pollen germination through modulation of the hyperpolarization-activated Ca^{2+} channel in the plasma membrane of *Arabidopsis* pollen (Wu et al. 2007).

The accumulation of intracellular signalling molecules causes the modulation of enzyme activities or gene/protein expression and in growing pollen tubes in determination of the growth direction. As the growing pollen tube has to be guided to the ovule for fertilization, a meticulous communication between male and female organs and tissues is essential (see also Chap. 8). In tomato pollen (*Solanum lycopersicum*), two plasma membrane-localized LRR-RLKs (leucine-rich repeat receptor-like kinases), *Le*PRK1 and *Le*PRK2, were identified which are involved in the regulation of pollen germination and pollen tube growth (Löcke et al. 2010). They form heterodimers and are components of a multimeric protein complex in which the *Le*PRKs presumably have a key role in transducing exogenous signals through the plasma membrane of the growing pollen tube. Receptor-like kinases have been identified in various plant species and in *Arabidopsis*, the RLK family includes >600 members, with the LRR-RLKs constituting the largest group (Osakabe et al. 2013). Receptor-like kinases (RLKs) and LRR-RLKs have been identified in lily pollen (Pertl-Obermeyer et al. 2014) and RLKs in canola pollen (Sheoran et al. 2009) and in *Arabidopsis* pollen (Holmes-Davies et al. 2005). Again, proteome analysis of the entire membrane compartment or a particular signal transduction pathway enables the researcher to investigate the dynamics of all components of the pathway in a single experiment instead of single components in many experiments.

11.3.2 Membrane Proteome in Cell Wall Biosynthesis

The complex plant cell wall structure is built and maintained by diverse proteins involved in cell wall synthesis, modification and secretion. The major structural and functional constituents of the walls are hemicelluloses, cellulose, pectin and lignin, whose relative content varies depending on the species, tissue and cell development and growth stages (Kim and Brandizzi 2014). The Golgi and plasma membranes are the two main sites where non-lignin cell wall constituents are synthesized.

Upon contact with the stigma, the pollen grain has to transmit the pistil tissue and grow to the ovary to deliver the sperm cells for fertilization (Heslop-Harrison 1987). For all of these functions, the pollen tube cell wall plays an important regulatory and structural role (see also Chaps. 3 and 8). The secondary cell wall layer of pollen tubes consists of two layers, the outer formed by pectin and the inner formed by callose. Depending on the species, cellulose microfibrils have been found to be associated either with the outer pectic or with the inner callosic layer (Chebli et al. 2012). The pollen cell wall provides structural support during development, and its functional integrity has to be maintained during pollen tube growth. Several enzymes involved in cell wall synthesis like subunits of the PM-localized large multimeric cellulose synthase complexes (CESA), the callose synthase (CALS) and members of the pectin (methyl)esterase family have been identified in tomato, lily and *Arabidopsis* pollen (Paul et al. 2016; Pertl et al. 2009; Holmes-Davies et al. 2005; Dai et al. 2006). Additionally, cell wall component modifying proteins, such

as UDP-glucose-, UDP-galactose-, UDP-glucuronate- and UDP-xylose-epimerase, are also involved in cell wall synthesis and were identified in these pollen proteome studies.

11.3.3 Membrane Proteome in Protein Trafficking and Protein Biosynthesis, Folding and Modification

The secretory pathway consists of numerous functionally interlinked organelles. The first organelle of the secretory pathway is the endoplasmic reticulum (ER) in which proteins are synthesized and assembled for export to the Golgi apparatus. The Golgi apparatus then collects membranes and luminal cargo from the ER for further processing and sorting to distal compartments which include the trans-Golgi network (TGN), vacuoles and the plasma membrane (Kim and Brandizzi 2014). The secretory pathway is of importance to pollen especially for pollen tube elongation. Tip-directed trafficking of membrane vesicles and proteins involves pairing of SNAREs (Soluble N-ethylmaleimide-sensitive factor attachment protein receptors) to facilitate the membrane fusion. Functional classification divides SNAREs into vesicle-associated and target membrane-associated SNAREs (v- and t-SNAREs). Alternatively, the structural classification groups SNAREs as Q- and R-SNAREs owing to the occurrence of either a conserved glutamine (Q) or arginine (R) residue in the centre of the SNARE domain (Tyrrell et al. 2007). Generally, t-SNAREs correspond to Q-SNAREs, and v-SNAREs correspond to R-SNAREs. In that context, SNAREs and SNARE-associated proteins were identified in lily, tomato and *Brassica napus* pollen (Pertl et al. 2009; Paul et al. 2016; Sheoran et al. 2009). Additionally, ADP-ribosylation factor (Arf) and secretion-associated and Ras-related protein (Sar) are major regulators of vesicle biogenesis in intracellular trafficking controlling the assembly of coat protein to facilitate budding of COPI and COPII vesicles, respectively (Memon 2004), and were identified in tomato, *Arabidopsis* and lily pollen (Paul et al. 2016; Holmes-Davies et al. 2005; Pertl et al. 2009). Rab GTPases are known to associate with various organelle membranes and assist vesicle trafficking between ER and Golgi, trafficking of secretory vesicles and exocyst formation (Cheung et al. 2002; de Graaf et al. 2005; Hala et al. 2008), and in lily pollen, Rab-type small GTPases could be detected in pollen grains and growing pollen tubes (Pertl et al. 2009), as well as in tomato pollen (Paul et al. 2016) and in *Arabidopsis* pollen (Holmes-Davies et al. 2005). As expected GDP dissociation inhibitor proteins, GTP-binding proteins, clathrin, clathrin adaptor proteins, COP family proteins and dynamins, all involved in protein and membrane trafficking, were identified in pollen.

ER-localized members of the calreticulin family, which act as calcium-binding chaperones promoting protein folding, oligomeric assembly and quality control in the ER, were identified in several pollen proteome studies in *Arabidopsis* (Holmes-Davies et al. 2005; Noir et al. 2005; Sheoran et al. 2006; Zou et al. 2009), lily (Pertl

et al. 2009), tomato (Sheoran et al. 2007; Paul et al. 2016), canola (Sheoran et al. 2009) and rice (Dai et al. 2006, 2007a). Proteins involved in protein biosynthesis, such as ER-associated ribosomal proteins, elongation factors and prohibitins, were also found. Prohibitins play a crucial role in mitochondrial biogenesis, protein processing and transcriptional control (Van Aken et al. 2010). BiPs (luminal binding proteins) are ER-localized members of the HSP70 family and are known to bind to misfolded, underglycosylated or unassembled proteins whose transport from the ER is blocked. Oligosaccharyltransferase (OST) is a membrane protein complex in the ER that transfers a sugar oligosaccharide from dolichol to nascent proteins. All these proteins, including some other sugar transferases or disulphide isomerases as well as different heat shock protein family members and chaperones, which are involved protein folding and modification, were identified in these pollen proteome studies.

11.3.4 Membrane Proteome in Ion and Nutrient Transport

The plant plasma membrane (PM) is the outermost membrane of the cell that functions as interface with the extracellular environment for exchange of information and substances. The PM is a dynamic structure. A highly active vesicle transport to and from cytoplasmic organelles allows a rapid modification of the plasma membrane composition in response to stimuli by triggering downstream signalling events. The composition of the plasma membrane very often varies with the cell type, developmental state and environment, resulting in several combinations of different protein classes such as transporters, channels, receptors and signalling components.

In general, the PM H^+ ATPase is an important housekeeping enzyme generating an electrochemical H^+ gradient across the plasma membrane for the transport of nutrients and ions into the cell through various channels or carrier proteins (Palmgren 2001). Pollen grains of many species can be easily cultured in diverse synthetic culture media, and during in vitro cultivation, an acidification of the germination medium could be observed due to H^+ extrusion from the pollen grains (Lang et al. 2014 and references therein). It was demonstrated that the PM H^+ ATPase is responsible for the medium acidification (Pertl et al. 2010). In accordance to physiological studies showing an effect of the fusicoccin-activated or the vanadate-inhibited PM H^+ ATPase on pollen grain germination frequencies (Rodriguez-Rosales et al. 1989; Fricker et al. 1997; Pertl et al. 2001; Sun et al. 2009), the importance of the PM H^+ ATPase during the early state of germination, e.g. transition from the 'quiescent' to the hydrated state of pollen grains, is supported by the increase in PM H^+ ATPase proteins within the first 10 min of pollen grain culture (Pertl et al. 2009). Additionally, it has been demonstrated that in pollen grains of *Lilium longiflorum*, osmoregulation occurs via modulation of the PM H^+ ATPase activity (Pertl et al. 2010). An increase in PM H^+ ATPase activity and an increase in membrane-associated 14-3-3 proteins were detected upon hyper-osmolar treatment of pollen grains suggesting a modulation of its activity by phosphorylation and subsequent binding of 14-3-3 proteins. 14-3-3 proteins are well-characterized

modulators of the PM H^+ ATPase (Svennelid et al. 1999; Fuglsang et al. 1999; Maudoux et al. 2000). All studies showed a direct correlation between the PM H^+ ATPase activity and the pollen germination as well as tube growth and thus demonstrate an active PM H^+ ATPase as a prerequisite for successful pollen germination and tube growth. Surprisingly, besides lily pollen, proteins encoding a PM H^+ ATPase were only identified in proteome studies of tomato pollen (Paul et al. 2016) and of rice pollen (Dai et al. 2006). Compared to other organelle membranes, PM is less abundant, and therefore, the PM H^+ ATPase is easily missed in proteome analyses not using membrane-enriched fractions or 2D DIGE for protein fractionation.

Two other H^+-translocating proteins, the V-type ATPase and the H^+ pyrophosphatase (H^+ PPase), are more abundant and could be identified in several pollen species. Both proteins are known to reside in the tonoplast and endomembrane compartments of pollen (Mitsuda et al. 2001; Padmanaban et al. 2004; Dettmer et al. 2005). V-ATPase subunits were found in *Arabidopsis* pollen (Noir et al. 2005; Holmes-Davies et al. 2005; Sheoran et al. 2006; Zou et al. 2009), in canola pollen (Sheoran et al. 2009), in rice pollen (Dai et al. 2006, 2007a) as well as in tomato and lily pollen (Paul et al. 2016; Pertl et al. 2009). The overall high abundance of these subunits reflects the functional relevance of the formation of vacuoles during pollen germination and their role in acidifying the vacuole lumen. H^+ pyrophosphatases were identified in pollen of lily, tomato and *Arabidopsis* (Pertl et al. 2009; Paul et al. 2016; Noir et al. 2005; Sheoran et al. 2006), but not in rice pollen. These tonoplast-localized proton pumps play a central role in maintaining proton homeostasis.

Another group of proteins known to be essential for pollen development are Ca^{2+}-ATPases. They have a high affinity for Ca^{2+} (0.1–2 μM) (Sze et al. 2000) and are members of the P-type ATPase superfamily (Axelsen and Palmgren 1998). In plants, Ca^{2+} -ATPases can be subdivided into two phylogenetic groups, the autoinhibited-type Ca^{2+}-ATPases (ACA) and the endoplasmic reticulum-type Ca^{2+}-ATPases (ECA), which both are regulated by calmodulin (CaM). Both ECAs and ACAs have been found at the PM, tonoplast and endoplasmic reticulum (Sze et al. 2000). It has been shown that *A. thaliana* ACA7 is a plasma membrane protein that has an important role during pollen development, possibly through regulation of Ca^{2+} homeostasis (Lucca and León 2012). In *Arabidopsis* ACA9 is expressed almost exclusively in pollen, and knockout of ACA9 leads to significant impairment of pollen tube growth (Schiott et al. 2004). Surprisingly, ACAs and ECAs were only detected in proteome studies of tomato pollen (Paul et al. 2016) and lily pollen (Pertl et al. 2009).

Voltage-dependent anion channel (VDAC) is the most abundant protein in the mitochondrial outer membrane which mediates the transport of metabolites (succinate, citrate, malate, ATP and ADP) between mitochondria and the cytoplasm. VDAC proteins belong to a small multigene family: the *Arabidopsis* genome contains five putative VDAC genes, and in poplar (*Populus trichocarpa*) ten genes are found. Microarray assays showed that AtVDAC2, AtVDAC3 and AtVDAC4

are expressed in pollen (Homblé et al. 2012), and recently, it has been shown that VDAC members are also important for vegetative and reproductive growth, while T-DNA knockout mutants of AtVDAC2 showed retarded growth and abnormal pollen development (Tateda et al. 2012). The varying number of genes may indicate a diversity of VDAC functions in plants. Several studies demonstrated that VDAC can also be found in non-mitochondrial cell membranes, such as in the membrane of peroxisomes, glyoxysomes and chloroplasts as well as in the plasma membrane (Homblé et al. 2012 and references therein). VDAC family members were identified in proteome analyses of tomato pollen (Paul et al. 2016), rice pollen (Dai et al. 2006, 2007b), mature *Arabidopsis* pollen (Holmes-Davies et al. 2005) and lily pollen (Pertl et al. 2009).

The uptake of K^+ is important for tube growth by probably balancing the osmotic potential of the cytosol and the turgor pressure during rapid tube elongation (Benkert et al. 1997; Winship et al. 2010). Generally, K^+ influx is caused by voltage-gated and acidic pH-sensitive inward rectifying K^+ channels (Griessner and Obermeyer 2003). So far, SPIK (=AKT6, AT2G25600) from *A. thaliana* (Mouline et al. 2002) and an AKT1-like K^+ channel (LilKT1) from *L. longiflorum* are the only inward rectifying K^+ channels that have been identified in pollen (Safiarian et al. 2015).

Compared to cytosolic or soluble proteins, the listed examples of identified membrane proteins and transporter are less abundant in the pollen, and their peptides are often missed during mass spectrometry analysis. This supports the view that appropriate membrane preparation and enrichment methods are necessary to investigate membrane proteins (Pertl-Obermeyer and Obermeyer 2013). More often, opposing techniques might have to be applied to identify all, even the lowest abundant membrane proteins by proteomics, to obtain a comprehensive image on the dynamics of membrane proteins in an organism.

11.3.5 Membrane Protein Complexes in Pollen

In addition to identification of single proteins, the knowledge about protein complexes and protein interaction partners becomes more and more important, especially for membrane proteins. Many examples showed that transmembrane signalling across the plasma membrane directly affects membrane proteins that are bundled in functional complexes. For instance, the interaction between LRR-receptor-like kinase BRI1 (brassinosteroid-insensitive 1) and BAK1(Serk3) (BRI1-associated kinase1 also known as somatic embryogenesis receptor-like kinase) is necessary to perceive brassinosteroid signals and transmit the signal to cytosolic components, and interaction between BAK1 and PM H^+ ATPases AHA1 and 2 together with cyclic nucleotide-gated channel CNGC17 is involved in transduction of phytosulfokine signals in *Arabidopsis* plants (Bücherl et al. 2013; Ladwig et al. 2015). Apart from molecular biology techniques to investigate protein-protein interactions, e.g. mbSUS, BiFC, etc., cross-linked and immunoprecipitated membrane protein complexes can be analysed by proteomic methods (Liu et al.

2015). So far, membrane protein complexes had been investigated in lily pollen only (Pertl-Obermeyer et al. 2014). Membrane proteins were rapidly cross-linked by glutaraldehyde, and complexes containing the PM H^+ ATPase were purified using immunoprecipitation with specific antibodies against the pollen proton pump. Interestingly, a large number of receptor kinases and calcium signalling proteins as well as 14-3-3 proteins are closely associated in the plasma membrane with the H^+ ATPase, thus forming a functional complex that is able to react as fast as possible to external signals. This rapid cross-linking strategy is quite new for plant research but has now also been used to identify functional PIP (aquaporin) complexes in *Arabidopsis* roots (Bellati et al. 2016). This rapid cross-linking techniques allows to 'freeze' the proteins/complexes in a specific state after an external signal and is method with a high future potential to investigate dynamics of membrane protein complexes in a system showing fast responses to external signals and a dynamic growth behaviour: the pollen tube.

11.4 Conclusion and Perspective

So far, in most of the pollen proteome studies, single proteins and protein families have been listed or identified, and the pollen proteome has been compared to the proteomes of other tissues to reveal pollen-specific proteins or protein isoforms. In some studies, the differences in the proteome between two or three states, e.g. pollen grains and tubes, were investigated. However, the germination and growth of pollen tubes is a highly dynamic process. Pollen grains have to transit from a quiescent to an active phase after landing on the stigma surface and the tubes change growth speed or growth direction from 1 min to the other. To understand these dynamic processes, a much higher time resolution for pollen proteome studies is necessary to catch the proteins in the act. In the moment, we are just at the beginning of using the power of proteomic techniques to understand the dynamics of pollen tube growth. Furthermore, biological processes are carried out by interactions between several biomolecules, and especially membrane proteins may form functional units which allow a fast propagation of signals from one molecule to the other. Therefore, not only the identification of proteins involved in tip growth is important but also to know their interacting partners at specific moments. It might turn out that the proteins in the plasma membrane may have different interaction partners or are involved in different functional protein complexes, just depending on the physiological process to be performed, e.g. during straight growth, the PM H^+ ATPase may interact with other proteins than during cutting a corner. Proteome studies with a high temporal and spatial resolution in combination with interactome studies are needed in the future to reveal the role of functional membrane protein complexes in tip growth.

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Chapter 12
Pollen Metabolome Dynamics: Biochemistry, Regulation and Analysis

Thomas Nägele, Lena Fragner, Palak Chaturvedi, Arindam Ghatak, and Wolfram Weckwerth

Abstract The metabolome of an organism represents the readout of its biochemistry comprising numerous and tightly regulated metabolic pathways. Experimental analysis of the metabolome and its interpretation in a biochemically and physiologically meaningful context is focused by the research field of metabolomics which has become an integral part of many systems biological studies. Pollen development, germination and tube growth comprise numerous steps of metabolic regulation resulting in significant metabolome dynamics. To unravel involved regulatory molecular processes and to promote the understanding of developmental reprogramming and stress tolerance mechanisms in pollen, it is crucial to quantitatively resolve dynamics in the pollen metabolome. Since these dynamics affect various substance groups with different physico-chemical properties, different experimental platforms are needed for robust compound identification and quantification. It has been shown that developmentally and stress-induced metabolic reprogramming in pollen significantly affects the redox homeostasis as well as metabolism of carbohydrates, amino acids, lipids, polyamines, flavonoids and phytohormones. In this chapter, mechanisms of metabolic reprogramming are summarized and discussed in the context of pollen development and stress exposure. Finally, it is discussed how these metabolome dynamics can be resolved methodologically in order to unravel molecular physiological mechanisms of pollen development.

Keywords Metabolomics • Pollen development • Biochemistry • Metabolic network • Primary metabolism • Secondary metabolism

T. Nägele • L. Fragner • W. Weckwerth (✉)
Faculty of Life Sciences, Department of Ecogenomics and Systems Biology, University of Vienna, Althanstr. 14, 1090 Vienna, Austria

Vienna Metabolomics Center, University of Vienna, Althanstr. 14, 1090 Vienna, Austria
e-mail: wolfram.weckwerth@univie.ac.at

P. Chaturvedi • A. Ghatak
Faculty of Life Sciences, Department of Ecogenomics and Systems Biology, University of Vienna, Althanstr. 14, 1090 Vienna, Austria

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Abbreviations

ABA	Abscisic acid
ATP	Adenosine triphosphate
GA	Gibberellic acid
GABA	γ-Aminobutyric acid
GC	Gas chromatography
HXK	Hexokinase
Inv	Invertase
LC	Liquid chromatography
MS	Mass spectrometry
$NAD^+/NADH+H^+$	Nicotinamide adenine dinucleotide (oxidized and reduced form)
STP	Sugar transport protein
SuSy	Sucrose synthase
UV	Ultraviolet

12.1 Pollen Development and Tube Growth: A Brief Overview

Development of pollen, which represents the mature male gametophyte, is a complex and sequential process occurring in the anthers of flowers. In particular, two phases, microsporogenesis and microgametogenesis, can be differentiated (Owen and Makaroff 1995; Scott et al. 2004). The meiotic division of diploid pollen mother cells results in tetrads of haploid microspores during microsporogenesis. Subsequently, microspores are released from the tetrad, enlarge and produce a large vacuole (vacuolation) in the stage of microgametogenesis. This polarized microspore is asymmetrically divided during Pollen Mitosis I resulting in a bicellular stage of development with a vegetative and a germ cell. Finally, during Pollen Mitosis II, twin sperm cells are produced from the germ cell (for a detailed overview of molecular mechanisms, see, e.g. Borg et al. 2009). Depending on the species, the step of Pollen Mitosis II might occur within the pollen grain before anthesis (tri-cellular pollen, e.g. *Arabidopsis thaliana*) or within the growing pollen tube (bi-cellular pollen, e.g. *Lilium longiflorum*).

Following the phase of vacuolation and Pollen Mitosis I, the pollen grain undergoes a dehydration phase before anther opening (Pacini 2000). Prior to anther opening, starch reserves, which have been built during pollen growth and development, are degraded and interconverted to soluble sugars, such as sucrose, fructose and glucose as well as to pectins or polysaccharides (Pacini et al. 2006). According to the species, the degree of starch hydrolysis may vary significantly and even allows for the differentiation between starchy and starchless ripe pollen (Baker and Baker 1979). The composition of reserve, structural and soluble carbohydrates plays a central role in the determination of pollen water content and viability

during phases of pollen ripening, presentation and dispersal (as reviewed in Pacini et al. 2006). For example, pollen water content and turgor pressure depends on carbohydrate reserves and their interconversion which reduces or prevents water loss. Hence, although mature pollen grains from the same flower may differ significantly in their water and carbohydrate content (Firon et al. 2012), a tight regulation of carbohydrate metabolism is a crucial part of pollen development. This also comprises the carbohydrate supply within the anthers, for which it has been shown that disturbance may lead to male sterility (Dorion et al. 1996; Goetz et al. 2001; Hedhly et al. 2016). Anthers are structured heterogeneously and consist of the anther wall, locular fluid and the pollen grains. The anther wall includes the connective tissue as well as the locule surrounding cell layers which are the epidermis, endothecium, middle layers and tapetum. This fraction has been shown to be the site of sugar synthesis, storage, mobilization and secretion during pollen development (Reznickova 1983; Reznickova and Dickinson 1982; Reznickova and Willemse 1980; Clément et al. 1998; Staiger et al. 1994). Following the ripening process, pollen grains are presented and released after the locular fluid has disappeared (Keijzer 1987). When landed on the stigma surface, they germinate to produce a pollen tube. The pollen tube penetrates the stigma and grows through the style towards the ovary. Finally, it invades the embryo sac and releases two sperm cells. One of these sperm cells fertilizes the egg, while the other one forms the triploid endosperm together with two polar nuclei of the central cell.

The pollen-stigma interaction and the (fast) growth of the pollen tube represent tightly coordinated processes comprising numerous molecular entities and a complex signalling network (Liu et al. 2014; Cheung and Wu 2008, 2016; Reichler et al. 2009; Šamaj et al. 2006; Hiscock and Allen 2008). As outlined previously, the pollen-stigma interaction comprises pollen-pistil interaction ranging from the pollen capture to the growth of the pollen tube through the transmitting tissue of the stigma and the entry into the style (Hiscock and Allen 2008). Lipids have been found to be an essential factor needed for the penetration of the stigma by the pollen tube (Wolters-Arts et al. 1998). In particular, *cis*-unsaturated triacylglycerols were shown to be required for penetration of the stigma by tobacco pollen tubes (Wolters-Arts et al. 1998). As summarized recently, polar and neutral lipids, e.g. triacylglycerols, accumulate during the late phase of pollen development (Ischebeck 2016). Pollen lipids play diverse roles in development, germination and tube growth. In addition to their protective role during dehydration, they are further involved in the attachment of the pollen to the stigma as well as in membrane signalling during pollen tube growth (Murphy 2006). Furthermore, metabolism of lipids, fatty acids and waxes plays a central role in pollen wall development (Jessen et al. 2011; Jung et al. 2006; Qin et al. 2013; Wu et al. 2014; Quilichini et al. 2015). In general, mature pollen grains contain three wall layers, the outer exine, the inner intine and the tryphine, which is also known as the pollen coat consisting of lipids, flavonoids, proteins and aromatic compounds (for a detailed overview, see Shi et al. 2015). The tapetum layer in the anthers predominantly contributes to the formation of the exine and tryphine, while the microspore contributes to the intine formation. Typically, the intine consists of cellulose, hemicellulose and pectin, while the exine

is composed of sporopollenin, a polymer of covalently linked phenylpropanoid and lipidic monomers (Heslop-Harrison 1968; Shi et al. 2015). In summary, it is obvious that pollen wall development comprises various substances derived from numerous biochemical pathways which are regulated by a large number of enzymes, transcription factors and metabolites. Particular complexity of regulation arises from the interconnection of lipid and polysaccharide metabolism that needs a tight regulation in order to provide an adequate developmental reprogramming and, probably, also stress response during pollen development.

Following the germination of pollen and the successful penetration of the stigma, the pollen tube grows through the stigma and style towards the ovary and the ovule. The pollen tube is guided through the female tissue along its growth path in a process termed pollen tube guidance. This guidance process has been shown to involve multiple steps of control (Dresselhaus and Franklin-Tong 2013) and was suggested to be separated in a preovular and ovular phase (Higashiyama and Takeuchi 2015). In *Arabidopsis*, female tissue was found to provide brassinosteroids to pollen tubes along the growth path during preovular guidance (Vogler et al. 2014). In detail, the authors showed that the promoter of one of the key enzymes in brassinosteroids biosynthesis, CYP90A1/CPD, is highly active in cells of the tract that form the pathway for pollen tubes. In addition, they observed that pollen growth was significantly reduced in the reproductive tract of a CYP90A1-deficient mutant, *cyp90a1* (Vogler et al. 2014). Also other phytohormones were shown to affect pollen germination and growth. For example, auxin was shown to stimulate in vitro pollen tube growth (Chen and Zhao 2008), and a gibberellin-deficient *reduced pollen elongation1* was observed to exhibit reduced pollen tube elongation (Chhun et al. 2007). In addition to phytohormones, also other metabolic compounds were determined to play a role in pollen tube growth and guidance, e.g. the non-proteinogenic amino acid γ-aminobutyric acid, GABA. The *Arabidopsis POP2* gene was shown to encode a transaminase degrading GABA and contributing to a gradient leading up to the micropyle (Palanivelu et al. 2003). *pop2* flowers accumulated significantly higher levels of GABA, finally resulting in infertility due to growth arrest of the pollen tube or misguided pollen tubes in the *pop2* pistil (Palanivelu et al. 2003). A mechanism of GABA-mediated communication between style and pollen tube was suggested by Yu and colleagues who observed that exogenous GABA modulates putative Ca^{2+}-permeable plasma membrane channels of pollen grains and tubes (Yu et al. 2014), and recently the GABA receptor was found to be the anionic channel ALMT, with effects on pollen tube growth (Ramesh et al. 2015).

Although the described processes being involved in pollen development, germination and tube growth are far from being complete, they already indicate a complex interplay of numerous metabolic pathways and regulatory mechanisms. Particularly, with respect to stress exposure and a multifaceted plant-environment interaction, it is an experimental and theoretical challenge to resolve dynamics of pathway regulation and its metabolic output. Due to the wide range of metabolic compounds being involved, various experimental techniques, methods and platforms are needed which can cope with the wide range of the physico-chemical properties and tissue-specific abundance. The following chapters aim at exemplarily discussing the biological

function of central primary metabolites, secondary metabolites and phytohormones. Finally, for each compound class, applied techniques and experimental workflows are discussed with respect to their capability of resolving metabolome dynamics.

12.2 Primary Metabolome Dynamics During Pollen Development and Stress Exposure

Pollen development, pollen tube growth and the response towards environmental fluctuations involve and affect metabolism of sugars, organic acids, amino acids, polyamines as well as energy metabolism. Previous studies have clearly demonstrated that disturbance of primary metabolism, in particular of carbohydrates, severely affects pollen development and may even lead to male sterility (Dorion et al. 1996; Datta et al. 2002; Goetz et al. 2001; Zhu et al. 2015; David-Schwartz et al. 2013). Photoassimilates for the carbohydrate demands of the anther and the pollen are supplied by leaves and also by floral organs including the anther itself (Vu et al. 1985; Kirichenko et al. 1993; Clément et al. 1997). For *Lilium* it was shown that anther wall layers regulate pollen sugar nutrition during maturation (Clément and Audran 1995). Sucrose was determined to be the main sugar in young filaments as well as in desiccating mature pollen grains (Clement et al. 1996; Speranza et al. 1997). Metabolism of sucrose in sink organs crucially involves the cleavage catalysed either by sucrose synthase (SuSy) or invertase (Inv) enzymes. Sucrose synthase is a glycosyl transferase, converting sucrose, for example, into UDP-glucose and fructose, while invertase represents a hydrolase releasing only free hexoses, i.e. glucose and fructose. Invertase enzymes have been shown to exist in different isoforms which possess different biochemical properties and are located in diverse subcellular compartments (Sturm 1996, 1999; Tymowska-Lalanne and Kreis 1998). Soluble and cell wall-bound invertase isoforms have also been detected in lily anthers (Miller and Ranwala 1994; Singh and Knox 1984). Furthermore, when pollen were cultured in sucrose-containing medium, it was observed that sucrose was quickly converted into equimolar amounts of glucose and fructose indicating the presence of cell wall-bound invertase in the growing pollen tube (Ylstra et al. 1998). Together with starch also soluble sugars, which are stored in the mature pollen, are used for pollen tube growth. Previous studies have shown that pollen tubes can grow at a very high rate. For maize, growth rates of approximately 1 cm h^{-1} have been reported (Mascarenhas 1993; Barnabas and Fridvalszky 1984), while in *Arabidopsis* growth rates of 5.3 $\mu m\ min^{-1}$, i.e. 0.3 mm h^{-1}, were observed (Wilhelmi and Preuss 1996). This rapid growth rate directly implies the need for energy supply, e.g. by stored carbohydrates or by carbohydrate secretions from the stylar canal (Labarca and Loewus 1973), to support respiration and cell wall growth. Due to its symplastic isolation from surrounding tissue, membrane transporters are required for nutrient import into the pollen tube. While the *Arabidopsis* sucrose transporter AtSUC1, which is localized to the plasma membrane of pollen tubes,

has previously been shown to play a crucial role in pollen germination (Sivitz et al. 2008), sucrose might also be hydrolysed by cell wall-associated invertase releasing the free hexoses fructose and glucose (Singh and Knox 1984). Recently, a member of the SUGAR TRANSPORT PROTEIN (STP) family in *Arabidopsis*, AtSTP10, was functionally characterized (Rottmann et al. 2016). Analysis of in vitro-grown pollen tubes showed a glucose concentration-dependent downregulation of *STP10* expression which was found to disappear in pollen tubes lacking the sugar sensor hexokinase 1 (HXK1) indicating a regulatory link between the glucose uptake system and the hexokinase pathway (Rottmann et al. 2016).

Significant dynamics of the sucrose-hexose ratio were also observed to differentiate the metabolic signature before and after pollen germination (Obermeyer et al. 2013). Obermeyer and colleagues found hexose levels to increase significantly during pollen growth, while sucrose concentration clearly decreased. These sugar dynamics were also persistent after application of antimycin A, an inhibitor of the mitochondrial electron transport chain limiting ATP production. Similar to previous studies (Mellema et al. 2002; Gass et al. 2005), the authors observed an increase of ethanol concentration immediately after application of antimycin A being due to rerouting of pyruvate to ethanol fermentation (Obermeyer et al. 2013). In addition, GABA dynamics were found to change immediately after antimycin A application. Inhibition of the mitochondrial transport chain and related ATP production resulted in an increase in GABA levels which the authors discussed in the context of deregulation of GABA biosynthesis. Based on their experimental observations, Obermeyer and co-workers hypothesized that the inhibition of the mitochondrial transport chain leads to an accumulation of reducing equivalents, i.e. $NADH/H^+$. This accumulation might be prevented by inducing ethanol formation via pyruvate decarboxylase and alcohol dehydrogenase explaining the reduced pyruvate levels (Obermeyer et al. 2013). Finally, to sustain pyruvate levels, biosynthesis of GABA would contribute to its regeneration and, at the same time, would consume reducing equivalents, thereby affecting the reprogramming of the redox homeostasis in growing pollen tubes.

GABA has been shown to be involved in numerous metabolic processes in plants, comprising roles in stress-induced signalling, pH and redox regulation and maintaining the energy as well as the carbon/nitrogen homeostasis (for an overview, see Fait et al. 2008). Together with proline, which represents another glutamate derivative, the role of GABA in sexual reproduction of angiosperms was summarized recently (Biancucci et al. 2015). While the specific role of proline during pollen development still has to be evaluated, several authors suggest that it plays a central role in protection of mature pollen during dehydration (Székely et al. 2008). Interestingly, Obermeyer and co-workers observed a clearly distinct dynamic behaviour of glutamate and proline concentrations, i.e. substrate and product of proline biosynthesis, when inhibiting the mitochondrial electron transport chain: after 3 h of inhibition, glutamate levels were reported to be similar to that of non-treated pollen tubes, while proline levels were found to dramatically increase due to antimycin A application (Obermeyer et al. 2013). This hints towards a possible regulatory interaction of GABA, proline and glutamate metabolism, possibly

comprising a previously discussed GABA shunt (Fait et al. 2008; Obermeyer et al. 2013).

Glutamate metabolism and associated concentration dynamics are crucially involved in the coordination of plant carbon/nitrogen metabolism. In addition to serving as a substrate for the above-mentioned derivatives, proline and GABA, glutamate participates in numerous further metabolic interconversions, e.g. as an amino group donor for the biosynthesis of other amino acids. Furthermore, glutamate serves as a metabolic substrate for the biosynthesis of ornithine and arginine which are precursors for the biosynthesis of the polyamines putrescine, spermidine and spermine being involved in many plant stress tolerance reactions (Alcázar et al. 2010; Alcázar and Tiburcio 2016; Sengupta et al. 2016). Also for pollen development and tube growth, it was shown that polyamines play various physiologically relevant roles. Polyamines are involved in regulation of developmental steps occurring during microsporogenesis; they play a role in the context of the quiescent state, pollen viability, rehydration and tube emergence as well as in the pollen-pistil interaction during fertilization and self-incompatibility. A more detailed summary of these various molecular and regulatory roles can be found elsewhere (Aloisi et al. 2016). During pollen tube growth, polyamines were shown to be involved in the organization and assembly of the cytoskeleton as well as cell wall deposition (Del Duca et al. 2009; Di Sandro et al. 2010) which seems, at least partly, to be mediated by transglutaminase activity. In pollen tubes of *Arabidopsis*, spermidine was found to be involved in the activation of Ca^{2+} channels, thus directly affecting the polarized growth of the pollen tube apex (Wu et al. 2010). Further, spermine was found to inhibit pollen tube elongation, and it was suggested that the observed degradation of nuclear DNA, and related cell death, was induced by Ca^{2+}-activated signalling or by an alteration of the cellular redox homeostasis (Aloisi et al. 2015).

12.3 The Interface of Primary and Secondary Metabolism in Pollen: A Central Role for Flavonoids

The secondary metabolome of plants comprises a vast variety of chemical structures that are characterized by diverse physico-chemical properties, physiological functions and ranges of concentrations challenging current bioanalytical approaches. In the context of pollen development, fertility and stress response, flavonoids have been shown to be crucially involved. Flavonoids comprise diverse classes of compounds such as flavonols, flavones, anthocyanins and isoflavonoids (Winkel-Shirley 2002). Flavonols were found to play a central role in pollen germination. Mo and colleagues observed that a lack of chalcone synthase, which catalyses the first step of the phenylpropanoid pathway leading to flavonoids, disrupts pollen fertility and flavonoid synthesis in maize and petunia (Mo et al. 1992). In another study, Ylstra and co-workers described a high abundance of flavonols and mRNA

of chalcone synthase in male and female reproductive organs of *Petunia hybrida* (Ylstra et al. 1994). The authors could show that due to anti-sense inhibition of the chalcone synthase gene activity, flavonol biosynthesis was blocked completely rendering these plants self-sterile. Such a central role of flavonoid biosynthesis was also shown for tomato where RNA interference silencing of chalcone synthase resulted in parthenocarpic fruits and impaired pollen tube growth (Schijlen et al. 2007). Interestingly, in *Arabidopsis* fertility was not affected by the disruption of the synthesis of active chalcone synthase indicating a species-specific effect of flavonoids (Burbulis et al. 1996). Yet, as summarized previously (Taylor and Grotewold 2005), these plants were affected in seed set and in vitro pollen tube growth (Ylstra et al. 1996; Kim et al. 1996), suggesting that flavonoids might enhance pollen tube growth but lacking flavonols can be compensated in their function by other metabolic compounds. In a recent study, this idea was supported by the finding that *Arabidopsis* plants lacking pollen-specific flavonols due to a defective glycosyltransferase were still fertile (Yonekura-Sakakibara et al. 2014).

Flavonoids are, together with substances like alkanes, steryl esters and oleosins, abundant in the coat of mature pollen. One possible function of flavonoids on the pollen coat might be the protection against damage due to UV radiation (Winkel-Shirley 2001; Pacini and Hesse 2005). Among the substances of this pollen coat, the structural characteristics of the phenylpropanoids hydroxycinnamic acid amides and flavonol glycosides are highly conserved in angiosperm pollen (Fellenberg and Vogt 2015). For *Zea mays* and *Petunia hybrida*, coat flavonoids have been shown to be involved in pollen germination and tube growth processes (Napoli et al. 1999; Mo et al. 1992). They are discharged on the pollen surface upon cell death of tapetum cells which accumulate endoplasmic reticulum-derived flavonoids (Hsieh and Huang 2007).

12.4 Signalling and Developmental Regulation: Phytohormones in Pollen

The regulatory networks which are involved in metabolic adjustment as well as the coordinate response of pollen metabolism during different developmental stages and environmental stresses comprise a remarkable number of molecular instances and signalling mechanisms. Particularly in the context of pollen tube/tip growth, the second messenger Ca^{2+} has emerged as a central regulator (for a detailed overview, see Franklin-Tong 1999; Steinhorst and Kudla 2013). Comprehensive models have been suggested integrating concentration dynamics of cytosolic Ca^{2+}, apical exocytosis of cell wall material, regulation of stretch-activated Ca^{2+} channels, inositol polyphosphates, reactive oxygen species, etc. (Steinhorst and Kudla 2013). Further, for *Arabidopsis*, a recent summary and comparison of transcriptome studies on pollen revealed an average value of more than 6000 genes being expressed in mature pollen (Rutley and Twell 2015). Hence, bringing together this multigenic

attribute with the comprehensive and highly interlaced structure of signalling networks and with the highly dynamic physiological output of developing pollen reveals a complex picture of regulation. Phytohormones have been shown to play a central role in the coordination and regulation of these networks affecting various steps of pollen development, tube growth and stress response.

Auxin belongs to the very central phytohormones playing an important role in promotion of cell elongation and cell division. Also in the context of pollen development, auxin was described to play a crucial role. Feng and colleagues found auxin flow in anther filaments to be critical for pollen grain development (Feng et al. 2006). In another study, auxin synthesized in anthers was suggested to coordinate anther dehiscence and pollen maturation using auxin receptor triple and quadruple mutants (Cecchetti et al. 2008). Furthermore, external application of auxin was observed to stimulate in vitro pollen tube growth (Chen and Zhao 2008; Wu et al. 2008) and, hence, seems to directly affect the signalling network of tube growth regulation. Mutant pollen being defective in PIN8, a pollen-specific auxin transporter, was claimed to be affected in germination when compared to the wild type (Bosco et al. 2012; Ding et al. 2012). Due to the fact that PIN8 is localized in the endoplasmic reticulum, this may indicate a link of the intracellular auxin homeostasis to the development of the male gametophyte.

Another phytohormone which has been described to play a central role in pollen development and stress response is gibberellic acid (GA). GA is involved in tapetum differentiation and initiation in tapetum programmed cell death which was described recently to occur via a GA-regulated transcriptional activator (Plackett et al. 2014; Aya et al. 2009). Singh and colleagues provided evidence that GAs are required for normal growth of the pollen tube by ectopically expressing a pea GA 2-oxidase2 cDNA in *Arabidopsis* resulting in reduced pollen growth (Singh et al. 2002). Based on studies in rice (*Oryza sativa*), it was suggested that GA de novo synthesis is preliminary for pollen germination and elongation (Chhun et al. 2007). While these observations clearly indicate a central role of GAs in pollen development and tube growth, they were also found to be involved in temperature stress response. During exposure to continuous heat stress, GA content was observed to positively correlate with pollen viability in two rice cultivars with a differential stress susceptibility (Tang et al. 2008). In contrast, cold stress was found to result in a reduction of bioactive GAs, particularly in susceptible anthers by the repression of GA biosynthesis genes (Sharma and Nayyar 2016; Sakata et al. 2014).

In addition to the GA-associated regulatory effects on different levels of molecular organization, it is also the crosstalk with other phytohormones that increases the coverage and complexity of phytohormone-based signalling to a very comprehensive level. One example of such a crosstalk is the DELLA-mediated interaction with signalling components of several phytohormones (Claeys et al. 2014). DELLA proteins represent transcriptional regulators and are conserved repressors of growth. GAs have been shown to regulate gene expression by promoting degradation of DELLA proteins (Murase et al. 2008). Other phytohormones, e.g. auxin, jasmonic acid or brassinosteroids, are interacting either directly with GAs, e.g. by transcriptional regulation, or indirectly, e.g. by post-translational regulation of

DELLAs (Claeys et al. 2014). Brassinosteroids were shown to promote *Arabidopsis* pollen germination and growth in a dose-dependent manner (Vogler et al. 2014). By expression analysis, Vogler and colleagues observed a highly active promoter of a key enzyme in brassinosteroid synthesis in cells of the reproductive tract forming the pathway from the stigma to the ovule (Vogler et al. 2014). An interesting regulatory interaction between hormone and carbohydrate metabolism was suggested for jasmonic acid, which was proposed to control water transport into the anther, perhaps via induction of the *AtSUC1* gene (Ishiguro et al. 2001). A strong interaction and influence on the central carbohydrate metabolism has also been unravelled for abscisic acid (ABA), which, together with GA, is relevant for the carbohydrate supply to the tapetum and microspores (De Storme and Geelen 2014). Particularly with regard to stress response, several studies have indicated a strong interaction between sugar- and ABA-mediated signalling (Gibson 2004; Dekkers et al. 2008). For example, ABA was shown to repress the expression of anther cell wall-associated invertase in wheat (*Triticum aestivum*) which resulted in a perturbation of sugar metabolism and a reduced level of hexoses in developing spores (Ji et al. 2011). This ABA-induced perturbation was directly related to an observed increase in drought sensitivity, exemplifying the central role of ABA in stress response of pollen. In rice, increased ABA levels in cold-stressed anthers were shown to interfere with apoplastic sugar transport inducing pollen abortion (Oliver et al. 2007). In accordance with the observations in wheat, exogenous ABA treatment revealed an effect on gene expression of cell wall invertase and monosaccharide transporter, which was accompanied by increased pollen sterility.

Although these examples of chosen regulatory interactions are only a very small part of the whole hormonal network, which has been unravelled in numerous studies, they clearly indicate the comprehensive impact on pollen physiology, development and stress tolerance. Finally, to be able to draw a reliable picture of those processes and to unravel interactions between hormonal regulation and observed dynamics in the metabolome, adequate bioanalytical techniques and integrative platforms with a comprehensive metabolic coverage are desirable.

12.5 Experimental Analysis of Metabolome Dynamics in Pollen

Although the discussed metabolic pathways and the involved substance classes represent only a part of the metabolism, they are highly interlinked, and, hence, their interdependent dynamics will significantly shape experimental results. This necessitates accurate and, at the same time, comprehensive experimental approaches to reveal a reliable picture of pollen metabolome dynamics during pollen development, tube growth and stress exposure. Particularly with regard to stress exposure, which very often leads to a strong accumulation or decrease of metabolite concentrations, suitable methodological workflows and analytical platforms are needed to enable the

reliable resolution of those dynamics. During the last two decades, the development and application of hyphenated high-throughput technologies, the so-called 'omics' technologies, have initiated the idea of the experimental multilevel analysis of an organism's molecular organization (Weckwerth 2011). The functional interpretation of the resulting high-throughput data represents the motivation for systems biology research ultimately aiming at the development of comprehensive and physiologically reliable models of molecular organization. Metabolites and their concentration dynamics play a crucial role in the development and validation of such models as they represent the output of regulation in biological systems. In their entirety, those metabolites constitute the metabolome of an organism. Finally, information about metabolome constitution and dynamics is essential to interpret the response of an organism to environmental changes or genetic perturbation (Fiehn 2002).

The experimental analysis of metabolome constitution and dynamics is focused by the research field of metabolomics, comprising steps of metabolite extraction, detection, identification and quantification. Metabolome analysis of pollen or pollen tube samples represents a challenge in several aspects. First, sampling of pollen or pollen tubes within the same or similar developmental and nutritional stage represents a difficult task. In contrast to leaves or roots for which developmental and nutritional states can often be estimated by eye, optical tools are necessary for pollen. Especially for the early stages of pollen development, it is essential to find a practicable and reliable method to separate, e.g. pollen mother cells from tetrads without the risk of changes in the metabolome. Recently, Dupl'áková and colleagues developed a protocol for separation of four pollen developmental stages, the uninucleate microspore, bi-cellular pollen, tri-cellular immature pollen and mature pollen grain applying a discontinuous Percoll concentration gradient (Dupl'akova et al. 2016). Using a Percoll gradient is advantageous because it does not penetrate the pollen grain, does not change the osmotic pressure, is not metabolized and, as a consequence, does not affect the biological function of the separated pollen.

A second challenge in pollen metabolomics is the washing step before metabolite extraction. It is necessary in order to remove pollenkitt and other hydrophobic compounds on the pollen coat. This washing step may be performed using hexane as described previously (Obermeyer et al. 2013).

Third, due to their small size and the (relatively) robust pollen wall, the grinding process has to be performed in a very thorough manner. Again, in contrast to other plant material, the control by eye is very limited in this step. A further very critical step is the metabolite extraction itself. While the targeted analysis of a special class of substances might facilitate the extraction procedure, e.g. using ethanol extraction for soluble sugars (Nägele et al. 2012) or hot water extraction for sugar phosphates (Sekiguchi et al. 2004), the combined and quantitative extraction of as many as possible hydrophilic and hydrophobic substances from one sample is much more challenging. Although a basic procedure for the integrated extraction of metabolites, proteins and RNA was developed already more than a decade ago (Weckwerth et al. 2004; Valledor et al. 2014) and has previously been applied to extract the primary metabolome of lily pollen (Obermeyer et al. 2013), such extraction methods need to be continuously developed and improved to increase the metabolic coverage. After

a successful extraction of metabolites, the fourth challenge is the very high dynamic range of metabolites within a tissue but also between developmental stages, which are differentially metabolic active. For instance, the polar fraction of an extract can contain low abundant primary metabolites, like amino acids and 100-fold higher levels of sucrose. This effect is even much stronger if very low abundant signalling molecules, like phytohormones, are analysed.

For the unbiased analytical assessment of the metabolome, chromatography coupled to mass spectrometry has become a central approach (Weckwerth 2003). Focusing on the quantitative analysis of dynamics in the parts of the metabolome which were discussed in the previous chapters, i.e. primary metabolites, flavonoids and phytohormones, a combination of gas chromatographic (GC) and liquid chromatographic (LC) separation coupled to mass spectrometric (MS) detection represents a suitable approach (Weckwerth 2003; Scherling et al. 2010; Doerfler et al. 2013). While chemical derivatization enables the GC-MS-based identification and quantification of central primary metabolites comprising soluble sugars, carboxylic acids and amino acids (Fragner et al. 2014), for larger and thermally instable molecules, e.g. secondary metabolites like flavonoids, LC-MS is the method of choice (Stobiecki and Kachlicki 2013; Doerfler et al. 2013; Scherling et al. 2010). An interesting combination of methods has been highlighted recently for the quantification of phytohormones. As summarized by Fu and colleagues, although LC-MS can directly analyse plant hormones, a previous chemical derivatization, which is usually applied for GC-MS, can increase the sensitivity up to 1000-fold improving the quantification of low concentrated phytohormones (Fu et al. 2011). Together with methods which have been developed for extraction and enrichment of plant hormones (Du et al. 2012), this bioanalytical procedure might even enable the robust and comprehensive quantification of trace amounts in pollen grains and tubes.

Finally, one of the central challenges in metabolomics, playing also a crucial role for pollen analytics, is the resolution towards the single-cell level. Recent approaches have indicated the difficulties in these approaches, which, for example, are due to the enormous dynamic range comprising only a few molecules up to millions of molecules per cell. Yet, these approaches have also clearly shown that difficulties in interpretation, e.g. due to dilution effects from tissue-based sampling, might be by-passed by single-cell techniques (Misra et al. 2014).

12.6 Conclusion and Perspective

The resolution of the pollen metabolome and the interpretation of its dynamics in the context of developmental and stress-related molecular processes represent a challenge in the research field of metabolomics. This challenge arises not only from limitations in chromatography, detection and quantification but also from sampling, extraction and biochemical validation. Integrative research platforms merging microscopy and imaging techniques with hyphenated omics techniques

and physiological assays represent a promising constellation which can overcome such experimental limitations. Conclusively, integrative approaches will promote our current understanding of how stress conditions affect pollen morphology, physiology and biochemistry and how this impacts the reproductive success of plant species.

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Part VI
Modeling Tip Growth

Chapter 13
Derivation and Use of Mathematical Models in Systems Biology

Robert W. Smith and Christian Fleck

Abstract Systems Biology has brought together researchers from biology, mathematics, physics and computer science to illuminate our understanding of biological mechanisms. In this chapter, we provide an overview of numerical techniques and considerations required to construct useful models describing natural phenomena. Initially, we show how the dynamics of single molecules up to the development of tissues can be described mathematically over both temporal and spatial scales. Importantly, we discuss the issue of model selection whereby multiple models can describe the same phenomena. We then illustrate how reaction rates can be estimated from datasets and experimental observations as well as highlighting the "parameter identifiability problem". Finally, we suggest ways in which mathematical models can be used to generate new hypotheses and aid researchers in uncovering the design principles regulating specific biological mechanisms. We hope that this chapter will provide an introduction to the ideas of mathematical modelling for those that wish to incorporate it into their research.

Keywords Hypothesis generation • Mathematical modelling • Model selection • Parameter estimation

List of Abbreviations

AIC	Akaike information criterion
CME	Chemical master equation
FBA	Flux balance analysis
ODE	Ordinary differential equation

R.W. Smith • C. Fleck (✉)
Laboratory of Systems and Synthetic Biology, Wageningen UR, 6700 EJ Wageningen, The Netherlands
e-mail: robert1.smith@wur.nl; christian.fleck@wur.nl

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PDE	Partial differential equation
qRT-PCR	Quantitative real-time polymerase chain reaction
SBML	Systems biology mark-up language
SSA	Stochastic simulation algorithm

13.1 Introduction

The use of mathematics to help understand the emergence of biological phenomena has occurred for over a 100 years. However, through recent advances and an ever-closer collaborative effort between theoretical and experimental biologists, the field of Systems Biology has come to prominence. The history of Systems Biology can be traced back to Alfred Lotka at the start of the twentieth century. Through numerical analysis of chemical reactions that produce damped oscillations, Lotka observed that conditions matching those of larger biological systems may be able to sustain stable periodic rhythms (Lotka 1920). This led to the development of models describing population dynamics that are now regularly taught as part of mathematics courses (Murray 2002a). Importantly, Lotka's aim was not merely to obtain an expression that can describe oscillations (such as a trigonometric function: sine or cosine), but to obtain an understanding of how oscillations can emerge through interactions between individual components within a system.

Over the last century further comparisons of the mathematics describing behaviour on a systems level were made. Consequently, scientists such as Ludwig von Bertalanffy aimed to derive a *General System Theory* whereby many different systems could be described by the same mathematical structure (von Bertalanffy 1968). In fact, some of the arguments made by von Bertalanffy in the 1960s are still prevalent today:

> Modern science is characterised by its ever-increasing specialisation, necessitated by the enormous amount of data, the complexity of techniques and of theoretical structures within every field. Thus science is split into innumerable disciplines continually generating new subdisciplines. In consequence, the physicist, the biologist, the psychologist and the social scientist are, so to speak, encapsulated in their private universes... This, however, is opposed by another remarkable aspect... Independently of each other, similar problems and conceptions have evolved in widely different fields.

Such a quote, essentially, highlights the *modus operandi* of Systems Biology: to bring together the biologist, the physicist, the mathematician, and the computer scientist to deal with the masses of experimental data currently being produced and understand phenomena that emerge from biological systems. In Systems Biology one aims at predictive models, but it should be made clear what is actually meant by this term. Any correlation can be used for the purpose of a probabilistic prediction. However, what is meant in many cases is the construction of a genuinely explanatory model. The prediction should also hold for the manipulated system, which requires that the model captures changes of specific molecular components internal to

the system. Thus, an understanding of the internal causal structure is needed to offer mechanistic explanations of system phenotypes (Westerhoff and Kell 2007; Brigandt 2013). This is a demanding task, requiring the integration of mathematical-modelling efforts, data detailing molecular interactions, and information on the physics of cellular structures (Mogilner et al. 2012).

There are a number of recent publications and books that have summarised aspects of the Systems Biology field and show how Systems Biology approaches can be implemented to solve biological problems (Kitano 2002a,b; Klipp et al. 2005). Some reviews highlight how spatial signals or organ development can be linked to intracellular networks, that have themselves been covered in books by Kholodenko (2006), Alon (2007), and Brady and Benfey (2009). On the other hand, mechanical forces that influence the growth of organs have been treated mathematically using methods that are generally independent of internal cellular processes (Goriely and Tabor 2008). Finally, for an overview of mathematical models describing a wide range of temporal and spatial biological phenomena, we direct interested readers to the excellent books by J. D. Murray and R. Phillips et al. that go into greater depths of mathematical analysis than the reviews listed above (Murray 2002a,b; Phillips et al. 2013).

In this chapter, we aim to supplement the reviews and book chapters listed above by considering the range of potential steps and questions that occur throughout the creation of a mathematical model within Systems Biology. We start by discussing model creation and how different types of model can be used to answer different biological questions. Then, we provide an overview of methods to infer kinetic rates within a biological system. This should, in principle, leave the user with a model that provides an accurate depiction of their biological data. Finally, we highlight methods to analyse a model and how to extract new understanding or experimental hypotheses about a biological network. Whilst we primarily consider what is referred to as the 'bottom-up' approach to Systems Biology (namely that we start with a limited amount of information and look to build upon this until our model is able to describe biological phenomena), we direct readers to other sources, such as Klipp et al. (2005), for more information about 'top-down' methods (whereby the causes of biological phenomena are unearthed from high-throughput 'omics' data).

13.2 Creating a Model

13.2.1 Prior Knowledge: What Is the System, the Data, the Question?

As with the start of any project, the most important consideration is what hypothesis does one wish to test, or what new understanding about the biological system does one wish to gain. The universal model from which all possible questions could

be answered would be impossible to handle, even if it would exist. This is both a practical and an epistemological constraint. The behaviour of complex systems is often only understandable if one finds the correct level of description. In many cases the details of a system are not important and a coarse-grained description of the interactions between the constituent of a system is better to explore its behaviour. It is often the case that a rich yet structured picture emerges only if one asks the correct questions by allowing for controlled errors.

Furthermore, the question itself (and the modelling style used, as we shall see later) is constrained by the information and data that is available. A model reflects what is known about the system under inspection and what particular questions are asked. A seemingly simple reaction like receptor–ligand binding can be quite complicated and difficult to understand in detail, where the binding is governed by a combination of steric, electrostatic and van der Waals forces (Gilson and Zhou 2007). However, in many cases the binding process can be described by a second order reaction; all the details of the binding process are subsumed in a rate constant. The same is true, e.g. for protein degradation. In most models it is described by first order reaction kinetics, which is equivalent to a spontaneous decay of the protein, where in reality it is a sequence of reaction steps. The modelling of protein degradation by first order reaction kinetics is in many cases sufficient. However, if one is interested in the details of the degradation process itself, the description has to be expanded from a simple first order reaction to a system of coupled ordinary differential equations.

The type of available data also matters for the decision on the modelling approach. For example, one may have data that describes different levels of biology: temporal (time-dependent) or spatial dynamics of system components within single cells, changes in component concentration within a cell population over a period of time, concentration gradients through a tissue layer or across different cell types, and physiological readouts of a biological phenomena with limited knowledge about the components that cause them. Furthermore, such data could be measured across a range of environmental conditions or varied transgenic systems, allowing one to compare how external and internal changes or perturbations impact the system.

Each of these data sources can allow for a range of different questions to be asked. For example:

- how does variability between single cells ultimately impact upon tissue generation or phenotypic responses?
- how are temporal changes in component concentration, that in turn impact physiological responses, related to environmental fluctuations?
- upon perturbing the concentration of a component, how is the spatial distribution of other components or tissue layers altered?
- what potential cellular mechanisms could lead to non-linear physiological growth rates?

Additionally to the analysis of new data, one should also consider any available prior knowledge, both within the biological system of choice or from evolutionarily linked systems. This can have several positive effects upon the modelling strategy

chosen for the study. For example, imagine that temporal changes of system components had been obtained but the researcher knew, qualitatively, about the spatial localisation of components through a tissue from published research. One could then construct a spatio-temporal model that captured the new data in detail and provided the broad pattern of expression across the spatial domain described by published observations. Another example would be combining information from different system perturbations. For example, a previous study may have implicated a particular component by observing altered responses in mis-expression studies. If another component was found to also alter the physiological response in new experiments, then a model could incorporate and link both of these components within a single system to explain biological phenotypes. Thus, combining data from multiple sources across a range of different scales can help model construction and analysis.

Finally, if a biological phenomena has been observed but little is known about the system responsible, then one possibility would be to look at analogous networks from different species or related systems—i.e. envisioning general systems principles for similar biological responses. A number of cases exist in plant biology. For example, simple models of the plant circadian clock (that coordinate daily physiological rhythms with the environment) were originally constructed assuming a similar network structure to that of the theoretical Goodwin oscillator that produces stable oscillations given certain kinetic rates (Goodwin 1965; Locke et al. 2005). By building on this simple theoretical system, a large number of components have been implicated in the regulation of plant circadian rhythms over the last decade (Pokhilko et al. 2012; McClung 2014). A further example has occurred through comparison of flowering phenotypes of different plants. The network that controls day-length-dependent flowering in short-day flowering rice, *Oryza sativa*, has been found to share components similar to the long-day flowering model plant *Arabidopsis thaliana*. By comparing system perturbations, researchers were able to understand how network connections differed between the two plant species, providing different flowering phenotypes despite having similar cellular components (Blumel et al. 2015). Thus, by viewing models as a general description of biological phenomena, rather than a description of very particular biological responses, one is able to elucidate a range of information about related systems that can act as starting points in more detailed examinations of a newly studied biological network.

Given a model and a hypothesis to test, one can now determine whether the current understanding of a biological system is correct or not. As described above, models integrate the current knowledge about a system and aim to answer specific questions. While deriving the mathematical model one idealises the actual biological situation and one, necessarily, simplifies certain aspects of the system. Usually, the derivation of a mathematical model is an iterative process. If the model describes the data and predicts correctly the manipulations—success. But, maybe, more interesting are model failures, because they point at not-well understood elements of the system; specific failures of the models may predict new regulatory interactions or components that can be tested by experimentation.

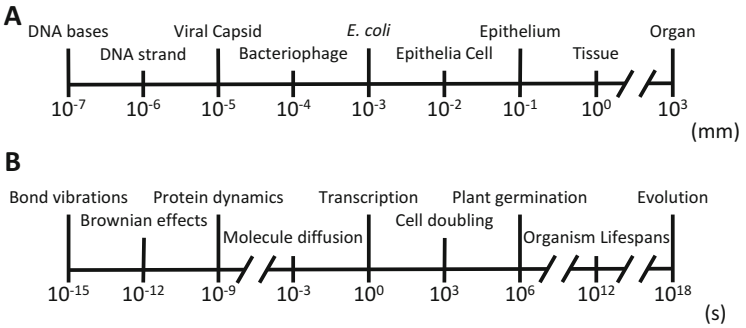

Fig. 13.1 Spatial and temporal scales of biological systems. An illustration of the different scales that biology covers (**a**) spatially and (**b**) temporally from the smallest molecules to populations

13.2.2 Model Characteristics: Which Model Is Suitable to Answer the Biological Question

Upon the decision of which level of biology one wishes to examine, the next step is to decide upon the modelling strategy required. As suggested in the previous section, this decision is limited by the data that the researcher possesses and what research questions are to be answered. In this section, we shall describe the mathematics and assumptions of different techniques from modelling individual molecules up to tissue level systems over both temporal and spatial dimensions (Fig. 13.1). For further information about these modelling strategies we refer readers to (Murray 2002a,b; Paulsson 2004; Kholodenko 2006; Gillespie 2007; Phillips et al. 2013). Whilst we shall not cover here large scale steady state models, such as those commonly found when analysing metabolic networks, we shall point out how these are a special case of dynamic systems and refer readers to Orth et al. (2010) for more details.

13.2.2.1 Assessing the State of a Single Interaction

At the basis of all biochemical reactions is the interaction between single molecules within a cell. In relation to Fig. 13.1, this implies that we are interested in reactions occurring on both temporal and spatial nano-scales. Here, we will introduce some of the important concepts to describe the effects of forces on such molecular interactions, but direct interested readers to the book by Phillips et al. for a more thorough treatment of the examples introduced here (Phillips et al. 2013).

A system is in thermodynamic equilibrium when it is in thermal, mechanical, and chemical equilibrium. This means there is no net flux of energy and matter between the system and its environment (Rao 2004; Phillips et al. 2013). A

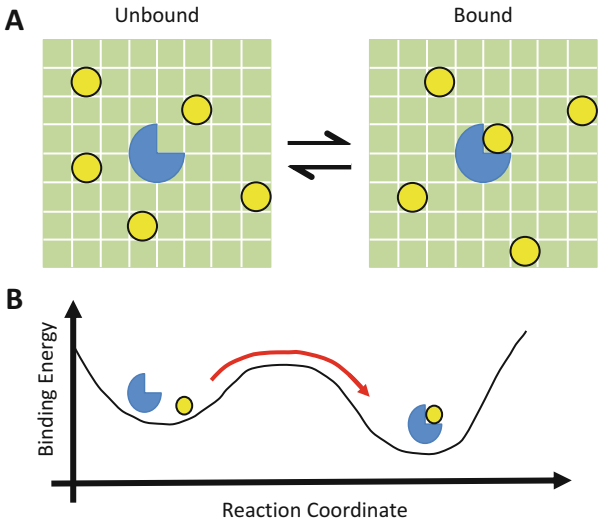

Fig. 13.2 Ligand binding to a receptor protein with a cell. (**a**) An illustration of the state switching between unbound and bound ligand with the receptor. Only one ligand can bind the receptor at any given time and this is influenced by its position within the cell. (**b**) An example of the double well energy landscape that such a system may generate. In this instance the states of unbound and bound ligand represent the minima in the binding energy landscape. The *red line* illustrates the gain of energy by the ligand–receptor complex to move from unbound state to bound state

steady state of a system is more general; it is a fixed point of the system in which the influx and the efflux are in balance, but exchange of matter and energy is possible. A thermodynamic equilibrium is a special kind of steady state, i.e. every thermodynamic equilibrium is a steady state but not vice versa. Biological organisms always exchange matter and energy with their environment and therefore they are never in thermodynamic equilibrium unless they are dead. However, it is in many instances well-justified to consider a particular process to be in equilibrium, which is often the case if the time-scales of the relevant biological process, e.g. gene expression, and the process in question, e.g. binding of transcription factors, are very different. In this case one can use the wealth of concepts developed for equilibrium systems.

For example, imagine ligand molecules that can bind to receptor proteins (Fig. 13.2a). There are two possible states for the ligands, either bound or unbound, and with each of these states an energy state is related (Fig. 13.2b). The equilibrium state (i.e. how many of the ligands are bound or unbound) minimises the free energy of the system (Fig. 13.2b), where the free energy is the difference between the internal energy U and the product of temperature T and entropy S ($F = U - TS$). Note that for the sake of simplicity we do not distinguish between Helmholtz and Gibbs free energy (Rao 2004; Phillips et al. 2013). The entropy of a system measures the number of its microstates compatible with the macrostate of the system. For

example, consider two receptors and two ligands. There is one microstate related to the macrostate with 100% of the ligands bound, one microstate related to the macrostate where 100% of the ligands unbound, and two microstates related to the macrostate where 50% of the ligands bound. In general, given N ligands and receptors, the number of microstates related to the macrostate with k ligands bound is $W(k) = N!/(k!(N-k)!)$. The entropy S of the receptor–ligand system is given by $S = k_B \ln(W)$ (k_B is the Boltzmann constant). If U_u and U_b are the binding energies of the unbound and bound state, respectively, then the free energy of the system with N receptors/ligands and k ligands being in the bound state is given by:

$$\frac{F(k)}{k_B T} = N\frac{U_u}{k_B T} + k\frac{U_b - U_u}{k_B T} - \ln(W(k)).$$

The question is which k, i.e. which number of bound ligands, minimises F? The first term on the right-hand side of this equation is a constant, which corresponds to the free energy of the system with all ligands unbound. To calculate some numbers, we set $N = 100$. In case $U_b = U_u$ the obvious result is $k = 50$. For $U_b - U_u = 0.5\, k_B T$ one finds $k = 62$ and in case $U_b - U_u = k_B T$ one obtains $k = 73$.

There is another instructive way of looking at the above example. The state which minimises the free energy is the most probable state of the system. Other states are possible as well, but with lesser probability. The probability of finding the system in state k (short for having k ligands bound) is given by Phillips et al. (2013):

$$P(k) = \frac{e^{-F(k)/k_B T}}{\sum_{k'=0}^{N} e^{-F(k')/k_B T}}.$$

Using the expression for $F(k)$ given above and doing some algebra one obtains:

$$P(k) = \frac{N!}{(N-k)!k!}\frac{e^{-k(U_b-U_u)/k_B T}}{(1+e^{-(U_b-U_u)/k_B T})^N}.$$

The probability of finding the system in state $k = 73$ for $U_b - U_u = k_B T$ is $P(73) \approx 0.09$, while for $k = 50$, $P(50) \approx 5 \times 10^{-7}$. What does this mean? If one does an experiment counting the number of bound states, in only 9% of the cases one will find *exactly* $k = 73$ bound receptors. But in 98% of the cases the number of bound receptors will be between $k = 63$ and $k = 83$.

Thus, starting from analysing microscale reactions between single molecules, we are able to understand snapshots of molecule populations containing mixed states through concepts of equilibrium statistical mechanics. Based on such ideas, several interesting results can be derived, including Hill functions that are often used to approximate the binding of transcription factors to promoters and the regulation of gene expression (Bintu et al. 2005; Phillips et al. 2013).

Using equilibrium approaches to analyse and describe a biological system can be very powerful, but time does not appear in these methods. There is no information of how long a process takes, how long one needs to wait in an experiment

to reach equilibrium, or how long to measure to obtain sufficient statistics. If temporal and spatial information is needed on small scales molecular dynamics simulation can serve as a computational microscope, revealing the workings of biomolecular systems at a spatial and temporal resolution that is often difficult to access experimentally (Dror et al. 2012). On a coarse-grained or mesoscopic level the chemical master equation provides a description of what happens within molecule populations over time and space (Gillespie 2007).

13.2.2.2 Modelling Small Molecule Numbers in Single Cells

When examining the changes in small populations of molecules in single cells, we need to refer to stochastic processes. For stochastic processes it is impossible to know at any time the *exact* state of the system (besides the initial state). It is only possible to make statements about the probability to find the system in a given state at a given time. The time development of a system can be described by an equation for the time development of this probability. On a mesoscopic description level one is not concerned with forces or energies (like the microscopic level), but rather with the probability that a given change of the system occurs within a certain small time interval. The equation for the probability is a balance equation; it is concerned at each time point with a gain and a loss in the probability to find the system in a given state. As an example let us consider the ligand–receptor binding from the previous section. To simplify matters we analyse the situation for one ligand and one receptor ($N = 1$). We examine the reaction between state 0 (unbound) and 1 (bound):

$$0 \underset{k_2}{\overset{k_1}{\rightleftharpoons}} 1$$

The probability $P(1, t+\Delta t)$ of finding the ligand in the bound state at time $t+\Delta t$ is based on the probability at time t and the transition probabilities to either move from state 0 to 1 (gain) or *vice versa* (loss):

$$P(1, t+\Delta t) = P(1,t) + \underbrace{k_1 \Delta t P(0,t)}_{\text{gain}} - \underbrace{k_2 \Delta t P(1,t)}_{\text{loss}}$$

Dividing by Δt and taking the limit $\Delta t \to 0$ yields a differential equation for $P(1,t)$:

$$\frac{dP(1,t)}{dt} = k_1 P(0,t) - k_2 P(1,t).$$

This type of gain-loss equation for the probability is called the master equation. In steady state, i.e. when $t \to \infty$ we find: $P(1, t \to \infty) = k_1/(k_1 + k_2)$. Comparing this result to the result for $P(k = 1)$ from the previous section yields:

$$\frac{k_1}{k_2} = e^{-(U_b - U_u)/k_B T},$$

which relates the mesoscopic scale (reaction rates) to the microscopic scale (binding energies). For a birth and death process (Ø denotes the empty set; the molecules appear out of nothing—production—and vanish into nothing—degradation):

$$\emptyset \xrightarrow{k_1} X \xrightarrow{k_2} \emptyset,$$

the corresponding master equation reads:

$$\frac{dP(n,t)}{dt} = \underset{\text{gain : production}}{k_1 P(n-1,t)} + \underset{\text{gain : degradation}}{k_2(n+1)P(n+1,t)} - \underset{\text{loss : production}}{k_1 P(n,t)} - \underset{\text{loss : degradation}}{k_2 n P(n,t)}$$

This equation describes the time development of the probability to find n molecules at time t and needs to be solved such that it obeys the initial condition $P(n,t_0) = P_0(n)$. To find an equation for the mean or average number of molecules $\langle n \rangle$ one multiplies the master equation by n and sums over all possible values for n. This gives rise to:

$$\frac{d\langle n \rangle}{dt} = k_1 - k_2 \langle n \rangle$$

The equation for the average of the stochastic process is identical to the deterministic equation one obtains using mass-action kinetics (Klipp et al. 2005). Thus, the average obtained from the master equation and the result from the mass-action kinetics agree. This only holds for linear systems; for non-linear reactions, such as protein–protein binding, the equation for the mean obtained from the master equation differs from the equation derived from mass-action kinetics. Additional assumptions, e.g. high molecule abundance, are required to find congruity between the stochastic and the deterministic description (Gardiner 2004).

For a general chemical reaction system the *chemical master equation* (CME) describes on a mesoscopic level the change of the chemical distribution. However, although being an exact description for the probability to find a given chemical composition of the biological system at a given time it is very difficult to obtain analytical solutions. This is due, in part, to biological networks breaking detailed balance through synthesis and degradation reactions. Numerical solutions to the CME can be obtained by using a variant of the Gillespie algorithm or the Stochastic Simulation Algorithm (SSA; see Gillespie (2007) for a review). However, to obtain information about the probability distribution several thousands of similar simulations need to be performed. Without computational parallelisation this process is thus highly time-consuming for complicated biological networks, but could be of use for small systems such as those created in the field of Synthetic Biology.

Since the CME is difficult to solve and computationally intensive to simulate for larger systems, several approximative schemes have been developed. Among these the linear-noise approximation and the Chemical Langevin Equation are widely used (van Kampen 1981; Gillespie 2000). Whilst we shall not go into the details of such a process, we would like the reader to note that this technique has allowed users to obtain accurate numerical estimates to the solution of the CME (Grima et al. 2011; Thomas et al. 2013).

13.2.2.3 Dynamics in Cell Populations

Ordinary differential equations (ODEs) are the most common modelling technique found in Systems Biology studies, and this is mainly reflected by the data that is available detailing cellular processes. For example, temporal evolution of system components is measured across a cell population, e.g. by quantitative real-time PCR (qRT-PCR), such that any internal fluctuations in the system are cancelled out. Consequently, a number of software packages have been developed to allow for the easy construction and simulation of such models (e.g. COPASI), whilst computer languages are under constant evolution to share these models around the Systems Biology community (e.g. SBML) (Hucka et al. 2003; Hoops et al. 2006).

From a mathematical perspective, the temporal dynamics of a reaction system can be described by the system of coupled ODEs:

$$\frac{dX_i}{dt} = f_i(\mathbf{k}, \mathbf{X}(t)), \tag{13.1}$$

where $\mathbf{k}$ is the vector of kinetic rates (or *parameters*) that determine the evolution of the *variables* X_i (the number of molecules of type i per unit volume), f_i is a function that relates the kinetic rates and components of the system with the regulation of X_i ($\mathbf{X}$ is the vector of components X_i). The function f_i can take many different forms depending on the reactions taking place within the system and will be, in general, a non-linear function of the X_i's. We provide a few examples of such functions in Table 13.1 that can be summed together to form a complete ODE of synthesis, complex formation, and degradation rates.

The description using ODEs does not capture *any* stochastic effects; it is a solely deterministic description of the system under inspection. This means that

Table 13.1 Example forms of function f_i from (13.1)

Biological function	Reaction	Mathematical function
Synthesis (e.g. transcription)	$\emptyset \xrightarrow{k_s} X_1$	$\dot{X}_1 = k_s$
Molecule-dependent synthesis (e.g. translation)	$X_1 \xrightarrow{k_t} X_2$	$\dot{X}_2 = k_t X_1$
Degradation	$X_1 \xrightarrow{k_d} \emptyset$	$\dot{X}_1 = -k_d X_1$
Complex formation	$X_1 + X_2 \xrightarrow{k_{ca}} X_3$	$\dot{X}_{i\neq 3} = -k_{ca} X_1 X_2$ $\dot{X}_3 = k_{ca} X_1 X_2$
Complex dissociation	$X_3 \xrightarrow{k_{cd}} X_1 + X_2$	$\dot{X}_{i\neq 3} = k_{cd} X_3$ $\dot{X}_3 = -k_{cd} X_3$
Saturation (e.g. protein–promoter interactions)	$\emptyset \xrightarrow{k_s X_p} X_1$	$\dot{X}_1 = \frac{k_s X_p}{k_m + X_p}$ [a]
		$\dot{X}_1 = \frac{k_s X_p^n}{k_m^n + X_p^n}$ [b]
Reversible switching (e.g. phosphorylation)	$X \underset{k_2}{\overset{k_1}{\rightleftharpoons}} X_p$	$\dot{X} = k_2 X_p - k_1 X$

Note: $\dot{X} = \frac{dX}{dt}$
[a] Known as Monod kinetics
[b] Known as the Hill function

for a given set of parameters $\mathbf{k}$ and initial conditions $\mathbf{X}(t = 0)$ the result will always be the same. One of the advantages of ignoring stochastic factors is that systems constructed by ODEs can be analysed in an easier fashion to obtain direct relations between certain biological rates and the emergence of system properties, such as oscillations. However, solving and analysing (e.g. how does the dynamic of the system depend on the parameters $\mathbf{k}$?) a system of coupled non-linear ordinary differential equations can be a very challenging task. Therefore, one may seek to simplify the mathematical description such that the important features of the system remain sufficiently accurately described. For example, if a subset of the modelled processes occur at much shorter time-scales to the rest of the system, then their dynamics can be assumed constant ($\dot{X}_i = 0$) and thus greatly reduce the number of differential equations. We shall not provide any detailed methods here, these can be found in Murray (2002a), but we will highlight one classic example where an ODE system was simplified to produce a well-known relationship: Michaelis–Menten kinetics.

The Michaelis–Menten reaction is

$$E + S \underset{k_r}{\overset{k_f}{\rightleftharpoons}} C \xrightarrow{k_c} E + P,$$

where E is an enzyme, S is a substrate, P is the product produced by the enzyme-substrate complex, C, and k_j are the biological rates describing reversible complex formation and P synthesis.

This system can be written in the following ODE form:

$$\begin{aligned}
\frac{dE}{dt} &= (k_r + k_c)C - k_f ES, \\
\frac{dS}{dt} &= k_r C - k_f ES, \\
\frac{dC}{dt} &= k_f ES - (k_r + k_c)C, \\
\frac{dP}{dt} &= k_c C.
\end{aligned}$$

An important facet to this reaction is that the enzyme conservation law is maintained such that the total amount of enzyme ($E + C$) does not change with time. Thus, if E_0 is the initial concentration of enzyme, then $E + C = E_0$ for all time.

At this point, one of the two assumptions can be made as to the time-scales present within the system. The first is that the complex C is in instantaneous equilibrium (i.e. that binding of E and S is fast) which implies that $(k_r + k_c)C = k_f ES$. Notably, this is similar reasoning to the rationale seen previously when comparing statistical approaches of gene regulation to using kinetic rates in the CME. The second is that the dynamics of C occur much more slowly than those

of E and S. Mathematically, this implies that C is always at a *quasi-steady state* and, hence, $dC/dt = 0$.

Ultimately, by setting $E = E_0 - C$, both approximations lead to the same mathematical conclusion, namely

$$C = \frac{E_0 S}{K + S},$$
$$\frac{dS}{dt} = -\frac{E_0 S}{K + S},$$
$$\frac{dP}{dt} = \frac{V_{\max} S}{K + S},$$

where $V_{\max} = k_c E_0$ and $K = (k_r + k_c)/k_f$. Thus, by making two assumptions—the conservation law of E holds and that a subset of the biological processes occurs faster than the rest of the network—a system of four equations has been reduced to two ($\dot{S}$ and $\dot{P}$). Note that these equations describe the dynamics of the system at larger times correctly, after an initial equilibration time such that $\dot{C} \approx 0$ holds. If one is interested in the early time development of the system the full system needs to be considered.

We end this subsection with a quick note about larger scale models often encountered when modelling metabolic networks. Whilst these are not often encountered in Systems Biology studies of pollen tip growth they are a well-studied subclass of ODE models. The equation is formulated in matrix notation

$$\frac{d\mathbf{X}}{dt} = \mathbf{N}\mathbf{v}(\mathbf{X}),$$

where $\mathbf{N}$ is the stoichiometry matrix of a reaction set and $\mathbf{v}$ is a vector of fluxes that depend on the concentrations of components $\mathbf{X}$ within the system.

These networks, generally, are very large and can be time-consuming to analyse numerically. Thus, researchers assume that enzymatic reactions occur at a much faster time-scale compared to observable changes in phenotypes of an organism (e.g. growth rate). This implies that the set of differential equations can be set to zero such that

$$\mathbf{N}\mathbf{v}(\mathbf{X}) = 0.$$

This leaves us with a large set of linear equations from which a solution $\mathbf{v}^*$ can be found. Many methods have been derived to find the possible solutions of these equations that satisfy particular conditions and are mainly based on a process known as Flux Balance Analysis (FBA). We do not go into the details of these methods here, but the review by Orth et al. (2010) gives a general overview of the principles for interested readers.

13.2.2.4 Processes Across the Spatial Domain

Thus far we have concentrated on analysing the temporal changes within networks, however, many biological processes also vary across the spatial domain. Such networks and chemical gradients lead to phenomena like cell division, tissue generation, organ elongation, and skin coat patterning. For more examples we recommend the reader to look at the book by Murray (2002b). Here, we will briefly introduce the reaction–diffusion equation that mathematically describes a particular class of self-regulated spatial phenomena (Kondo and Miura 2010).

The question underlying spatial patterning of cellular tissue is how genetically identical cells can exhibit differentiated behaviour. A conceptually easy possibility is by using boundary layer information. A morphogen is produced at the boundary of the tissue and due to finite stability of the morphogen a gradient is established. Depending on the distance from the boundary cells experience disparate concentrations of the morphogen and by a threshold mechanism differentiate into different states (Wolpert 1996; Kondo and Miura 2010). In this scheme no feedback between cells is required. The challenge lies in the explanation of the threshold mechanism. Another possibility to achieve a spatial pattern is through the exchange of information between cells, which modifies the chemical reactions. One way to exchange information—or to achieve spatial coupling between cells—is by secretion of molecules. In many cases this can be described by a diffusion-like transport of molecules across the tissue. Using Fick's Laws, the flux of a concentration q across spatial domains is related to the diffusion coefficient by

$$J(x,t) = -D\frac{\partial q}{\partial x},$$
$$\frac{\partial q}{\partial t} \sim -\frac{\partial J(x,t)}{\partial x} = \frac{\partial (D\partial q/\partial x)}{\partial x} \equiv \nabla(D\nabla q),$$

where $\nabla = \partial/\partial x$ (or in three dimensions $\nabla = (\frac{\partial}{\partial x}, \frac{\partial}{\partial y}, \frac{\partial}{\partial z})$) that tells us how the concentration of q changes across a spatial step Δx. D is the *diffusion coefficient* that could be constant or depend on time and space. Adding this diffusion term to a chemical reaction system as described in the previous section results in a reaction–diffusion equation:

$$\frac{\partial \mathbf{q}}{\partial t} = \mathbf{f}(\mathbf{q}) + \nabla(D\nabla\mathbf{q}),$$

where $\mathbf{f}$ describes the reaction kinetics. This is referred to as a *partial differential equation* (PDE). Because the reactions are typically non-linear, reaction–diffusion systems are mostly non-linear PDEs, which need to be solved numerically.

In the absence of reaction kinetics, $\mathbf{f}(\mathbf{q})$ above, the boundary conditions of the system will determine the final pattern. In the presence of reaction kinetics, different patterns can be obtained depending on the system parameters such as the initial

conditions, the boundary of the system, and the kinetic rates. The diversity in these values leads to the rich variety of patterns observed across biological systems.

A number of other reaction–diffusion systems have now been studied due to the prevalence of patterns in biological systems. Generally, one can say that long-ranged inhibition and short-ranged activation of diffusing systems are necessary ingredients to produce stationary patterns as are seen in biology (Kondo and Miura 2010). For a system of two interacting and diffusing chemicals one can show that exactly two classes of pattern forming networks exist: activator-inhibitor and substrate-depletion (Murray 2002b). Many examples exist in the literature and interested readers can find further details in the textbooks by Murray and Edelstein-Keshet (including Edelstein-Keshet (1988) and Murray (2002b)).

13.2.2.5 Mechanical Descriptions of Tissue Growth

Thus far we have concentrated on methods of modelling individual molecules up to concentrations across temporal and spatial scales. Ultimately, these processes lead to the growth of tissues and organs. For example, in the case of pollen tip elongation, dynamic changes in ion concentration and subcellular localisation can have an impact on growth rate and organ development (Kroeger et al. 2008; Kato et al. 2010). However, once an organ has developed, mechanical forces start to play a role in growth dynamics, for example, friction and elasticity of the tissue surface (Goriely and Tabor 2008; Fayant et al. 2010). These sets of forces are different to those outlined at the start of this section (e.g. thermal forces). As with our previous discussions on the effects of forces in biological systems, we refer interested readers to Phillips et al. (2013).

If we go back to our previous description of biological interactions on the microscale, the system is in equilibrium states upon the minimisation of internal energy. When referring to mechanical growth of a tissue we are interested in *potential energy* or, rather, the amount of energy required for the organ to do 'work' and grow. Thus, a tissue is in mechanical equilibrium if the forces acting upon it are balanced and the potential energy is minimised. This implies that

$$\sum_i \mathbf{F}_i = 0,$$

where $\mathbf{F}_i$ is one of the i forces acting on the spatial structures of the tissue. These forces could include such effects as elasticity, elongation, weight, and friction.

The strain exerted on an extending tissue by a force can, for purely elastic material, be described by Hooke's Law which states that the force required to stretch the growing tissue is proportional to the displacement of a position along the tissue

$$F = -k\Delta x,$$

where F is the force, Δx is the displacement, and k is some positive constant. This can be rewritten as

$$\frac{F}{A} = E\epsilon,$$
$$\epsilon = \frac{\Delta L}{L_0} = \frac{L - L_0}{L_0},$$

where A is the cross-sectional area of the tissue, ϵ is the strain that describes the fractional extension of the tissue, and E is Young's modulus that represents how stiff the tissue is. In general the relationship between stress (force per unit area) and strain (relative displacement) in three dimensions is described by a tensor equation, i.e. the scalars stress and strain are replaced by second-rank tensors (Jones and Chapman 2012). Further complication can stem from the fact that the biological material is not purely elastic, but rather yields under stress, which renders the material to be visco-elastic (Jones and Chapman 2012). Moreover, often biological materials exhibit non-linear stress–strain relationships, for which the linear approach sketched above is only valid for small relative displacements of the tissue (Jones and Chapman 2012).

An important factor for mechanical growth is the strain energy density that (for a linearly elastic material in one dimension) takes the form

$$W(\epsilon) = \frac{1}{2}E\epsilon^2 = \frac{1}{2}E\Big(\frac{\Delta L}{L_0}\Big)^2.$$

This function provides us with an estimate for the total stored strain energy contained per unit volume of the growing biological material. These relationships can be extended to relate growth across two- or three-dimensional spaces to forces exerted on the tissue from different directions. In the case of pollen tip elongation, such external forces have been shown to have an effect on tip shape and speed of elongation (Goriely and Tabor 2008; Kroeger et al. 2008). Phillips et al. present several examples showing how to calculate and analyse these functions for the effects of forces on growing biological material (Phillips et al. 2013).

In this section, we have covered some basic principles that can be used to model biological systems at different levels, from interactions between individual molecules to population level dynamics of system components in a cell to forces acting on tissue growth. Choosing the right modelling approach is a challenging task and requires knowledge about the corresponding mathematics, the physics, the biochemistry, or more generally, the biology of the system. Regardless of the modelling strategy employed in a study though, model analysis and the estimation of biological rates follow similar principles. However, before we turn our focus to these issues, we shall discuss one caveat to modelling, notably that if different models can describe the same phenomena, which model is correct?

13.2.3 Non-uniqueness of Models and Model Selection

In the previous section, we highlighted the methods used for modelling networks in Systems Biology across a range of different spatial and temporal scales. However, one should consider (and remember) that models are built with a specific purpose in mind and are constrained by the prior knowledge of the system. This raises an interesting epistemological question about the comparison of models. For example, could the same biological phenomena be described by multiple models? If so, is there a way of determining which model is more useful to obtain new biological insights? Could a different model exist to describe the same system and more?

Whilst this issue has not been fully realised yet in a number of Systems Biology fields, one such example where these ideas have been considered is that of the plant circadian clock. Due to the nature of obtaining qRT-PCR measurements from plant tissues (i.e. a population of cells), models of the circadian clock have been constructed using ODEs and the Langevin equation. Since the first mathematical model was published in 2005, a number of revisions to the model have been made as larger amounts of data have become available and incorporated into the mathematical analysis (see Locke et al. (2005, 2006), Guerriero et al. (2012), and Pokhilko et al. (2010, 2012)). Consequently, the number of components and biological rates has shot up from <10 components and approximately 30 parameters, to nearly 30 components and over 100 parameters over the course of these model iterations. Notably, due to high levels of interest in the dynamics of plant circadian behaviour, one version of the plant circadian clock model was obtained independently by two research groups working with similar data and similar assumptions (Locke et al. 2006; Zeilinger et al. 2006).

Recent work has aimed to elucidate the basic core structure of the circadian clock that can describe the available datasets in a qualitative manner (De Caluwé et al. 2016). By reducing the model to 4 core subunits, the system size decreased to 9 components and 34 parameters. This core minimal model responded in similar manner to the data upon altered environmental conditions and when the system was perturbed through transgenic alterations. Whilst this may suggest that the larger, more complex models are *overfitting* the real biological system (i.e. the system is so complex that it can describe any simple systems), this is not really the case. Larger systems are able to describe a whole range of genetic perturbations in detail that reduced or coarse-grained models cannot due to the lack of appropriate mechanisms and components.

So which model is 'best'—the minimal model or the more complex and detailed system? Importantly, the answer to this question depends on the research problem that one wishes to solve. For example, if the user was interested in understanding large scale effects of genetic perturbations within the system, or wished to understand how their new component could be incorporated into the current models, then using the more complex systems would be appropriate. However, for a conceptual understanding or if one wished to understand more qualitative effects, such as how an output of the network would be altered in different experimental settings, then the coarse-grained model would be easier for use.

Notably, mathematical and statistical methods of model comparison have been developed over the years. These methods range from the Akaike Information Criterion (AIC) to computing the probability of a model reproducing data given specific biological rates to characterising *Pareto fronts* that analyse a models ability to fulfill multiple different requirements (Akaike 1974; Friel and Pettitt 2008; Vyshemirsky and Girolami 2008; Simon 2013). For the purposes of understanding the evolution of biological networks, and how to manipulate them for the needs of Synthetic Biology, studying a range of model systems in conjunction (thus analysing a range of positive and negative model traits) promises to be a highly fruitful avenue of research over the coming decades.

13.3 Identification and Estimation of Biological Rates

Once a model has been constructed that describes the biological processes deemed important in producing specific responses, the next step in development is to obtain estimates for the rates that describe synthesis, degradation, complex formation, etc. As with obtaining equations by which a network is described, the parameter values used in simulations are also constrained by the available data. In this section, we shall discuss some of the key issues around parameter estimation and highlight to readers the principle ideas behind these concepts.

13.3.1 Experimental Variation of Data

The first step before attempting to estimate any parameter values is data collection with which to compare model simulations. As is well understood in experimental design, variation within a dataset can occur through two sources—intrinsic biochemical fluctuations (as is often captured mathematically by the CME) and external fluctuations (such as those in the environment or due to experimental equipment). Consequently, if one wished to match model simulations to data from a specific experimental condition, you would wish that the intrinsic variability is small. Thus, you would have confidence that you can find a specific set of parameters that describe this data. Alternatively, if the data is highly variable, then this would negatively impact the chances of finding a single optimal parameter set. Similarly, since changing experimental conditions can lead to alterations in biological networks, data for parameter estimation would ideally come from a single set of experimental conditions. Mixing of datasets across different experimental set-ups could lead to further variation in parameter estimates and the researcher cannot guarantee that the underlying system of equations do not need altering between different experimental conditions.

Another source of variation in data collection can occur through species comparisons. To illustrate this issue, we shall draw on one pertinent example. Let us assume that a large network of metabolic reactions is known but that there are limited amounts of experimental data obtained to aid parameter estimation. This

is often referred to as an *underdetermined* problem, i.e. there is not enough data available to get good estimates of the biological rates (Orth et al. 2010). In some instances, large online databases have been developed that contain a wide range of experimentally measured catalytic rates and equilibrium constants (Schomburg et al. 2004; Flamholz et al. 2012; Wittig et al. 2012). Thus, if a model was being built to understand metabolism of a relatively understudied species, one could be pragmatic and obtain estimates for a large number of rates from closely related species. This assumes, of course, that the two species are evolutionarily close such that the underlying metabolic networks for the two species are relatively similar.

13.3.2 Parameter Estimation Methods

Using the available data, one needs to find a method of estimating the system parameters such that the model simulations match with what is observed experimentally. This could either be done by manual tweaking of parameter values within the model or through a more automated and unbiased approach. There is a wide range of literature related to this problem (Simon 2013; Raue et al. 2014). Here we shall go through the basic principles of how to obtain estimates for the parameter rates of a model given specific data.

Arguably, the most important facet of this procedure is to construct a scoring function that is *smooth* and has defined finite *maxima* or *minima*. By smooth, we mean that if one was to plot the scoring function in a multi-dimensional surface that no discontinuities exist. This means that for a given set of parameter values some finite score definitely exists and that no jumps within the surface occur. By finite maxima or minima, we mean the scoring function cannot extend to the realms of positive/negative infinity when calculated on a computer. The introduction of symbolic numbers (such as infinity in many computer software packages) can lead to problems when automating the optimisation process such that score values between different iterations are numerically compared.

In principle, one can construct a scoring function to match a particular model feature (such as oscillatory behaviour or relaxation to a steady state after an external pulse) or to measured data values. In the following we shall discuss the case where a modeller has data available to compare the model against. One of the simplest scoring functions is the calculation of the sum-of-squared residuals

$$C = \sum_{i=1}^{n} \frac{(x_i - y_i)^2}{\sigma_i^2},$$

where x_i is the simulation at a given datapoint, y_i is the measured datapoint, and σ_i^2 is the variance of datapoint y_i. The score, C, is calculated as the sum over the n measured datapoints. Importantly, one should notice that as the model simulations $\mathbf{x}$ get closer to the data $\mathbf{y}$ (denoted as $\mathbf{x} \rightarrow \mathbf{y}$) then $C \rightarrow 0$. Hence, the subset of parameter values where $C = 0$ are the set of potentially correct biological rates for the system under study. Importantly, the origins of such a scoring function can

be found in the derivation of likelihood probabilities for Gaussian distributions, i.e. $C \sim \log P(\mathbf{k}|\mathbf{x}, \mathbf{y})$ for parameter set $\mathbf{k}$ found given the model simulations $\mathbf{x}$ that is being matched to data $\mathbf{y}$ (Raue et al. 2009).

Due to the complexity of high-dimensional parameter spaces (that have as many dimensions as the number of parameters being estimated), one wishes to explore this space and obtain scores C to determine where the optimal parameter set lies. One possible method of doing this is by calculating C for all possible parameter combinations in an appropriately discretised parameter space, which is highly time-consuming from a computational perspective and infeasible for high-dimensional parameter spaces. Another option would be to use a Latin Hypercube sampling method (McKay et al. 2000), whereby the evaluated parameter sets are evenly distributed through parameter space, and calculate the scores C. This gives the user information about the global structure of the parameter space, but may also indicate subregions where the global optimum is likely to exist for closer inspection. One final option would be through the use of automated optimisation algorithms whereby, given an initial starting point in parameter space, the algorithm updates itself towards the direction of an optimal solution where $C \rightarrow 0$.

Minimisation functions generally have the following steps (see Fig. 13.3 for a pictorial overview):

- Pick random starting point in multi-dimensional parameter space, $\mathbf{k}_0$.
- Simulate the model using $\mathbf{k}_0$ and calculate C_0.

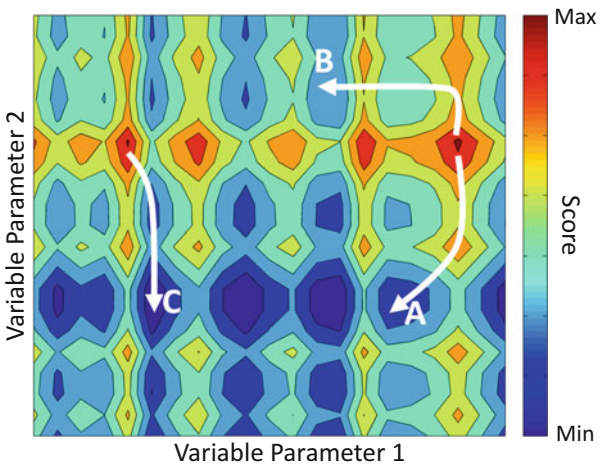

Fig. 13.3 An example of two-dimensional multimodal parameter space. Given variation in two parameters, optimisation routines aim to find the global minimum (*dark blue*) within the search space. However, due to the complexity of some mathematical models, several minima could exist. Here, we show three illustrative examples whereby the optimisation starts at a high-scoring parameter set (*red regions*) before moving towards (**a**) a low-scoring local minima, (**b**) a high-scoring local minima, and (**c**) the global optimum. What this highlights is that, depending on where within the search space an algorithm begins, the likelihood of finding the optimum result also changes

- Set i = 1.
- Optional: set $C_{\text{threshold}}$ as some threshold that C must be lower than for $\mathbf{k}$ to be considered optimal.
- While $C_{i-1} > 0$ (or $C_{i-1} > C_{\text{threshold}}$):
  Pick $\mathbf{k}_i$ as perturbed parameter set of $\mathbf{k}_{i-1}$;
  Simulate the model using $\mathbf{k}_i$ to calculate C_i;
  If $C_i < C_{i-1}$:
  Set i = i+1.
  Else if $C_{i-1} \leq C_i$:
  Do not change i.
  Else if $C_i = 0$ or $C_i < C_{\text{threshold}}$:
  Stop the algorithm as you have found the optimal parameter set.

Thus, one can see that with each iteration of the algorithm, the optimal parameter set is only updated when the score is less than the previous best result. Hence, upon reaching zero or the manually chosen threshold for optimal parameter sets, the algorithm stops and the optimal $\mathbf{k}$ can be obtained.

Multiple computational methods have been created to improve the accuracy and reliability of parameter estimation. These methods range from multi-start minimisation, whereby the minimisation procedure above is started from multiple different random positions to cover as much of the multi-dimensional parameter space as possible, to methods based on the principles of random walks, such as Simulated Annealing, whereby the jump to a new parameter set or region of parameter space is determined probabilistically. The interested reader can find details in (Simon 2013). One interesting point to make, though, is that the scoring function above can be generalised in two ways to incorporate multiple experimental conditions. In the following subsection we shall introduce these ideas.

13.3.2.1 Multi-Experiment Fits

The first way in which the scoring function can be generalised is to describe the match between data obtained from several experimental conditions and multiple model simulations. This can be used, for example, in cases where an input signal into a model is altered but the underlying network structure and biological rates should remain unchanged. Thus, one can rewrite the scoring function to be

$$C = \sum_{j=1}^{m} C_j$$

$$= \sum_{j=1}^{m} \sum_{i=1}^{n_j} \frac{(x_{i,j} - y_{i,j})^2}{\sigma_{i,j}^2}.$$

Hence, one is not just taking into account the n_j datapoints in experiment j, but also what takes place in m different experiments. The advantage of using multiple

datasets is that this can result in the parameter space being constrained to subregions where both datasets are described equally well. In large systems, this is particularly important as the parameter space in some dimensions may be relatively flat (i.e. that the parameter could be any value without altering the score C).

13.3.2.2 Multi-Objective Optimisation

A second generalisation to the scoring function above is to include weights within our multi-experiment fit. Therefore

$$\begin{aligned} C &= \sum_{j=1}^{m} w_j C_j \\ &= \sum_{j=1}^{m} w_j \sum_{i=1}^{n_j} \frac{(x_{i,j} - y_{i,j})^2}{\sigma_{i,j}^2}, \end{aligned}$$

where w_j is a vector of weight values given to particular comparisons to experimental conditions. Again, the advantage to doing this is that the parameter space can be constrained in such a way that only optimal parameter sets in particular subregions are considered.

What is interesting to note is if the optimisation procedure is carried out for multiple weight vectors, w_j. The resulting multi-dimensional space of $C(\mathbf{w})$ values forms what is known as a Pareto front. Pareto fronts are often used in other engineering disciplines where one wishes to consider trade-offs between multiple different optimal situations. Whilst this principle is only just starting to be used for biological problems, it can provide researchers with an interesting view of their systems (see Shoval et al. (2012) for an example). For example, given a specific biological system, model, and dataset, one could obtain a range of parameter sets but find that some are robust to environmental perturbations but sensitive to genetic manipulations, whilst other parameter sets have the opposite properties. Thus, researchers will understand how to manipulate their biological system for future applications depending on the functions they want the system to achieve (e.g. robustness to environmental variation).

13.3.3 Parameter Identifiability Problem

Upon obtaining your optimal parameter set, post-analysis of the parameter search space can be informative in deciding which experiments should be conducted in future to obtain more accurate information about the biological system of choice. As eluded to above, if a particular direction within parameter space is flat, this implies

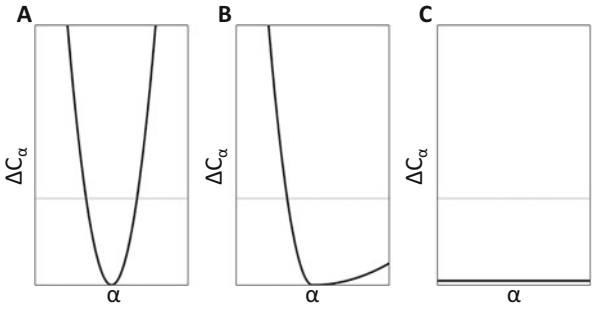

Fig. 13.4 Parameter identifiability as observed by re-optimising parameter α. (**a**) The parameter α is identifiable since fixing the value of α in both directions (increasing and decreasing) and re-optimising all other parameters increases the optimal score beyond a given threshold (*dashed line*). (**b**) The parameter α is practically non-identifiable since ΔC_α only increases above the threshold when α is decreased. (**c**) The parameter α is structurally non-identifiable since ΔC_α does not increase above the threshold regardless of α being increased or decreased

that the parameter can take any value without altering the scoring function C. Such a case leads to an *identifiability* problem, i.e. this parameter is non-identifiable from the current datasets being used during optimisation (Raue et al. 2009). Following the theory produced in Raue et al. (2009) (and sources therein), non-identifiability of a parameter can be assessed by looking at how the scoring function C changes as one parameter, α, is fixed and all others are re-optimised. If the difference between new and previous optimal scores ($\Delta C_\alpha = C_\alpha^{\text{new}} - C_\alpha^{\text{opt}}$) is less than a specific threshold, then the parameter is non-identifiable.

The question is whether any further experiments could be conducted that would render this parameter identifiable (Fig. 13.4). There are two forms of non-identifiability:

- Practical non-identifiability (Fig. 13.4b)
  ΔC_α is less than the threshold in one or both directions. This implies that the confidence interval is infinite. In principle, this means that the amount and quality of the data is insufficient to obtain a good estimate for this kinetic rate.
- Structural non-identifiability (Fig. 13.4c)
  ΔC_α is perfectly flat. This implies that a functional relationship between model parameters exists. To cure this one needs to change the model by taking into account qualitatively new data (e.g. from a new experimental condition).

Thus, by assessing multiple parameter fits between a model and data, one can understand where information is lacking about a given biological system and design experiments to appropriately remedy the situation. In the next section, we shall assume that a model has been constructed and an optimal, identifiable parameter set has been obtained. We shall then discuss how principles of biological phenomena can be validated and assessed to provide new insights into biological mechanisms.

13.4 Model Analysis, Validation, and Experimentation

At this point, the researcher should have a complete mathematical model with optimal rates that allow for simulations to closely match measured data. However, this is not the end of the modelling process. What we have currently is a model that describes the data that we already know. The next step is to assume that this model represents the idealised biological system—at least to the extent that it captures the relevant features—and to determine what new insights into the network can be made through analysis. The analysis of mathematical models does not necessarily follow a strict protocol and different researchers have different preferred techniques. Hence, although there have been some preliminary attempts at automation, we shall discuss here our own opinions on how model analysis can be performed (MacDonald et al. 2011; Rausenberger et al. 2011; Song et al. 2012; Rybel et al. 2014; Gabor and Banga 2015; Seaton et al. 2015).

13.4.1 Validating a Model Against Data Unused in Optimisation

An important aspect of mathematical analysis is the *predictive power* of the model. In principle, this means that although the model has been constructed to match a particular dataset, the model should still be able to match newly obtained data not used during model development and optimisation. Consequently, there are three possible results:

- Case 1: the model quantitatively matches the validation dataset.
  This situation implies that the model (and the obtained kinetic rates) is a sufficient representation of the biological system.
- Case 2: the model qualitatively matches the validation dataset.
  The model captures the main effects, but there is an error either in the model or the obtained parameter set.
- Case 3: the model does not match the validation dataset.
  In this case, the model is, in some way, structurally incorrect and is missing elements. Thus, our initial idealisation of the biological system being studied is incorrect.

Arguably, cases 2 and 3 are the most interesting for researchers. These cases require further research and new experiments to be conducted to improve the model's accuracy. This leads to an improved understanding of how biological systems function.

So how should one divide their datasets into *test* data (used in parameter optimisation) and *validation* data? This question often has no answer and differs from researcher to researcher, but in principle one requires the test dataset to be

large enough that each parameter of the system can be adequately predicted but no more than that. Such a dataset could incorporate measurements obtained from *wild-type* or *unperturbed* conditions plus measurements from a small subsection of genetic perturbations. The validation dataset could then include measurements from the rest of the genetic perturbations plus data from different experimental conditions that would alter any model inputs.

13.4.2 Obtaining Experimentally Testable Hypotheses

Upon finding that model simulations do not match the validation dataset, the aim of the researcher is to find out why and whether any modifications to the model would allow one to describe both the test and validation data. As with model construction there are two issues at play. In the first, one could look to re-optimise the biological rates in a multi-experiment fit by incorporating the validation data into the test dataset. The resulting model would then need to be validated against new data to determine whether the model is describing biological reality. In the second case, one should look at whether the mathematical model needs to be altered in order to describe both the test and validation data. It is these steps that provide researchers with the opportunity to obtain new biological insights.

Let us say that, based on previous knowledge and assumptions, a model has been created that is able to match the biological process of an unperturbed system. However, simulations of a genetic perturbation do not match the experimental data. There could be two potential cases: the model shows no response to a genetic perturbation whereas experiments show changes to (e.g. increased) expression levels of a model component, or; the model shows the opposite response to those observed experimentally (decreased simulated expression compared to observed experimental increases). The first example could be rectified by a re-optimisation of the system parameters to produce a stronger simulated response upon network perturbations. In the second case, though, it is likely that the model is missing a particular type of regulation—in this instance, where the model simulates decreased expression given a genetic perturbation but data shows increased expression, then it is possible the network is missing some form of feedback regulation.

Only by computationally experimenting with the mathematical model can the range of plausible missing mechanisms be found. Such experimentation is quicker and cheaper than performing similar tests experimentally in the lab (a more practical advantage of mathematical modelling). Furthermore, if one is able to find a simple solution to match model simulations with experimental data, then this can be tested in the lab with extra genetic perturbations or conducting experiments under new conditions.

13.4.3 Design Principles: Relating Model Variables/Parameters to the Emergence of Biological Phenomena

By this point, one should now have a model (with corresponding parameter estimates) that can describe all the available datasets. As stated above, we can now believe that the mathematical system is an idealised version of biological reality. Consequently, one may be interested in more philosophical discussions. For example, what is the mechanism present in the model that is the most important for the emergence of a specific biological phenomena? What is the core model structure required that maintains the desired response? These questions can be highly illuminating for a number of reasons, but arguably the most important reason is that one can relate simple theoretical mechanisms to biological phenomena.

To highlight this point, we refer back to our earlier example of biological oscillators. The most recent mathematical model of the plant circadian clock is a highly complex system comprising multiple feedback mechanisms (Pokhilko et al. 2012). However, it has been known for nearly 50 years that stable oscillations can arise in simple negative feedback systems comprising of three components with a reaction that produces a sufficient amount of time delay within the network (Goodwin 1965). This has been experimentally shown by the creation of a synthetic oscillator in *E. coli* (Elowitz and Leibler 2000). Thus, why is such a complex network required for circadian regulation of plant function? It turns out, though, that the plant circadian model can be conceptually simplified to a model that is highly reminiscent of the synthetic oscillator (Pokhilko et al. 2012). Furthermore, model analysis has highlighted that this simple mechanism is able to qualitatively describe large amounts of data obtained from experiments in plants (De Caluwé et al. 2016). Thus, it appears that the circadian clock in plants (and potentially in other species) has evolved from a simple core structure to a more complex mechanism.

13.5 Conclusions

In this chapter we have attempted to cover as many of the topics that we feel are important to construct accurate and useful mathematical models of biological systems. We hope that one can observe that models can be constructed for a range of reasons, whether that be to design new biological systems or to understand the emergence of phenomena in existing networks. Crucially, the critical point of model construction is to determine which modelling technique one can use given the data that is available. Thus, it is vitally important that one understands the basic assumptions behind different mathematical concepts in order to create a meaningful coarse-grained model. Upon making this choice, the following steps of model construction and analysis are fairly homogeneous for all model types. Whilst modelling has proven useful in many areas of science and industry, we hope that this

introduction will aid the development of future models to elucidate the biological mechanisms required for pollen tip elongation.

Acknowledgements Given the large field of mathematical modelling in biological systems we would like to apologise to any readers who feel that we have neglected important references. The references contained herein are those that the authors believe would provide a useful introduction to interested readers. RWS is funded by FP7 Marie Curie Initial Training Network grant agreement number 316723. CF is funded by HFSP Research grant RGP0025/2013.

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Chapter 14
A Fresh Look at Growth Oscillations in Pollen Tubes: Kinematic and Mechanistic Descriptions

Milenka Van Hemelryck, Roberto Bernal, Enrique Rojas, Jacques Dumais, and Jens H. Kroeger

Abstract Pollen tubes exhibit rapid polar growth that can display either stationary (steady) or oscillatory elongation rates. The oscillatory mode of growth provides a window into the network of interactions regulating the morphogenesis of these cells. Empirical studies of oscillatory pollen tube growth have sought to uncover the sequence of cellular events that constitute one oscillatory cycle, while other studies have attempted to formalise the principal causal interactions into computational feedback models. In this chapter, we first review the phenomenon of oscillatory tip growth from a kinematic standpoint. Three key kinematic features have emerged from our analysis: (1) oscillatory cells dominate at high elongation rates, (2) well-defined symmetrical and asymmetrical modes of oscillation are observed, and (3) the oscillation cycle of most pollen tubes unfolds over a fairly well-defined distance, independently of the average elongation rate. We then discuss some mechanistic models aiming to explain oscillatory growth and evaluate their ability to account for the observed kinematic features. Although some of these models have reached a fairly high degree of sophistication, none account for the whole range of kinematic behaviour reported in pollen tubes. We conclude with some suggestions of how current models could be improved.

Keywords Pollen tube growth • Oscillations • Modelling

M. Van Hemelryck • R. Bernal
Universidad de Santiago de Chile, Av. Ecuador 3493, 9170124 Santiago, Chile

E. Rojas
Stanford University, 443 Via Ortega, Stanford, CA 94305, USA

J. Dumais
Universidad Adolfo Ibáñez, Av. Padre Hurtado 750, Viña del Mar, Region V, Chile

J.H. Kroeger (✉)
Raymor Nanotech, Boisbriand, Québec, J7H 1R8, Canada
e-mail: jkroeger@raymor.com

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14.1 Introduction

Oscillatory responses are observed in many biological processes including the firing of node cells in the heart, glycolytic oscillations in yeast and muscle, intracellular calcium waves in plant and animal cells and the circadian oscillation of gene products (Goldbeter 1996; Novak and Tyson 2008). Damped oscillations are also central to the function of man-made feedback systems such as thermostats. Generally speaking, these oscillations can be viewed as a regulatory mechanism to attain homeostasis, i.e. to maintain stable internal conditions that ensure an adequate behaviour (Goldbeter 1996; Feijó et al. 2001; Novak and Tyson 2008; Portes et al. 2015).

The discovery of oscillatory growth in pollen tubes has motivated researchers to exploit this response to piece together the different feedback interactions that control polar growth. Although there is still debate as to whether oscillatory growth is adaptive in pollen tubes or whether it is even part of the normal in vivo morphogenesis of these cells (Iwano et al. 2009), there cannot be any doubt that oscillatory growth is a powerful tool to analyse the molecular and biophysical control of cell morphogenesis. Like a mutation, oscillatory growth gives us a window into the control systems that govern pollen tube morphogenesis.

Many measurable cytological changes associate with oscillatory growth, including changes in Ca^{2+} and proton concentrations (Pierson et al. 1996; Holdaway-Clarke et al. 1997; Messerli et al. 2000; Lovy-Wheeler et al. 2006), wall thickness (McKenna et al. 2009) and the cytoplasmic concentrations of enzymes (Hwang et al. 2005; Cárdenas et al. 2006). To understand how these different elements interact to produce specific growth patterns, many studies have focused on the phase relationships between oscillating physiological events and pollen tube growth rate (Hepler et al. 2006). These phase relationships give us valuable information about the plausible causal link between cellular processes.

Although papers analysing specific aspects of oscillatory tip growth abound, few studies have attempted a systematic analysis of the kinematics of pulsatile cells and stated explicitly the implications for tip growth models. The objective of this chapter is to present an overview of the phenomenon of oscillating tip growth. We begin with a detailed analysis of the range of kinematic responses observed in lily pollen tubes and follow with a survey of the feedback interactions that could be the basis of the oscillatory response.

14.2 The Phenomenon of Oscillatory Tip Growth

Although kinematic observations for pollen tube elongation are available in the literature, no single paper is of the scope necessary to reach general conclusions about the most basic kinematic descriptors, such as the probability of oscillation, the range of oscillations possible and their specific amplitude, period, etc. Therefore, in

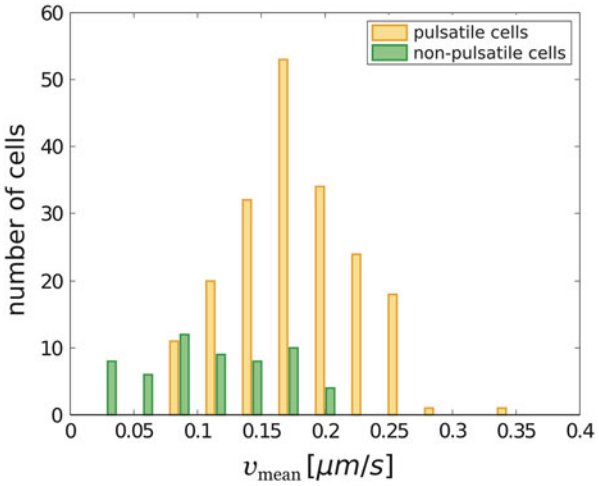

Fig. 14.1 Distribution of the average elongation rate of lily pollen tubes

this section we complement published kinematic results with unpublished results obtained from 251 pollen tubes from Asiatic lily cultivars (*Lilium* sp.). The experimental conditions and computational tools used to obtain these results are presented in Box 14.1.

Oscillatory tip growth has been reported in a number of systems besides pollen tubes (Lopez-Franco et al. 1994; Monshausen et al. 2007), but inspection of the elongation rates in non-pollen systems such as fungal hyphae or root hairs reveals very few instances of oscillations approaching the regularity observed in pollen tubes. In fact, most non-pollen reports show time series more akin to experimental or biological noise than true oscillations. We may thus ask why pollen tubes should distinguish themselves in that respect. A possible explanation comes from kinematic analyses of pollen tubes themselves where it has been noted that only pollen tubes exceeding a certain elongation rate exhibit oscillations (Rojas et al. 2011). In the case of lily pollen tubes, the minimum critical value for oscillation is approximately 0.05 μm s^{-1}. Whereas pollen tubes elongating at four times this critical value are common (Fig. 14.1), no other plant system appears able to exceed it (Williams et al. 2016). Therefore, pollen tubes may oscillate because they elongate faster than any other tip-growing cells found in plants. Interestingly, all lily pollen tubes elongating at more than 0.2 μm s^{-1} are observed to oscillate (Fig. 14.1).

The characteristics of the oscillatory elongation rate of pollen tubes depend largely on the species under observation. In particular, the pollen tubes of *Lilium longiflorum* (diameter of 16–20 μm) grown in vitro display sinusoidal oscillations with a period of 15–60 s and average elongation ranging from 0.1 to 0.4 μm s^{-1} (Pierson et al. 1995, 1996; Holdaway-Clarke et al. 1997; Messerli and Robinson 1997; Messerli et al. 1999; Rojas et al. 2011). In contrast, the pollen tubes of *Nicotiana tabacum* (diameter of 7.7–11.8 μm) and *Petunia hybrida* (diameter of

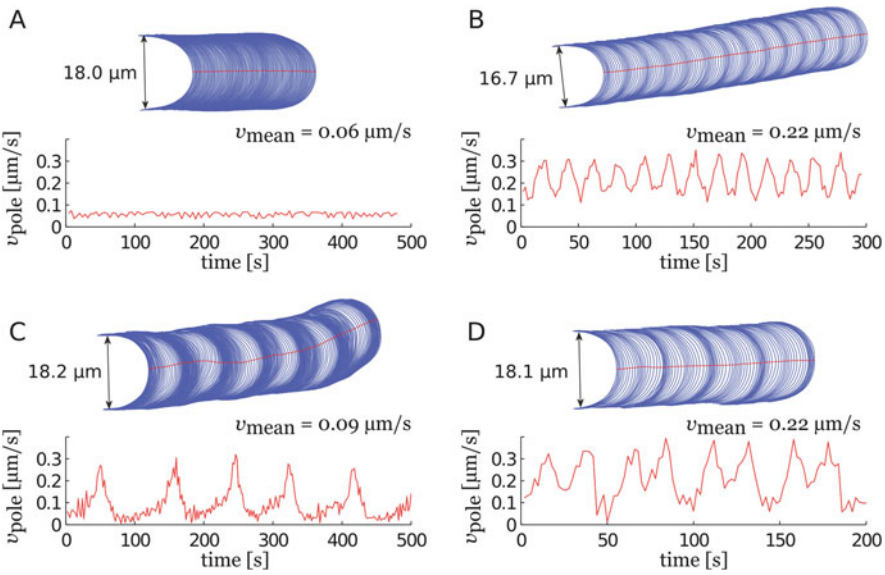

Fig. 14.2 Different growth rate profiles of cells under the same experimental conditions. (**a**) A stationary (steady) cell. (**b**) A symmetric pulsatile cell. (**c**) An asymmetric pulsatile cell. (**d**) A pulsatile cell with multiple peaks

9–13.2 µm) observed in vitro display abrupt peaks in the elongation rate (lasting 10–20 s) at intervals of several minutes (lasting 4–8 min) and an average elongation rate ranging from 0.02 to 0.08 µm s^{-1} (Geitmann et al. 1996; Pierson et al. 1995).

The characteristics of the oscillatory cycle appear to depend also on the 'internal state' of the cell. For example, lily pollen tubes grown under identical conditions fall into four groups according to the shape of their elongation rate profile (Fig. 14.2). Pollen tubes can grow at a steady rate, with minor random fluctuations (<0.03 µm s^{-1}) around an average speed (Fig. 14.2a), or can exhibit pulsatile growth (Fig. 14.2c–d). The most frequent form of oscillation is symmetric with a 'sinusoidal' or 'triangular' waveform (Fig. 14.2b). Another type of oscillation is characterised by an asymmetric waveform (as measured by the skewness of the distribution, Box 14.1). In this type of cells, the growth rate is characterised by an initial exponential increase, followed by an abrupt decline in speed (Fig. 14.2c). The amplitude of these oscillations is relatively high with respect to the average growth rate (Table 14.1). Finally, some pollen tubes present two or more peaks during oscillation (Fig. 14.2d). Among the cells analysed, 169 showed symmetric oscillations, 25 asymmetric oscillations and 57 stationary cells, while a handful of cells adopt more complex modes of oscillation.

The characterisation of oscillations as symmetric and asymmetric provides a natural grouping for two well-defined populations of cells (Fig. 14.3). Using the probability density function for the tip velocity, we find that for symmetric oscillations, cells are found with equal probability elongating at rates superior or

Table 14.1 Representative average elongation rate, amplitude and period of oscillation for different types of cells

	Oscillating		
	Symmetric	Asymmetric	Stationary
Mean elongation rate (± RSD[a]) [μm s^{-1}]	0.19 (±31%)	0.12 (±69%)	0.11 (±16%)
Mean amplitude (± RSD[a]) [μm s^{-1}]	0.08 (±25%)	0.14 (±13%)	
Mean period (± RSD[a]) [s]	35 (±14%)	80 (±9%)	

[a]Relative standard deviation (internal)

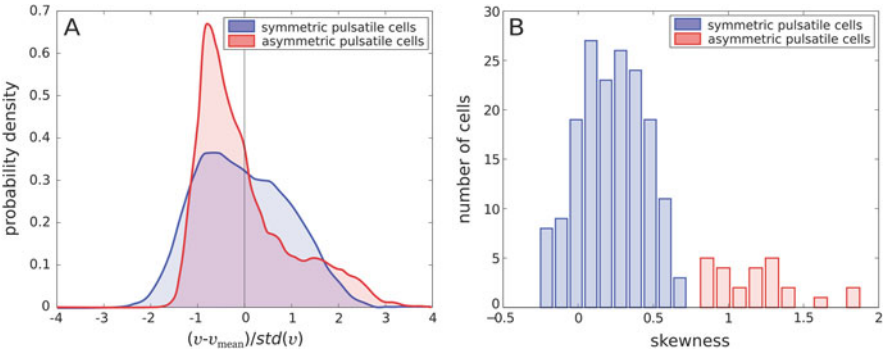

Fig. 14.3 Characteristic velocity distribution of symmetrically and asymmetrically oscillating cells. (**a**) Histograms showing the probability density function of all normalised velocities for symmetric and asymmetric cells. (**b**) Histogram of skewness for all cells observed. Cells oscillating symmetrically have small skewness (skew < 0.8), while cells oscillating asymmetrically have large skewness (skew > 0.8)

inferior to the mean elongation rate (Fig. 14.3a). In contrast, asymmetric oscillations are characterised by long periods in which the cell is elongating at a rate inferior to the mean elongation rate and brief periods spent at very high velocities (Fig. 14.3a). Symmetric and asymmetric pulsatile cells also show significant differences in the period and amplitude of oscillation as well as the mean elongation rate (Table 14.1). The mean period and the mean amplitude of oscillation of symmetric cells are approximately half the value observed in asymmetric cells. This difference may imply a mechanistic difference between these two types of oscillation.

Globally, our observations indicate that for high average elongation rates (v_{mean}), the period of oscillation (p) is shorter (Fig. 14.4). The dependence of p on v_{mean} is well fitted with the relation $p = l/(v_{\text{mean}} - v_0)$, where $l = 5.3$ μm and $v_0 = 0.03$ μm s^{-1}. The value of v_0 matches rather closely the minimum elongation rate observed experimentally in pollen tubes. An inverse relationship between the average growth rate and the period of oscillation is indicative of an oscillation cycle unfolding over a well-defined distance. We find that the product of p and v_{mean} gives a characteristic length scale of 6.5 ± 2.1 μm, which is slightly shorter than the radius of the pollen tubes observed in this study (8.7 μm). While it was argued that this length scale could emerge from simple mechanistic interactions during tip

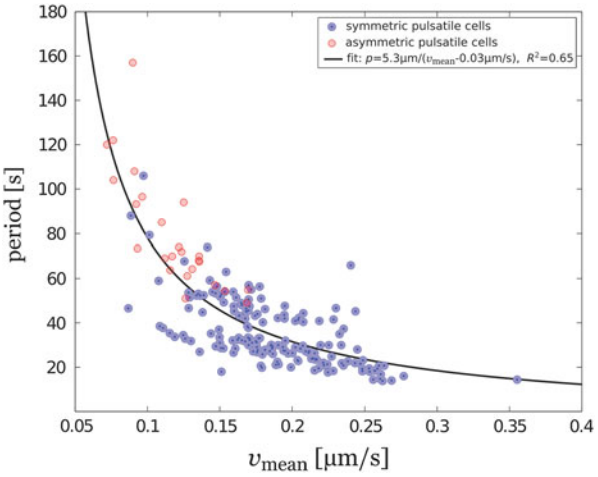

Fig. 14.4 Relationship between the period of oscillation and the elongation rate of lily pollen tubes

growth morphogenesis (Kroeger and Geitmann 2013; Rojas et al. 2011), more work remains to be done to establish a solid mechanistic basis for the relation observed in Fig. 14.4.

In summary, models of pollen tube growth should explain three major kinematic observations: (1) the dominance of oscillatory cells at high elongation rates (Fig. 14.1); (2) the different modes of elongation—stationary (steady) growth, symmetric oscillations, asymmetric oscillations and 'excited' oscillations (oscillations having two or more peaks in growth rate, Fig. 14.2)—as well as their specific characteristics (Fig. 14.3, Table 14.1); and (3) the inverse relationship between the period of oscillation and the average growth rate (Fig. 14.4).

Box 14.1 Methodology

Pollen grains were obtained from Asiatic lily cultivars (*Lilium* sp.). Grains were germinated in growth medium containing 15 mM MES buffer, 1.6 mM H_3BO_3, 0.1 mM $CaCl_2$, 0.1 mM KCl and 7.5% sucrose, adjusted to pH 5.3 with KOH. For microscopic observations, a square chamber made with dental polymer was affixed to a glass slide. A thin layer of growth medium supplemented with 1% low melting point agarose was deposited inside these chambers. While the agarose was still in a molten state, pollen grains were deposited onto the surface. After approximately 40 min, the chamber was filled with growth medium and covered with a cover slip. In this way, pollen tubes grew along the gel-liquid interface rather than in and out of the imaging plane, which was ideal for imaging.

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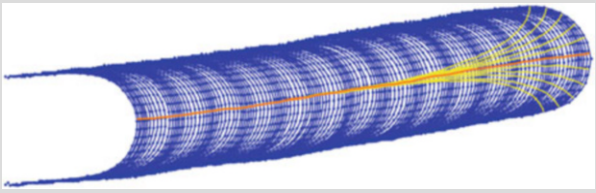

Fig. 14.5 Outlines of an elongating pollen tube (*blue*) and orthogonal trajectories (*yellow*) used to locate the pole axis (*orange*)

All cells were imaged through a 40x objective with bright-field microscopy on an inverted microscope (Olympus IX81). Images were acquired using a Thorlabs camera (DCC1545M-GL). Microscope and camera were controlled with the Micromanager software. Pollen tubes were imaged at time intervals of 2 or 4 s starting at different times after inoculation. Image analysis was performed using ImageJ and Matlab. A custom routine was written to extract the cell outlines and compute from them the pole of the cell and the cell's elongation rate. First, a bright-field image sequence was loaded into Matlab. The user could then select approximate contour points of the first image of the sequence. A cubic spline interpolation was used to obtain regularly spaced points of the outline. Afterwards, the routine maximises the fit between the splined contour and the cell edges in the image and then tracks the evolution of the cell outline for the entire stack. To this end, the normal vectors were computed for each point of the outline. These normal vectors were then used to take small steps orthogonal to the cell contour in order to predict the position of the next contour.

To determine the pole of the cell, orthogonal paths were drawn from the last cell outline to the first one in such a way that the distances to the next outline were minimised. The growth axis (pole) was defined as the longest trajectory between the first and the last cell outline (Fig. 14.5). The tip velocity (rate of elongation) was computed based on the displacement of a five-point neighbourhood around the pole of the cell. The width of the cell was measured at ten different positions along the axis of the cell using the software ImageJ. The cell width was defined as the average of those measurements.

To evaluate the period and amplitude of the velocity oscillations, we first selected time windows within which a cell displayed a single type of oscillation. Our analysis was based on an average of six consecutive growth peaks for asymmetric oscillations and 12 consecutive growth peaks for symmetric oscillations.

(continued)

The period of oscillation was obtained by taking the average of the time interval between successive growth rate peaks. The amplitude was calculated as half the difference between the maxima and minima in growth rate over one cycle and then averaged over all growth cycles in the selected window. For the purpose of amplitude calculation, Savitzky-Golay filtering was applied to smooth the growth rate data prior to calculation. The method fits, using least squares, a local polynomial regression of degree k, with at least $k + 1$ equally spaced points. The resulting polynomial is used to produce a smoother curve. This approach tends to preserve the relative maximum and minimum of the peaks (Savitzky and Golay 1964). However, for the measurement of the period, an average filter works better than the Savitzky-Golay filtering because it reduces noise further. Finally, symmetrically and asymmetrically oscillating cells were identified using the skewness: $\text{skew} = \left(\sum_{i=1}^{N} \frac{(v_i - v_{\text{mean}})^3}{N}\right) / \left(s^3\right)$, where v_{mean} is the average elongation rate for the cell, s is the standard deviation and N is the number of data points. A skewness of 0.8 was used to distinguish symmetrical (skew < 0.8) and asymmetrical (skew ≥ 0.8) oscillations.

14.3 Models

14.3.1 Elements of Pollen Tube Growth Models

Most mechanical descriptions of pollen tube elongation start with the assumption that turgor pressure provides the force for tip-confined cell expansion (Lockhart 1965; Ortega 1990; Fayant et al. 2010; Dumais et al. 2006; Winship et al. 2010). Turgor, the pressure in the cytoplasm, is dependent on the difference in osmolarity between the intra- and extracellular media. The pressure difference across the cell wall is given by

$$\Delta P = \Delta \Pi - \frac{\mathrm{d}V/\mathrm{d}t}{L_p A_{\text{mem}}} \tag{14.1}$$

where ΔP and $\Delta \Pi$ are the differences between internal and external pressure and osmolarity, respectively, while $\mathrm{d}V/\mathrm{d}t$ is the rate of change of the intracellular volume, L_p is the hydraulic conductivity and A_{mem} is the membrane area over which water enters or leaves the tube (Nobel 2009). The dynamics of osmolarity are determined by water, ion and metabolite fluxes, which were modelled by Liu et al. (2010) as well as by Hill et al. (2012). In fact, metabolite fluxes as well as synthesis or decay of metabolites are often neglected in the current models. Several experiments were carried out in order to investigate whether variations in turgor

pressure may be the cause of the oscillations in pollen tube growth rate. So far, there is no evidence that internal hydrostatic pressure variations are the cause for pollen tube growth rate oscillations. Although turgor pressure is high in pollen tubes (e.g. mean value in *Lilium longiflorum* of 0.2 MPa (Benkert et al. 1997)) and is necessary for growth, pressure probe measurements and microindentation (Zerzour et al. 2009) show that turgor pressure does not change significantly during growth, despite obvious changes in the pollen tube growth rate. According to microindentation experiments by Geitmann and Parre (2004), the cell wall at the tube tip is softer than the wall of the shank, i.e. there is a gradient in the cell wall's mechanical properties. A recent study by Vogler et al. (2013), however, explained how this apparent difference can be explained by the geometry of the tube, rather than the intrinsic cell wall properties.

During tube elongation, the cell continually deposits and integrates new material into the cell wall. The new material necessary to sustain the cell wall is contained in secretory vesicles, formed in the Golgi apparatus and delivered to the tip, the location of pronounced cell wall elongation. The delivery and deposition of secretory vesicles (exocytosis), necessary to maintain the structural integrity of the cells during growth, is thus a required element of any tip growth model.

Unlike the cell wall in the tube shank, which is composed of pectin, callose and cellulose (Chebli and Geitmann 2007), the wall at the apex consists mostly of pectin, which is synthesised as methyl esters in the Golgi apparatus. The enzyme pectin methylesterase (PME), delivered by the same vesicles, can de-esterify the pectin and thus expose carboxyl residues, which will be cross-linked with calcium as the cell wall ages. This cross-linking stiffens the cell wall (O'Neill et al. 1990; Bosch and Hepler 2005). According to O'Neill, boric acid can also stiffen the cell wall by cross-linking rhamnogalacturonan II, a pectic polysaccharide. McKenna et al. (2009) observed oscillations in the secretion of pectin methylesterase (PME) and in cell wall thickness. Their measurements show that cell wall thickness and peak growth rate are phase-delayed by $-99°$. This relation between cell wall thickness and peak growth rate was used in the molecular model of pollen tube growth discussed below. Portes et al. (2015) explain that the variations in cell wall thickness result from an offset between growth rate and vesicle secretion. Furthermore, the advance of the cytosol seems to actually precede that of the cell wall.

Secretory vesicles are pulled along the actin microfilaments by myosin and move towards the cell's apex, where they are presumably released into the apical clear zone, and some of them are incorporated into the cell wall by exocytosis (Hepler and Winship 2015). The movement of the vesicles is part of, and is at least partially responsible for, cytoplasmic streaming. The flow pattern of the cytoplasm takes the shape of a fountain in gymnosperm pollen tubes and a reverse fountain in angiosperm pollen tubes. Actin microfilaments also play an important but poorly understood role in oscillatory growth. Geitmann et al. (1996) conducted experiments on pollen tubes of *Nicotiana tabacum*, where actin polymerisation was inhibited by cytochalasin D, and found that pollen tubes stop to oscillate.

As the paragraphs above suggest, the wealth of interacting components inside tip-growing cells leads to inherently complex reaction kinetics, mechanochemical

feedbacks as well as intricate signalling networks. Nevertheless, tip-growing cells and especially pollen tubes display some very robust phenomenology that lends itself to mathematical modelling. Notable attempts to explain the oscillations with biophysical models can loosely be classified according to the mechanism at the core of the oscillation. Kroeger et al. (2008, 2011), Kroeger and Geitmann (2013), Rojas et al. (2011) as well as Pietruszka (2013) consider tube growth rate to be at the core of the mechanism leading to oscillations. These so-called growth-based oscillators are put into question by Iwano's observation (2009) that Ca^{2+} can oscillate even in nongrowing tubes, as argued by Portes et al. (2015). Yan et al. (2009) build their oscillator around ROP1, F-actin and Ca^{2+}. Liu et al. (2010), Liu and Hussey 2014) base their model on ion dynamics and ion channels, while Zonia and Munnik (2008, 2009) consider hydrodynamics and turgor to be at the root of the oscillations.

The next sections will describe some of these models in further detail and explain what phenomenology they address.

14.3.2 Ion Concentrations as Pace Keepers

Ions and their fluxes are critical for turgor generation but also play an additional role by regulating many biochemical reactions in the pollen tube apex.

A strong and time-varying gradient in Ca^{2+} concentration is found at the apex, with a maximum in concentration close to the tip. Cross-correlation analysis of the oscillatory growth rate and the intracellular Ca^{2+} indicates that the intracellular calcium concentration peak follows, rather than leads, the growth rate peak by about $+10°$–$40°$ (Messerli et al. 2000). One explanation for the increase in calcium after the increase in growth rate is a calcium influx through stretch-activated calcium channels, which open when the growth rate increases and the plasma membrane is stretched (Dutta and Robinson 2004). It is important to mention that the presence of stretch-activated channels in pollen tubes remains contentious. The influx of calcium will affect F-actin and thus the cytoskeleton; the increase in calcium concentration activates villin/gelsolin and profilin, which are actin-binding proteins. Gelsolin fragments the F-actin, causing an increase in G-actin (Yokota and Shimmen 2006), whereas profilin retards the polymerisation of new microfilaments (Kovar et al. 2000). These processes would slow growth according to Cárdenas et al. (2008). A very similar scenario, but involving ROP1 Rho GTPase rather than villin, gelsolin and profilin, has been formulated into a mathematical model by Yan et al. (2009).

Finally, the extracellular Ca^{2+}, which also oscillates, is delayed in phase from intracellular Ca^{2+} by about $+100°$ and from growth rate by about $+135°$ (Holdaway-Clarke et al. 1997; Messerli et al. 1999). The extracellular Ca^{2+} can participate in the stiffness of the wall cell, and thus directly impacts elongation rate, by cross-linking the acidic residues of pectin (Steer and Steer 1989).

Pollen tubes also exhibit a gradient in pH, where the tip is slightly acidic and there is an alkaline band a few microns behind the tip. Feijó et al. (1999) show that there is a H^+ influx at the extreme apex of the pollen tube and an efflux in

the region of the alkaline band. Accordingly, the pH oscillates during oscillatory growth. The band becomes more alkaline in anticipation of the increase in growth rate, i.e. the peak in pH is phase-delayed by about $-100°$ with respect to the peak in growth rate (Lovy-Wheeler et al. 2006). Following the growth rate peak, the pH at the extreme tip decreases due to a H^+ influx, and the pH minima is phase-delayed by about $+70°$ with respect to the peak in growth rate. The position of the alkaline band coincides with the position of the cortical actin fringe. Additionally, two actin-binding proteins, ADF (actin-depolymerising factor) and AIP (actin-interacting protein), which are susceptible to changes of pH, are found in this same region. Lovy-Wheeler et al. (2006) suggested that an increase in the pH in the alkaline band activates these proteins, which in turn fragment the F-actin, expose new plus ends and promote polymerisation of new microfilaments.

Liu et al. (2010) model the ion movement inside the pollen tube and across the cell membrane. The ion fluxes depend on ion concentration gradients inside the tube and on voltage-gated ion channels across the membrane. An extension of their model, published recently (Liu and Hussey 2014), couples ion concentrations to vesicle fusion and secretion rate, cell wall viscosity and finally cell wall extension rate. This model of oscillation in the growing pollen tube is arguably the most complete description of the phenomena, as it encompasses most processes that are currently believed by the biophysical community to contribute to oscillations in growing tubes. However, the complexity of the model makes it difficult to predict from inspection whether it will reproduce the observations presented herein. Future work performed with the model will determine whether this is the case and, if not, which parts need to be altered to recover the observed trends. It must be noted that the model shows that ion currents can sustain oscillations even in the absence of tube growth, a phenomenon observed *experimentally* (Parton et al. 2003). The growth rate curves simulated using ion concentration as growth rate regulator can display very sharp peaks, i.e. a 'triangular' waveform (Fig. 14.2b). The sharp growth rate peaks are the result of a regulator that changes on a very short timescale. Indeed, ion concentration can increase over twofold within milliseconds (Gerstner et al. 2014) as a result of the rapid opening of the ion channels and the ensuing ion influx into the cytoplasm.

14.3.3 Constitutive and Kinematic Equations for Computational Modelling

We briefly describe a computational method, based on constitutive and kinetic equations, developed by Dumais et al. (2004, 2006), and later used to model pollen tube growth rate oscillations. It was one of the first 3D models of tubular growth and took advantage of the cylindrical symmetry of tubular cells around their long axis. This simplification, however, restricts the approach to cells growing in a straight line.

The building blocks for the method are constitutive and kinematic equations. Constitutive equations relate turgor stresses to the rates of wall extension. Once the

cell wall stresses are calculated from the cell geometry and translated into extension rates using the constitutive equations, kinematic equations are used to describe the movement of points on the cell wall. Kinematics are sets of equations which describe the motion of points, bodies or objects without consideration for the forces that may have caused the motion. To illustrate these concepts, the constitutive equation for the motion of a projectile relates the projectile's acceleration to the force of gravity. The kinematic equation states that the projectile moves in a parabola.

At the beginning of each iteration of the algorithm (that calculates the evolution in time), the cell wall curvature is analysed to yield the stresses and strains. These are fed into the constitutive equations, which are in turn fed again into the kinematic equations to calculate the movements of points along the cell wall surface. The velocity vector for each point on the cell surface is computed, and the new positions of the cell wall points at the end of each iteration form a new leading edge (Fig. 14.6). The method thus closely mimics the successive outlines of an elongating pollen tube and the trajectories seen in Fig. 14.5. The method was further developed by Rojas et al. (2011) to model oscillatory pollen tube growth. They show how chemically mediated mechanical expansion of the pollen tube cell wall leads to oscillatory growth.

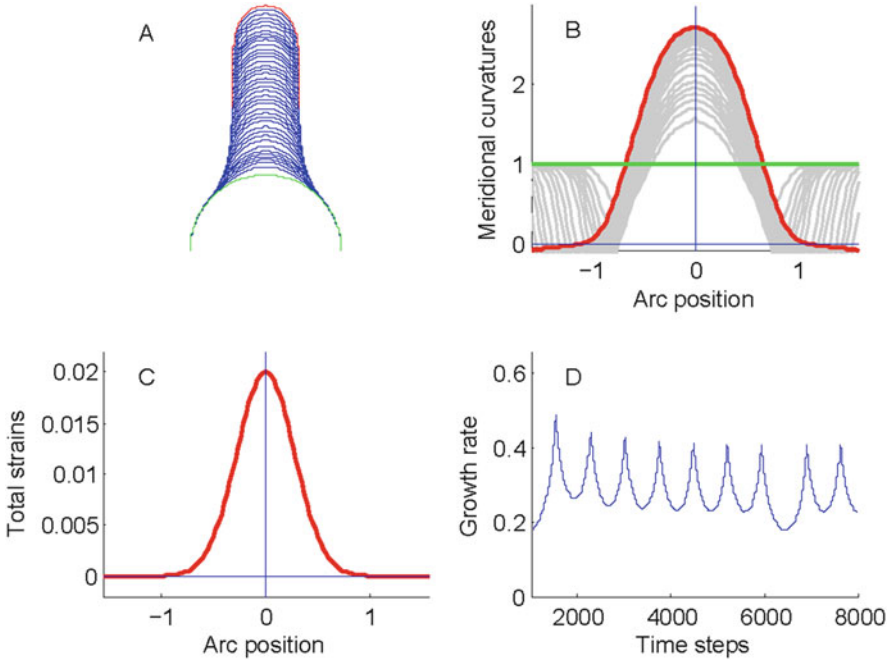

Fig. 14.6 Result of numerical simulations combining constitutive and kinetic equations. (**a**) Shape of the pollen tube as it elongates with oscillatory growth rate. (**b**) Meridional curvatures vs. arc position (meridional position *s*). (**c**) Total strain vs. arc position. (**d**) Growth rate as a function of time. All units are arbitrary following normalisation

14.3.4 A Molecular Model Explaining the Two Growth Modes of Pollen Tubes

While several mathematical models have attempted to explain the oscillatory dynamics of pollen tube growth, only one provides an explanation for two of the modes of growth described above: steady and oscillatory dynamics (Rojas et al. 2011). The tenets of this model that yield the dual behaviour are (1) turgor pressure drives cell wall expansion; (2) deposition of new wall material, i.e. pectin, softens the apical cell wall (the mechanism of this softening is proposed to be cross-link turnover due to de-esterification of nascent pectin); and (3) the rate of deposition of new cell wall material depends on the difference between the rate of cell growth and the rate of cytoplasmic streaming, such that the rate of deposition is lower for faster-growing cells.

According to this model, when the rate of cell growth is low compared to the rate of cytoplasmic streaming, cell growth rate is constant. However, when the average rate of cell growth is high compared to that of cytoplasmic streaming, cell growth essentially outpaces deposition. Because deposition is required for softening of the apical cell wall, this causes a period of reduced growth rate. During this period, new cell wall material is deposited in excess. After a characteristic time constant, this material is de-esterified, thereby softening the wall and leading to a burst in growth, completing the oscillatory cycle. It must be mentioned that this dynamic cell wall softening is contentious, as Vogler et al. (2013) determined that the apparent gradient in cell wall mechanical properties is a consequence of the cell wall geometry. Specifically, the larger inclination at the tip explains the lesser mechanical resistance experienced by the microindenter.

The molecular model predicts several key experimental results. Most importantly, it offers a mechanistic basis for the experimental observations that faster-growing cells are more likely to be oscillatory, while slower cells grow at a constant rate (Fig. 14.1). Second, it predicts the experimental distribution of the 'oscillatory wavelength', that is, the distance the cells grow during an oscillatory cycle. Last, the model predicts the phase relationships between the growth rate, cell geometry and cell wall thickness.

The success of this model for the dynamics of pollen tube growth, based on 'chemically mediated mechanical cell wall expansion', to explain a breadth of experimental observations necessitates further research to test and refine tenets of the model. In particular, the model proposes that de-esterified pectin temporarily softens the apical cell wall by eliciting 'cross-link exchange', whereby the nascent de-esterified pectin residues break existing cross-links by competing for their divalent calcium cations. Support for this mechanism comes from the observation that when de-esterified pectin is added exogenously to cultures of growing *Nitella axillaris*, a giant single-celled alga, the growth rate accelerates (Proseus and Boyer 2005). Similar experiments need to be repeated in pollen tubes. Also, by measuring the phase relationship between the thickness of the cell wall (McKenna et al. 2009) and the cell wall expansion rates, the rate of cell wall deposition can be inferred

(Rojas et al. 2011), and this calculation supports the proposition that the rate of cell wall deposition is proportional to the negative of cell growth rate. While there is currently no method for measuring the rate of deposition directly, such a method would enable the direct measurement of this relationship.

14.3.5 A 1D Model Amenable to Analytical Treatment

Using constitutive and kinematic equations of cell wall deformation enables a 3D model of pollen tube growth, as described above. This model allows to study the spatial distribution of cell wall bending, elongation and renewal through vesicle secretion. Simpler models serve different purposes. So-called toy models reduce a system to the bare essentials, enabling an analytical solution to the mathematical equations and yielding firm predictions for certain parameters. For example, a very well-known toy model of neuronal activity is the integrate-and-fire model that represents a neuron as a capacitor charged by ion currents and discharged upon reaching a certain threshold potential (Gerstner et al. 2014). For the present system, the toy model is a 1D representation of pollen tube growth containing just enough ingredients to reproduce oscillations. Its simplicity leads to a firm prediction for the relation between average growth rate and period of oscillation. However, the oversimplification of toy models prevents them from explaining phenomena outside of their scope.

The following one-dimensional model of pollen tube growth was originally proposed by Kroeger and Geitmann (2013). The main variable is the cell wall strain rate $\dot{\varepsilon}$, directly proportional to the difference between the cell wall stress and the yield stress $\sigma - \sigma_y$. The constant of proportionality is the inverse of the cell wall viscosity η:

$$\dot{\varepsilon} = \frac{1}{\eta}\left(\sigma - \sigma_y\right) \tag{14.2}$$

This equation was first proposed by Bingham and introduced to plant cell growth by Lockhart (1965), augmented by Ortega (1990) and used by Dumais et al. (2004, 2006) to model tip growth. Kroeger et al. (2011), Rojas et al. (2011), Pietruszka (2013) and Liu and Hussey (2014) later use it to model pollen tube growth rate oscillations.

To complete the 1D description of pollen tube strain rate and produce oscillations, a further set of equations is needed. They are the differential equations dictating the rate of change of the cell wall viscosity η, which determines the cell wall's resistance to turgor:

$$\frac{\partial \eta}{\partial t} = -\frac{R\eta_s}{h} + a_\eta\left(\eta_{eq} - \eta\right) \tag{14.3}$$

Here η_s is the viscosity of the secreted, new cell wall material, while h is the cell wall thickness and R is the rate of secretion of new cell wall material, in units of $\mu m\ s^{-1}$. Finally, η_{eq} is the equilibrium value of the cell wall viscosity towards which the value of η will eventually return to.

The oscillations in this representation of pollen tube growth are due to the change in cell wall viscosity η caused by the inclusion of soft new cell wall material (vesicle secretion or exocytosis) through secretion, at rate R. In the original work, the secretion rate is regulated by cytoplasmic ion concentration and thus ion fluxes. The model was completed by coupling the ion fluxes to the tube elongation rate: the ion fluxes were dependent on the cell wall strain rate through strain-gated ion diffusion. The underlying assumption was that the ion channels are stretch-activated.

The causal chain leading to a feedback loop was thus: elongation rate and cell wall strain → opening of ion channels → ion influx → change in secretion rate → change in elongation rate. As explained above in the section on ion fluxes, the very small timescale built into this model leads to sharp growth rate peaks, i.e. a 'triangular' waveform (Fig. 14.2b).

However, for the purpose of the analysis, we will disregard the precise mechanism that controls vesicle secretion. As described below, an approach based on the separation of timescales (also known as a slow-fast or asymptotic analysis) allows to approximate the correlation between average tube growth rate and period of oscillation (Fig. 14.4). Our target is to obtain an analytical result as close as possible to experimental results (Fig. 14.4). The calculation rests on the assumption that during the largest part of the growth rate cycle, the secretion rate R stays constant, which is a better assumption for an ion concentration regulation mechanism than for a linear relation as used in the molecular model.

Here we perform our first separation of timescales by assuming that the second term on the right-hand side of Eq. (14.3) changes much slower than the first term. This assumption is reasonable as in the original version the secretion rate R on the right-hand side of Eq. (14.3) depends on the ion concentration C, which changes very rapidly once the ion channels open. Indeed, ion channels can be conceived to be either open or closed and modelled using sigmoid-type functions involving exponentials. Resulting ion fluxes can lead to large increases in ion concentrations on very short timescales. While the link between sharp growth rate peaks ('triangular waveforms') and ion concentration bursts has not been established, Lassig et al. (2014) observe growth-dependent Ca^{2+} bursts in mutant pollen tubes, whose NAD(P)H oxidase RBOHH and RBOHJ functionalities were disabled. The current analysis assumes that for pollen tubes displaying triangular waveforms, the cell wall viscosity changes very slowly during the longest part of the growth cycle and then changes very suddenly once the pollen tube has reached a critical (peak) growth rate. The invoked mechanism is the opening of stretch-activated ion channels as the cell wall strain rate reaches a critical value. This mechanism is embodied by an exponential (sigmoid) function in the original work by Kroeger et al. (2011).

Furthermore, h, $\Delta\sigma = \sigma - \sigma_y$, R, η_s, η_c are assumed to be constants throughout the analytical treatment. We then linearise the solution to Eq. (14.3) and obtain

$$\eta(t) \approx \eta(0) - \frac{R\eta_s}{h}t \tag{14.4}$$

where $\eta(0)$ is the value of the viscosity at $t = 0$. The average strain rate $< \dot{\varepsilon} >$ is simply the integral over one period of oscillation divided by the period, as given by

$$< \dot{\varepsilon} >= \Delta\sigma < \eta^{-1} >= \frac{\Delta\sigma}{P}\int_0^P \frac{\mathrm{d}t}{\eta(t)} \tag{14.5}$$

where $\Delta\sigma = \sigma - \sigma_y$ and P is the period of oscillation. Combining Eqs. (14.3) and (14.4) and using a change of variable of integration, we obtain

$$< \dot{\varepsilon} >= -\frac{h\Delta\sigma}{PR\eta_s}[\ln(\eta)]_{n(0)}^{n(P)} \tag{14.6}$$

Next, we assume that at the beginning of a growth cycle ($t = 0$), the viscosity has a maximal value $\eta(0) = \eta_c + \frac{RP\eta_s}{h}$ and that immediately before the end of the cycle ($t = P - \varepsilon$), the viscosity reaches a minimal (critical) value which we will call $\eta(P - \varepsilon) = \eta_c$ and which triggers the opening of the stretch-activated ion channels. This embodies the assumption that the minimal cell wall viscosity (at which the ion channels suddenly open) is fixed and thus that the maximal, peak growth rate does not change despite changes in average growth rate or period of oscillation. The peak growth rate is physically limited at a value at which a significant fraction of the ion channels open. Furthermore, this assumption and our analysis split the growth cycle into two different intervals: (a) one long interval (from $t = 0$ to $t = P - \varepsilon$) during which the ion channels are closed and the cell wall viscosity gradually decreases from a high towards the critical minimal value and (b) one short interval (from $t = P - \varepsilon$ to $t = P$) during which the sudden ion influx leads to the interruption of secretion and a dramatic increase in cell wall viscosity. By linearising the equations for the gradual decrease of the cell wall viscosity during the long interval and equating the length of this long interval to the length of the growth cycle, we can analytically calculate a relation between period of oscillation and other measures such as the average growth rate.

Effectively, this analysis assumes that during changes in amplitude of oscillation or frequency, it is the value of the basal growth rate that changes rather than the peak growth rate. This assumption is partly supported by the findings of Lassig et al. (2014) that oscillating pollen tubes display either high-amplitude/low-frequency growth oscillation or low-amplitude/high-frequency oscillation. In this article, the authors also report growth-dependent Ca^{2+} transients. Modelling pollen

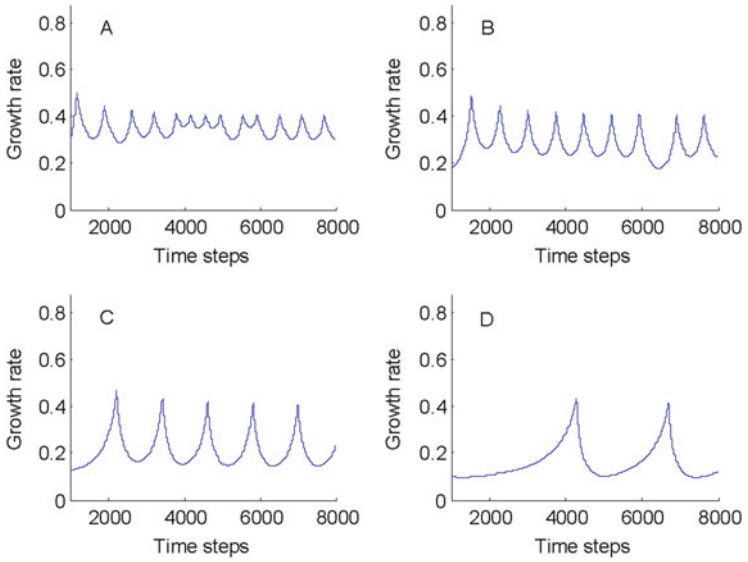

Fig. 14.7 Pollen tube growth rates (vs. time) from numerical simulations for different numerical parameters. Simulations reproduce that cells with shorter periods of oscillation grow at a higher average rate. Arbitrary units following normalisation

tube growth using this assumption of a fixed maximal growth rate can reproduce a similar behaviour as the one observed in this experimental study (Fig 14.7).

We now obtain

$$\bar{v} \propto < \dot{\varepsilon} > \simeq \frac{h\Delta\sigma}{PR\eta_s} \ln\left(1 + \frac{R\eta_s P}{h\eta_c}\right) \tag{14.7}$$

The inverse relation between average growth rate and period of oscillation, predicted by this analysis at first order, is very close to the trend emerging from experimental data shown in Fig. 14.4. This result can be interpreted schematically: the linear decrease in time of the viscosity η (Eq. 14.3) together with the assumption that the viscosity is reset to a high value once it hits a lower critical value produces a ratchet-like solution shown in Fig. 14.8. The inverse of the viscosity is proportional to the tube growth rate according to Eq. (14.1) and shown with the full line in Fig. 14.8. This growth rate profile bears some resemblance to an extremely skewed profile (Fig. 14.2c). Using the assumption that the maximal growth rate is constant despite changes in period of oscillation, we can see that shortening the period of oscillation (Fig. 14.8b) leads to higher basal and average growth rates, as compared to Fig. 14.8a. Pollen tubes with shorter periods of oscillation grow more quickly, on average, than pollen tubes with longer periods of oscillation. This more or less conserves the average distance grown during one growth cycle (Fig. 14.4).

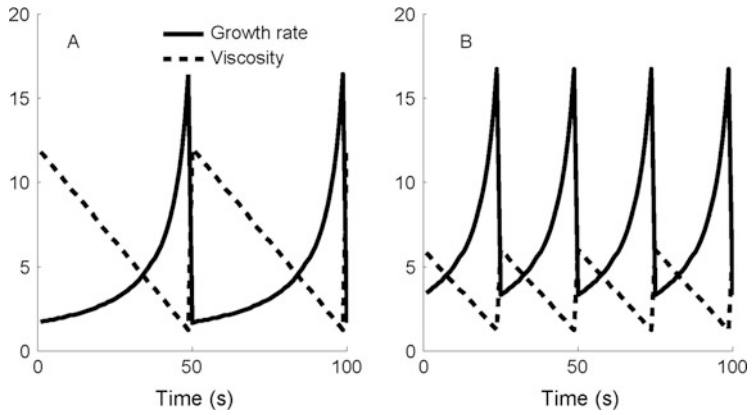

Fig. 14.8 Growth rate (*full line*) and viscosity (*dashed line*) as a function of time, assuming that the viscosity is a linearly decreasing function of t during the growth cycle and is reset at the end of the cycle. (**a**) Long period of oscillation and lower basal and average growth rates. (**b**) Shorter period of oscillation and higher basal and average growth rates

14.4 Conclusion and Perspective

We have discussed three key kinematic features of lily pollen tube growth: (1) oscillatory cells dominate at high elongation rates, (2) symmetrical and asymmetrical modes of oscillation are present, and (3) the oscillation cycle unfolds over a fairly well-defined distance. This has been called the characteristic wavelength of oscillatory tube growth. Furthermore, we have discussed two mathematical models reproducing oscillating growth curves and proposing explanations for these three features. A molecular model of oscillatory growth reproduces the onset of oscillation at a critical growth rate, offering the explanation that oscillation sets in when the growth rate outpaces cytoplasmic streaming and secretion becomes the bottleneck for growth. This model produces a sinusoidal waveform, which is displayed by the slower growing fraction of oscillating pollen tubes (Fig. 14.4). A second model rests on the assumption that secretion is mediated by ion concentration primarily. This model leads to sharper growth rate peaks, as displayed by the faster-growing fraction of pollen tubes (Fig. 14.4). Assuming a constant secretion rate over the largest fraction of the growth cycle leads to an analytical prediction of the correlation between average growth rate and period of oscillation, approaching an inverse relation.

We now begin to get an insight into the types of simulated kinematic responses that will be elicited from certain mechanisms. In the molecular model, the secretion rate has a linear response to a change in growth rate of the form $v_0 - v$. The further the growth rate deviates from the speed of the cytoplasmic flow (v_0), the stronger the change in secretion rate will be. It is not surprising that this linear function leads to a smoother 'sinusoidal' waveform (Fig. 14.2b). Mechanisms based on ion fluxes will

use an exponential response to changes in the growth rate (or cell wall strain rate) and thus elicit much sharper growth rate peaks ('triangular' waveforms). As multiple waveforms are observed experimentally, it appears that multiple mechanisms impact and thus regulate vesicle secretion during pollen tube growth, which is a critical aspect of pollen tube growth and function.

Starting with Weisenseel et al.'s (1975) discovery of oscillating ion fluxes in growing pollen tubes, a growing number of molecules, mechanisms and pathways participating in the regulation of oscillations in pollen tubes have been uncovered. As experimental techniques mature, our community will establish robust trends and correlations in experimental data sets, allowing us to refine our models until they reproduce this phenomenology. The scope of the models could be reduced to reproduce precise aspects of pollen tube phenomenology, rather than pollen tube growth as a whole. The modelling work done so far by our community to understand phase delays between different signals was the first step in this programme. Reproducing the spectra of pollen oscillation curves, i.e. the response as a function of oscillation frequency, expands this effort and paves the way for tighter integration of modelling and experimental work.

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Chapter 15
One Thousand and One Oscillators at the Pollen Tube Tip: The Quest for a Central Pacemaker Revisited

Daniel S.C. Damineli, Maria Teresa Portes, and José A. Feijó

Abstract Pollen tube growth and oscillations constitute a prime system to study polarized cell growth, warranting the expansion of mathematical models in recent years. Although it is unlikely to have a myriad of distinct independent oscillators at the pollen tube tip, the tendency to describe as many new oscillators as there are objects of study is likely to continue. The same process has happened for over 60 years of studies in circadian rhythms and, similarly, in other biological phenomena displaying oscillatory behavior. Here, we take the opportunity to revise pollen tube oscillator models with cautionary tales learned in other systems. Currently the nature of the core negative feedback loop generating the oscillations, as well as the role and regulation of Ca^{2+} and growth, seems elusive. The vast majority of mathematical models assume that intracellular Ca^{2+} is primarily controlled by stretch-activated channels and, thus, relies on membrane tension and growth. Counterpoints to these canonical assumptions will be given as to evidence the gaps in achieving a comprehensive view of pollen tube oscillations. Finally, inspiration stemming from systems involving intracellular polarity and chemotaxis motivates a proposal for the existence of two putatively coupled and yet distinct oscillatory systems involved in pollen tube growth: a signaling-based oscillator at the tip and a motion-based oscillator at the shank, which could be coupled by intracellular signals as Ca^{2+} waves. This proposition may account for the oscillations observed in nongrowing tubes, as both oscillators could be uncoupled in certain regimes, resolving the conundrum entailed by current model.

Keywords Apical growth • Chemotropism • Ion dynamics • Regulatory motifs • Negative feedback • Biochemical and biophysical oscillations • Mathematical models

D.S.C. Damineli (✉) • M.T. Portes • J.A. Feijó
Department of Cell Biology and Molecular Genetics, University of Maryland, College Park, 20742-5815 MD, USA
e-mail: damineli@umd.edu

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15.1 Introduction

Oscillations can be found in virtually all spatial and temporal scales of biological organization (Novák and Tyson 2008) ranging from the quantum level, as the femtosecond quantum beats reported in the photosystem II reaction center (Romero et al. 2014), to whole populations (Bjørnstad and Grenfell 2001; Blasius et al. 1999), as the ten-year cycle of hare/lynx populations or even hundred-year cycles as in the extremely long flowering times reported in bamboo (Renvoize 1991). Although oscillations may be caused by external factors, as periodic environmental changes, they can also emerge from the interactions between components of a biological system—that is, a bona fide oscillator (Novák and Tyson 2008). Even in the absence of an oscillating environment, these endogenous oscillators can generate rhythms with certain characteristics as an intrinsic period, amplitude, and phase flow (Fig. 15.1, Pikovsky et al. 2003).

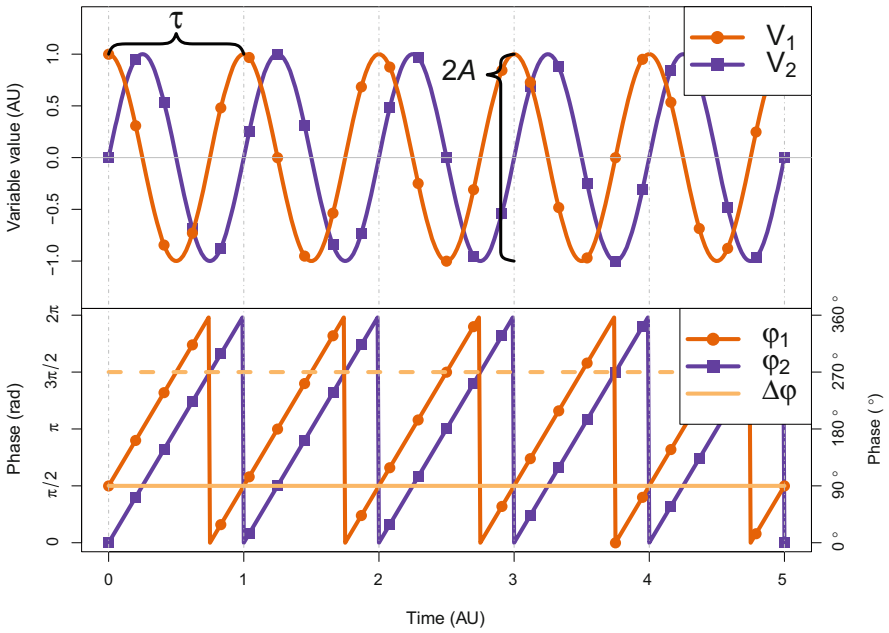

Fig. 15.1 Basic definition and landmarks of oscillations. The canonical example of an oscillation is a sinusoidal wave with a constant period, amplitude, and linear phase flow. The period (τ) is often measured as the time interval between two peaks of an arbitrary variable (here V_1 and V_2, in arbitrary units—AU), and the amplitude (A) as half of the range of values spanned, while the phase (φ) is the relative stage of the oscillation defined in a circle from 0 to 2π (or 0° to 360°), represented in the *bottom panel* (the y-axis is in fact circular, since $0 = 2\pi$). Two processes with the same period have a constant phase relationship or phase difference ($\Delta\varphi$), being 0 when both oscillations are in phase and π in antiphase. In the example, the oscillation marked with *circles* (φ_1) phase leads the one with *squares* (φ_2) by $\Delta\varphi = \pi/2$ (*solid line*), which can be alternatively interpreted as a phase lag of $\Delta\varphi = \frac{3\pi}{2}$, i.e., $2\pi - \pi/2$ (*dashed line*). Thus, the oscillation marked with squares is delayed by a quarter cycle, 0.25τ, or advanced by 0.75τ

Biochemical oscillators have been described in gene regulatory networks, protein interactions, metabolism, ion dynamics, membrane dynamics, growth, and other biophysical processes (Novák and Tyson 2008). These oscillators underlie several crucial aspects of plant development such as anticipation of periodic environmental changes (Hotta et al. 2007), triggering of specific developmental programs (Berridge et al. 2000; Parekh 2011) and spatial patterning (Moreno-Risueno et al. 2010). Yet, in some cases, their function is less clear or possibly even absent (Feijó et al. 2001). Oscillatory networks can evolve by common evolutionary processes and may be pervasive in biology not only due to their functional aspects but also because they appear to be easy to arise. Self-sustained oscillations require only a negative feedback loop, a "delay" or another source of "memory" and sufficiently nonlinear relationships between the network components with adequately balanced time scales (Novák and Tyson 2008). However, more often than not, the network of interacting elements generating the oscillations and their downstream influence are large and complex. This leads to an inevitable bias in measuring specific components and in interpreting the identity of the oscillators themselves, due to both methodological and theoretical limitations.

Pollen tube apical growth involves a number of oscillations, with the spatial control and temporal coordination of diverse cellular processes that can sustain one of the fastest growth rates known (Boavida et al. 2005; Damineli et al. 2017). Pollen tube growth rate can be itself oscillatory, having several cellular events associated with tip growth oscillating supposedly with the same periodicity, including tip-localized actin microfilaments (F-actin, Fu et al. 2001), membrane trafficking (Parton et al. 2001), cell wall banding patterns (Pierson et al. 1995), intracellular ion gradients (Holdaway-Clarke et al. 1997), and extracellular ion fluxes at the tip (Feijó et al. 2001). The joint rhythmicity in such a wide range of processes suggests the existence of a pacemaker that would dictate the periodicity of the oscillations. However, the identity of this underlying oscillator and its physiological role in in vivo conditions remain to be established, while precise quantification with the required spatiotemporal resolution is lacking. Thus far, the community has witnessed the proposal of central oscillators with distinct players, mostly featuring each author's object of study as a fundamental part in generating the oscillations. This raises the question as to whether this is simply a coincidence or if there is a reason to expect such bias when unraveling oscillatory system.

When present with a myriad of oscillatory behaviors, authors seem to either look for a common origin (lumpers) or to separate them into different entities (splitters), a tendency that is recurrent across many fields and made famous in biological classification (Endersby 2009). The field of chronobiology offers a rich epistemological account on the quest for a central circadian pacemaker (McClung 2000; Roenneberg and Merrow 2005), the so-called biological clock, which has led to technical developments and applications of interdisciplinary methods to disentangle possible coexisting oscillators. As it currently stands, neither tendency has proven to be absolutely correct since well-studied instances of both central pacemakers influencing many downstream components as well as different coexisting self-sustained oscillators (Bell-Pedersen et al. 2005), even within the same cell, abound

(van Ooijen and Millar 2012). Thus, it is not trivial to interpret diverse oscillations as stemming from a single or multiple oscillator system from first principles, as there are reasons to expect both. This renders the adoption of a pure lumper or splitter perspective inadequate, as we hope to illustrate. However, as it happened in chronobiology, the struggle to unravel an oscillatory system is likely to be first dominated by splitters describing what they think is the main pacemaker, usually coincidentally involving their favorite molecule/pathway/phenomenon. Eventually these processes may be clumped into a main oscillatory mechanism, while many may be dethroned as a downstream pathway or even as part of an independent oscillatory mechanism.

Here, reasons for either splitting or lumping oscillations are provided pivoting on the fact that they are easy to arise or are expected to propagate down large networks, respectively. The aim is to raise awareness of the most common pitfalls in the development of any field dealing with biological oscillations. After reviewing the structure and minimal requirements for an oscillatory network, together with challenges in inferring causality in these systems, the current models of pollen tube oscillations are discussed with evidence for a pacemaker or multiple oscillators. This motivates questioning the nature of the main feedback loop generating oscillations in models of the pollen tube tip and their potential functional significance. Finally, a brief overview of oscillations in the context of intracellular polarity establishment and guidance, particularly chemotaxis, is used to further illustrate the putative organization and function underlying oscillatory networks in pollen tubes. The conclusion features a proposal favoring the existence of two putatively coupled and yet distinct oscillatory systems involved in pollen tube growth, one mostly localized at the tip and related to signaling (signaling-based oscillator) and the other that influences tip growth but more localized at the shank, being related to actin polymerization and motion (motion-based oscillator), both of which can be coupled especially through calcium ion waves.

15.2 Understanding Biological Oscillators: Definition, Organization, and Fundamental Properties

The most basic way to distinguish the presence of a single (lumper view) or multiple (splitter view) oscillators in a system is the presence of a single or multiple frequencies in oscillations measured therein, more precisely incommensurable frequencies, which are not simple ratios of each other (Winfree 2001; Granada et al. 2011). This principle is, however, insufficient as biological systems are composed of complex networks of interacting components that can span different levels of organization and time scales. This causes theoretical and experimental challenges that are also source of confusion, having led authors to incur erroneous interpretations.

15.2.1 *Oscillations Are Not Necessarily Oscillators: Many Oscillations, One Master Pacemaker (Pro-lumping)*

Perhaps the most common misconception is that oscillations observed in a biological system are necessarily part of the mechanism that generates them, i.e., the oscillator per se. Often, oscillations occurring in phase or antiphase are immediately taken as evidence for direct positive or negative association, respectively, serving as basis to propose a tight regulatory dependence. However, this is not necessarily the case as the elements assayed might be part of independent downstream pathways or outputs of the oscillator (discussed further in Sect. 15.2.5 and Fig. 15.3). Thus, it is important to discriminate inputs and outputs from the core mechanism (top part of Fig. 15.2, McClung 2001; Roenneberg and Merrow 2001), which defines the intrinsic properties of an oscillator (Fig. 15.1). Output pathways enable the coordination of effector processes, often carrying the temporal signature of the core oscillator. However, they do not participate in the generation of oscillations and can show diverse phase relationships depending on delays and on which component of the oscillator drives them. Input pathways can influence phase, amplitude, and even period of the endogenous oscillations, but, nonetheless, they are not responsible for generating the rhythm itself. Thus, even in a simple oscillatory network, different components are expected to oscillate with potentially distinct characteristics. This point of view, in the absence of further information, favors the principle of lumping the oscillations observed in different components in a single oscillatory system, with a core mechanism and input/output pathways.

15.2.2 *Synchronization in Oscillator Populations: We Are Legion (Pro-splitting)*

In practice it is not trivial to disentangle the different pathways in an oscillatory system (Fig. 15.2), especially since the core oscillator itself can be complex, often having multiple feedback loops as reported in the regulation of circadian clock genes in plants (Greenham and McClung 2015). Furthermore, a single oscillator can be composed of other autonomous oscillators (middle box in Fig. 15.2), where oscillators with different intrinsic frequencies can synchronize with the ensemble adopting a common frequency (Winfree 2001; Pikovsky et al. 2003). This has been reported even in centralized pacemakers, as the suprachiasmatic nucleus in mammals, where different neurons couple to have a common collective rhythm in each nuclei (intercellular synchronization), and both nuclei interact as to yield the main internal circadian rhythm (inter-tissue synchronization, Roenneberg and Merrow 2001; Welsh et al. 2010).

In single cells, multiple oscillators have been proposed to interact but still generate their own rhythm independently. This is the case for the circadian oscillators in *Neurospora*, where the "main" oscillator based on a transcription-

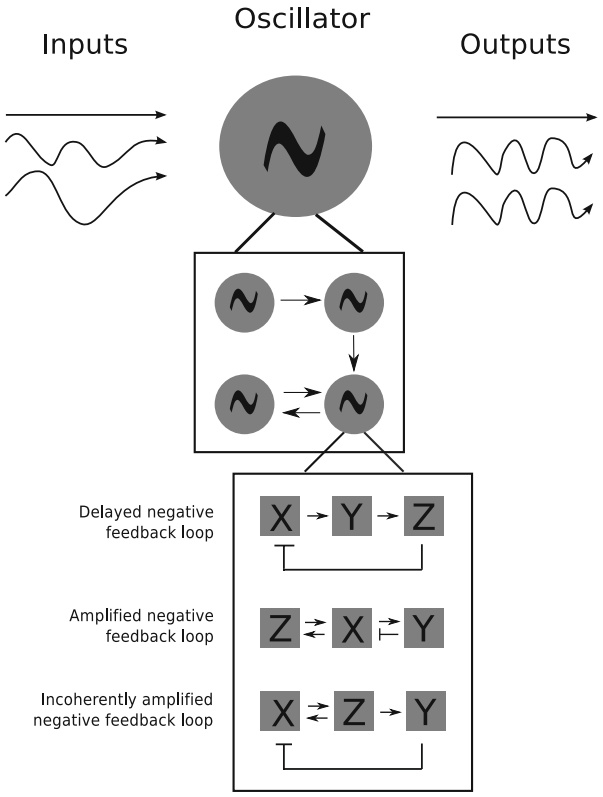

Fig. 15.2 Schematic representation of a generic oscillatory system. The simplest view of a pacemaker type of system (*top*) comprises input pathways, which may or may not be rhythmic, the core mechanism that is the oscillator per se, and output pathways that usually carry the temporal signature of the oscillator. However, multiple oscillators can interact and synchronize, behaving effectively as one and yielding a joint rhythm (*middle box*). Simple regulatory motifs underlying a single oscillator (*bottom box*) reflect the minimal requirements for endogenous oscillations to arise, as categorized by Novak and Tyson (2008)

translation feedback loop is centered around the frequency gene (FRQ), but there is evidence for other oscillators capable of generating rhythms independently and of responding to distinct cues, as the frequency-less oscillator (FLO), even though both interact (Bell-Pedersen et al. 2005). Yet, there are other potential oscillators that are not fully autonomous being named "slave" oscillators, since in isolation they produce rhythms that dampen out and disappear but are maintained in interaction with a self-sustained oscillator, as the FRQ-dependent one. A recurrent theme in the organization of circadian systems is the existence of an oscillator composed of a transcription-translation negative feedback loop coupled with a non-transcriptional oscillator, relying on posttranslational modifications as phosphorylation and oxidation (van Ooijen and Millar 2012). In fact, a circadian oscillator based on peroxide

production and peroxiredoxin has been described in an increasing number of species, with some authors considering it to be present in all domains of life, being perhaps an ancient circadian clock shared from cyanobacteria to mammals (Edgar et al. 2012). Thus, interacting oscillators composing a joint oscillatory system seem to be ubiquitous in biology, even within a single cell.

Synchronization in oscillator populations is a widespread phenomenon and has been shown to depend on general properties as inter-oscillator coupling and differences in intrinsic frequency and amplitude (Pikovsky et al. 2003; Granada and Herzel 2009; Abraham et al. 2010) but remains a major theoretical and experimental challenge to understand. Roughly speaking, synchronization occurs when factors driving oscillators together are stronger than the ones pushing them apart. Sharing a common periodic input and inter-oscillator coupling can promote synchronization, while conflicting inputs and differences in the intrinsic frequencies promote desynchronization (Pikovsky et al. 2003). This complicates the argument for lumping, since even a myriad of autonomous oscillators can behave as one and only reveal their distinct identities under special circumstances. Thus, the existence of synchronization between a number of oscillators strengthens the principle of splitting oscillations into separate entities especially since, as presented below, self-sustained oscillations can arise in very simple networks.

15.2.3 Regulatory Motifs: How Oscillations Arise (Minimum Requirements)

While the material basis of biological oscillators varies extremely, the interactions among network elements capable of generating oscillations follow general principles. Despite the challenges that the biological and mathematical complexity of oscillatory systems poses to experimental and theoretical studies, design principles for small networks have been proposed, as summarized by Novák and Tyson (2008). The authors classify networks into three essential motifs capable of generating oscillations, all based on negative feedback loops: delayed, amplified, and incoherently amplified negative feedback loops (lower box in Fig. 15.2). This classification scheme is exhaustive only for networks of three components with three or four links (without self-activation); however, often more complex networks can be accurately reduced to these simpler motifs (Novák and Tyson 2008; Forger and Kronauer 2002). Furthermore, even the addition of a single element and more links would severely complicate such a classification effort, since complex dynamics as chaos can occur. Chaos, in principle, is not easy to control probably being mostly avoided in regulatory networks (Novák and Tyson 2008), although a number of biological functions have been proposed to rely on complex chaotic dynamics (Schuster et al. 2002).

15.2.3.1 Minimal Oscillator

The fundamental requirement for oscillations to occur in a biochemical system is the existence of a negative feedback loop (Novák and Tyson 2008). A minimal oscillator could be composed of a single element, X, which inhibits its own production $X \dashv X$ (where "$\dashv$" denotes inhibition; Lema et al. 2000). However, if the inhibitory action is immediate, X would reach steady-state levels and oscillations would not occur, as in a homeostatic regulation. Thus, the action of X over its own production has to be delayed in order for X levels not to reach a steady state, as to always overshoot or undershoot the equilibrium. This requires a sort of "memory" in the system, that is, the rate of production of X depends on previous levels of X. In biological networks this is accomplished in essentially two ways, either by an actual time delay or by bistability, which features two possible stable states (or hysteretic switch).

15.2.3.2 Delayed Negative Feedback Loop

Time delays can occur due to transcription, translation, and protein transport but also simply due to interactions with other components (intermediate reaction steps) in a bigger loop, for example, in the regulatory motif $X \rightarrow Y \rightarrow Z \dashv X$ (where "$\rightarrow$" denotes activation, lower box in Fig. 15.2). This is the case of delayed negative feedback loops, defined by a series of three or more elements with an odd number of negative interactions (Novák and Tyson 2008), used to describe biological oscillators as circadian clocks (Goldbeter 1995), somitogenesis (Lewis 2003), and diverse signaling pathways (Hoffman et al. 2002; Monk 2003; Garmendia-Torres et al. 2007). Oscillations of a typical delayed negative feedback loop are shown in the time series in the top right of Fig. 15.3, obtained from simulations of the equations and parameter values presented by Novák and Tyson (2008, in their Supplementary Information S1 for Figure 5a).

15.2.3.3 Amplified Negative Feedback Loop

Bistability, the existence of two stable states, can occur in motifs containing positive feedback loops as in the amplified negative feedback loops, used to model many chemical and biological oscillators as the *Xenopus* embryonic cell cycle (Pomerening et al. 2005) and even to build synthetic ones (Stricker et al. 2008). For example, consider $X \rightarrow Y \dashv X$, where X acts as an activator and Y as an inhibitor. The addition of a positive feedback loop amplifying either the inhibitor or the activator could induce bistability. In the case where X is amplified by, say by Z ($Z \rightarrow X \rightarrow Z$), inhibition by Y in intermediate levels would lead to different effects on X depending if its levels were previously high or low (Novák and Tyson 2008; lower box in Fig. 15.2). Such history dependence, or hysteresis, is a notorious example of memory in dynamical systems (Strogatz 1994).

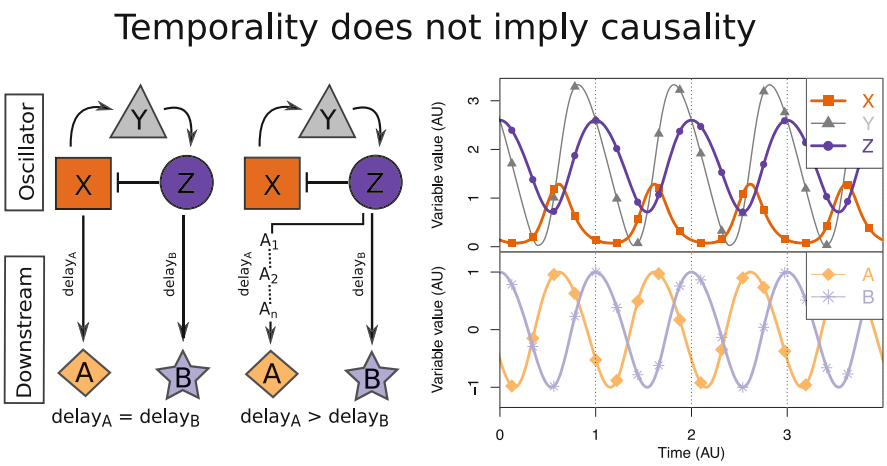

Fig. 15.3 Causally unrelated processes (*A* and *B*) that do not participate in the generation of oscillations could seem to be associated. This can be either due to their upstream control by different elements in an oscillatory system (*left scheme*) or due to different time delays (*right scheme*), leading to the same oscillatory behavior (time series on the right) given some simplifying assumptions. Much like "correlation does not imply causation," here the aim is to show *A* and *B* do not interact and are not part of the core oscillator, yet can seem to be intimately associated due to their temporal relationship. Thus, interpreting that *B* inhibits *A* (and *A* promotes *B*) may be tempting to some researchers, albeit false. Time series were obtained with simulations of the delayed negative feedback loop presented by Novak and Tyson (2008; Supplementary Information S1, Figure 5a). See text for further explanation

15.2.3.4 Incoherently Amplified Negative Feedback Loops

Positive feedback loops allow oscillations in other motifs with a different wiring (connections) also by conferring bistability to the regulatory mechanism. This is the case of incoherently amplified negative feedback loops, found in models of metabolism (Goldbeter and Lefever 1972), signaling (Meyer and Stryer 1988; Ciliberto et al. 2005), and even cAMP oscillations in slime mold cells (Goldbeter 1975). For example, in a two-component positive feedback loop ($X \rightarrow Z \rightarrow X$) embedded into a three-component negative feedback loop ($X \rightarrow Z \rightarrow Y \dashv X$), the amplification of X by Z is incoherent since it also promotes inhibition indirectly through Y (lower box in Fig. 15.2).

15.2.3.5 Other Requirements for Oscillations

The network topology per se, i.e., the arrangement of elements and their links, is not sufficient to guarantee the generation of oscillations, since they are also determined by the strength and time scales of the interactions. Nonlinearity in the reactions is a crucial factor, often implemented biochemically by mechanisms like

multimeric transcription factor binding, cooperative binding in allosteric enzymes, reversible phosphorylation and dephosphorylation, and stoichiometric inhibition (Novák and Tyson 2008). In addition, the time scales of the reactions must fulfill some constraints to allow oscillations and define its characteristics. For example, changing the rate of synthesis of a key component can lead a system to go from a single stable state to an oscillatory regime (Strogatz 1994; Novák and Tyson 2008), while further changes can alter properties as the period or amplitude of oscillations. However the effect of changes in any parameter will depend on the full network of interactions, being particular for each model.

15.2.4 Regulatory Motifs: Controlling Period or Amplitude

Other more complex regulatory motifs, with a greater number of components and interactions, can also show regular oscillations. Detailed models of the *Arabidopsis* transcription-based circadian clock reveal a complex oscillator that is apparently based on three interlocking negative feedback loops (Pokhilko et al. 2012) but features even more regulatory loops (Greenham and McClung 2015). Similar topologies, but with interlocked positive and negative feedback loops, were reported in *Drosophila* and mammals (Bell-Pedersen et al. 2005). However, it is difficult to achieve a general understanding of the role of different aspects of the network topology, such as the particular contribution of certain types of feedback loops.

Models of biological oscillators can be simplistically divided in those containing only negative feedback loops and those with positive and negative feedback. Tsai et al. (2008) investigated the role of the positive feedback in different oscillator models with computational methods. Varying the synthesis rate of a key component, the authors evaluated the range of frequencies and amplitudes of oscillations produced in simulations of diverse oscillator models. Oscillators composed of negative feedback loops showed oscillations with a narrow range of frequencies but with a wide range of amplitudes, being interesting for timekeeping functions such as circadian clocks (Tsai et al. 2008). The addition of the positive feedback allowed the oscillator to operate in a dramatically wider range of frequencies with little effect on the amplitude. Having constant amplitude and adjustable frequency may be crucial for the survival of an organism, as in the case of the cardiac pacemaker (Tsai et al. 2008). Furthermore, the frequency could be increased almost linearly with increases in the rate of synthesis. This could facilitate other cellular processes to control the period of the oscillations and even the action of natural selection, by enabling a gradual increase in period with a single kinetic rate. Finally, the range of parameters where oscillations occur is wider in motifs with positive and negative feedback (Tsai et al. 2008). This was interpreted as an indication of robustness since the oscillator would continue to function under larger variations in kinetic rates. All of these properties were enhanced by increases in the strength of the positive feedback, proposed to confer tunability (the ability to change, or tune) and robustness (the capacity to maintain stable) to these oscillators. The biological function of many

oscillators seems to rely on these properties, as in the case of the Ca^{2+} spikes, cell cycle, and somitogenesis (Meyer and Stryer 1988; Ciliberto et al. 2005; Gibb et al. 2010). In short, while negative feedback seems to confer robustness to frequency, positive feedback appears to allow robust amplitude and a tunable period.

15.2.5 Temporality Does Not Imply Causality

A fundamental lesson to be learned from the structure and dynamics of oscillatory networks is that, just as "correlation does not imply causation," temporality does not imply causality. Although this may sound as an ordinary point, it is a major source of confusion and misinterpretation. When studying biological processes that oscillate together with a constant phase relationship, many authors will likely be tempted to draw conclusions about their interaction and/or their role in generating the oscillations, especially if there are reasons to expect a causal relationship between them. The ubiquity of this problem in diverse fields motivated the emphasis on this point, which can be better visualized with a minimal example (Fig. 15.3). For that, a modified system based on simulations of the delayed negative feedback loop, as presented by Novák and Tyson (2008) in their Supplementary Information S1, was used.

Consider two processes of interest, A and B, which were assayed and found to oscillate with the same period, with peak levels of A diminishing as B rises. Then, B reaches its peak, while A is reaching its lowest point, which is followed by a progressive decrease in B until A increases again, and the cycle continues. This dynamic seems to indicate that B inhibits A, while A is responsible for producing B, with their reciprocal interaction generating this cycle. Although it could be the case, this explanation can be a mere product of confirmation bias. The example herein illustrates that not only A and B may not participate in the oscillations, they may also be completely independent. Two basic scenarios involving a simple oscillator can explain the apparent regulatory dynamics between both variables. Here, the actual oscillator that generates the rhythm is a delayed negative feedback loop comprising other three variables X, Y, Z, with $X \rightarrow Y \rightarrow Z \dashv X$, that were not considered in the anecdotal study (Fig. 15.3).

In the first scenario, different elements of the oscillator drive components of downstream pathways A and B, here $X \rightarrow A$ and $Z \rightarrow B$ (left scheme in Fig. 15.3), so that A and B are phase delayed due to the phase relationship between the upstream components X and Z. In this case A and B will have a large phase relationship even if the delay between the oscillator and downstream components is the same ($delay_A = delay_B$); however, their waveform is assumed to be the same for simplicity. In the second scenario, components of the downstream pathways A and B are driven by the same oscillator component Z (right scheme in Fig. 15.3) but with different delays ($delay_A > delay_B$) so that the phase relationship between A and

B resembles the one between X and Z. This could occur due to the time taken for a series of intermediary reactions (labeled $A_1, A_2 \cdots A_n$) to lead from $Z \rightarrow A$.

In addition, notice that even the temporality between A and B is ambiguous and compromises a causal narrative of their interaction since, without additional information, A could phase lead B by less than half a cycle or B could phase lead A by over half a cycle (as shown in the bottom plot of Fig. 15.1).

15.3 Oscillations at the Pollen Tube Tip: Pacemaker or Clockshop?

The quest for the central pacemaker generating oscillations in a biological system is not uncommon and has led to theoretical and experimental advances. Having in mind lessons learned in these past endeavors, the evidence and hypotheses regarding the generation of pollen tube oscillations and their putative function are reviewed in the following sections.

15.3.1 Evidence for a Pacemaker

It is generally assumed that the period of oscillations in pollen tubes varies between species and, to a certain extent, between individual pollen tubes but is mostly constant across the different oscillatory cellular processes (Feijó et al. 2001). This led authors to characterize the phase relationships and time delays between the different processes having growth as reference, often representing them in a circular diagram, which assumes that oscillations occurring within the same cell have identical periods (Holdaway-Clarke and Hepler 2003; Hepler et al. 2013). Growth and intracellular ion gradients have been reported to oscillate in phase, while extracellular fluxes of Ca^{2+} and H^+ are delayed by 15 s and 10 s, respectively (Feijó 1999; Michard et al. 2008). A computational approach to quantifying oscillations in Arabidopsis pollen tubes reported a synchronized regime where both $[Ca^{2+}]_{cyt}$ and H^+ influx are in phase, with a delay of approximately 4 s in relation to growth rate (Damineli et al. 2017). Other intracellular processes associated with growth were reported to maintain a constant phase relationship, for example, vesicle fusion that appears to occur in phase with growth, as evidenced by a decline in FM4-64-stained vesicles at the tip correlating with growth rate peaks (Parton et al. 2001). In addition ROP1 (Rho-like GTPases from plants 1) accumulation at the tip, which would influence vesicle fusion and actin polymerization, was described to oscillate with a phase difference of 90° with growth rate oscillations (Gu et al. 2003; Hwang 2005).

The existence of a common oscillatory mechanism is further supported by changes in oscillatory characteristics, such as period and amplitude, induced by

interfering with certain cellular components involved in tip growth (Feijó 1999; Portes et al. 2015). Inhibiting microtubule polymerization with colchicine decreased the period and amplitude of growth rate oscillations in *Petunia hybrida* and *Nicotiana tabacum* (Geitmann et al. 1996). Inhibition of Ca^{2+} channels in *P. hybrida* induced an increase in the period of oscillations or their abolishment, whereas in *Lilium formosanum* higher extracellular Ca^{2+} concentration was reported to increase period and decrease the average growth rate (Geitmann and Cresti 1998; Holdaway-Clarke et al. 2003). Interestingly, glutamate receptor agonists and antagonists induce changes in the amplitude of Ca^{2+} influx oscillations, with the agonist D-serine triggering oscillations of Ca^{2+} influx in previously non-oscillatory pollen tubes (Michard et al. 2011).

Thus, a central pacemaker at the pollen tube appears to be a parsimonious explanation considering the body of work available so far, which spans different species and a range of cellular processes, mostly reporting a single steady frequency within individual tubes with stable phase relationships between growth and other oscillations. However, there are major shortcomings in this interpretation. First, the spatial and temporal resolution available to most works, as well as the statistical techniques employed, does not allow a precise quantification of period and phase relationships through time, not enabling to exclude the existence of additional frequencies and alternative phase relationships, especially for less studied species like *Arabidopsis*. Second, given the complex interactions between processes involved in tip growth, the feedback mechanisms between the cellular components generating oscillations cannot be solely derived from descriptions of phase relationships and remain to be established (addressed in Fig. 15.3). Third, multiple self-sustained oscillators could be interacting generating a common rhythm (Fig. 15.2), in which case their composing elements and characteristics would have to be taken apart. The observation of multiple oscillations alone certainly does not warrant a splitter view, which would tend to pose the existence of as many oscillators as there are oscillations, as warned in previous sections. Thus, the question of which components comprise the separate autonomous oscillators stands.

15.3.2 Multiple Oscillators: Evidence for a Clockshop

The hypothesis of multiple interacting oscillators coexisting at the pollen tube tip is often alluded to; however, scarce experimental evidence actually supports it. The general idea is that pollen tube oscillations would be generated by a synchronized ensemble of oscillators, much like the clockshop hypothesis that contradicted the idea of a single main circadian clock (Winfree 1975, 2001), in which case they could be separated under certain conditions evidencing differences in their intrinsic behavior. Indeed, the period of oscillations in growth rate was lengthened upon inhibition of ATP production, which extinguished oscillations in NAD(P)H fluorescence—indicative of electron transport chain (ETC) activity (Rounds et al. 2010). In normal conditions, pollen tubes show synchronized oscillations with peaks

in ETC activity preceding growth rate; thus, the disruption seems to point to a degree of independence of the oscillator involving growth from ATP production and its rhythms. The authors suggest, however, that ETC is a self-sustained oscillator distinct from growth, a claim that would require further evidence. While the influence on the period of growth rate oscillations may indicate that ETC acts as a positive feedback loop (see Sect. 15.2.4), demonstrations of its ability to oscillate independently with a characteristic frequency (free running period, in circadian parlance) are needed. Another possibility for the clockshop hypothesis would be to find distinct frequencies in the undisturbed system, suggesting that the oscillatory ensemble is not synchronized, at least not always (Granada et al. 2011). In fact, the presence of distinct frequencies in growth, ion fluxes (Michard et al. 2008), and vesicle trafficking (Parton et al. 2003) oscillations was reported but needs further quantitative assessment. Thus, although multiple interacting self-sustained oscillators were speculated to coexist in pollen tubes (Kroeger and Geitmann 2013), the evidence suggesting that the pollen tube pacemaker is actually a clockshop remains circumstantial.

15.3.3 Modeling Oscillations: As Many Models as Objects of Study?

Many different modeling approaches on pollen tube growth and oscillations have been developed (reviewed in Kroeger and Geitmann 2012, 2013; Pietruszka 2013; Liu and Hussey 2014); however, a comprehensive view of the interactions between key cellular processes and the rhythm generation is still lacking. A critical point is that most models directly or indirectly rely on Ca^{2+} entering via stretch-activated channels (Hwang et al. 2008; Kroeger et al. 2008; 2011; Yan et al. 2009; Rojas et al. 2011; Kroeger and Geitmann 2013; Liu and Hussey 2014), serving as the main negative feedback sensing excessive growth through membrane tension and relaying a signal to reduce growth mostly through Ca^{2+}-induced actin depolymerization (or downregulation of polymerization). This contentious pathway has not been experimentally demonstrated to be involved in tip growth, posing a major limitation to the validation of most proposed models that, in addition, inherently require growth for oscillations to occur. In fact, oscillations observed in cytosolic Ca^{2+} concentrations in nongrowing tubes (Iwano et al. 2009; Damineli et al. 2017) challenge all oscillator models that rely on growth-associated events to generate rhythms or as necessary outputs that, to the extent of our knowledge, apply to all formal models until this date (Kroeger et al. 2008, 2011; Yan et al. 2009; Liu et al. 2010; Liu and Hussey 2011, 2014; Rojas et al. 2011; Kroeger and Geitmann 2013; Pietruszka 2013). Regardless of their validity, these different models reflect certain research scopes raising the question of whether they are mutually exclusive, compose distinct and possibly coexisting oscillators or, instead, point to different aspects of an all-encompassing oscillator.

15.3.3.1 Mechanical Models

Oscillations occur due to a negative feedback where elevated growth rates promote Ca^{2+} entry through stretch-activated channels, inducing actin depolymerization and inhibition of vesicle exocytosis, ultimately decreasing growth rate in the biophysical models of Kroeger et al. (2008), Kroeger et al. (2011) and Kroeger and Geitmann (2013). Predictions are compatible with part of the available experimental data, mainly the phase relationship between growth and Ca^{2+} influx (Kroeger et al. 2008), and propose the increase in oscillation amplitude and period with greater cell wall viscosity (Kroeger et al. 2011). Furthermore, Kroeger and Geitmann (2011) predict that increasing turgor pressure leads to an increase in average growth rate and decrease in oscillation frequency, an effect that would be more prominent in low turgor regimes. However, despite of possible limitations in resolution, turgor in the range of 0.1 to 0.4 MPa did not seem to affect the period of oscillations in *Lilium longiflorum* (Benkert et al. 1997) posing a challenge to this prediction until further evidence is presented.

In an elegant mechanical model of turgor-driven cell wall expansion via incorporation of de-esterified pectin cross-linked by Ca^{2+} binding, the negative feedback promoting oscillations was based on expansion inhibiting the delivery of exocytic vesicles (Rojas et al. 2011). Although not explicitly modeled, the rationale employed also relies on the opening of stretch-activated Ca^{2+} channels that would eventually inhibit actin polymerization and the deposition of secretory vesicles. The model yields predictions of the onset of oscillations after a critical growth velocity, phase relationships with cell wall thickening preceding elongation rate peak, which is then followed by cytosolic Ca^{2+} peaks, also explaining different pollen tube morphologies. The predictions are loosely in accordance with data, as cells with faster growth rate do appear more likely to oscillate, and the distributions of phase relationships, albeit broad, reproduce the order of events and encompass experimentally assayed values.

15.3.3.2 Polarity-Associated Model: The ROP1 Oscillator

Another modeling thread focuses on ROPs (Rho-like GTPases from plants, also known as RACs), particularly ROP1 that much like Rhos in other cellular systems (Edelstein-Keshet et al. 2013) has been implicated in polarity and guidance of pollen tubes. The activity of ROP1 at the tip has been reported to oscillate with the same period as growth but preceding it and would be responsible to regulate F-actin polymerization and vesicle trafficking, including the fusion of exocytic vesicles (Hwang et al. 2008; Yan et al. 2009). The negative feedback proposed in a nonmathematical model would occur by the fusion of the vesicles that would both dilute ROP1 at the delivery sites, as they do not contain it, and inactivate ROP1 since the vesicles carry the inhibitor REN1 (ROP1 enhancer 1), whose accumulation also precedes growth peaks at the tip but follows ROP1 activity peaks (Hwang et al. 2008; Wu and Lew 2013). Although not explored mathematically and yet to be

fully demonstrated biologically, a global inhibition by REN1 would not only be the basis of the rhythmic behavior but also spatially constrained ROP1 activity at the tip promoting polarity maintenance. Such a ROP1 oscillator was modeled mathematically with a negative feedback based on the activation of Ca^{2+} channels by the effector RIC3, which would promote calcium-induced calcium release and ultimately promote a rise in intracellular Ca^{2+} concentrations, either leading to the disassembly of F-actin (the canonical pathway discussed herein) or activation of negative ROP1 regulators like REN1 (Yan et al. 2009). The predicted phase relationships qualitatively match the expected sequence of ROP1 peaks followed by intracellular Ca^{2+} peaks, which would lead to F-actin depolymerization and growth rate suppression. Although not including growth explicitly, both models featured would entail an elongation of the plasma membrane due to vesicle fusion, sharing the problem of the other growth-based models of not addressing oscillations in the absence of growth.

15.3.3.3 Ion Dynamics Oscillator

A core oscillator based on ion dynamics, which would be integrated by the membrane voltage, is an appealing candidate model to circumvent the necessity of growth-dependent events to generate the rhythm, while in addition allowing fast spatial communication through different intracellular locations. Oscillations in the intracellular concentrations and extracellular fluxes of different ions at the tip, namely, Ca^{2+}, H^+, Cl^-, and K^+, were described in a model using the classical Hodgkin-Huxley type of formalism (Liu et al. 2010). The conductance of ion channels is affected by membrane voltage, featuring Ca^{2+} entry through voltage-gated channels (Michard et al. 2009) but also assuming voltage gating of H^+ and K^+ channels. Two compartments with different channel orientations were considered attempting to mimic the difference between tip and shank, successfully reproducing basic phenomena qualitatively, as the occurrence of oscillation at the tip but not in the shank and a difference in cytosolic concentrations. Although growth was not part of the mechanism generating the oscillations, it was considered to be coupled to ion dynamics by a power-law formalism that would entail oscillations in ions propagating to growth, thus still not addressing the observation of oscillations under growth arrest. An extension of the model included stretch-activated Ca^{2+} and K^+ channels, deepening the dependency of the model behavior on growth (Liu and Hussey 2014). This model further incorporated cell wall expansion, turgor pressure, and other hydrodynamic variables, proposing a context-dependent role of turgor by loosely characterizing different regimes of oscillations in turgor with small amplitude, below the resolution of available experimental methods, are one prediction of the model.

Unfortunately, these models are sufficiently complex not to lay bare the principles acting on the generation of the oscillations, so the core negative feedback loop of the oscillator and their fundamental characteristics remain unclear. While the effort of integrating all of these aspects involved in pollen tube growth is

commendable (Liu and Hussey 2011), the amount of parameters and assumptions renders the model premature, as the magnitude and direction of the fluxes are disputable and the specificity of the parameter values chosen lacks validation.

15.3.3.4 Modeling Limitations

Perhaps the biggest challenge in modeling oscillations in biological systems is ensuring the specificity of the predictions. Many models are expected to show oscillations since they arise in simple regulatory motifs (as discussed in Sect. 15.2.3), making large models with many variables and parameters extremely difficult to interpret. As a matter of fact, many complex models of biological oscillations can be accurately reduced to simpler models, as in the example of a five-variable model of the circadian clock that was effectively described by the classical two-variable Van der Pol oscillator (Forger and Kronauer 2002). Yet, most of the pollen tube models presented herein have a large number of equations and parameters, not incurring into quantitative data fitting. Since many of these equations are not established for the biological process they represent while the parameter values compatible to experimental data are broad, it remains unclear whether predictions are specific to the particular model under study or simply reflect general properties of oscillatory networks. Thus, it is currently difficult to evaluate whether one, multiple, or none of the models of pollen tube oscillators are correctly pinpointing the key players in generating the oscillations and regulating growth.

15.4 Searching for Function of the Oscillations: A Parallel with Polarity and Chemotaxis

Even with major experimental and theoretical developments in the study of pollen tubes, the structure of the oscillator and the biological functions of the ensuing oscillations remain to be determined. Oscillations are often dismissed as epiphenomenon, a by-product that has no bearing to function. This is a reaction expected from the scientific community until clear evidence of their biological role is undeniable, as it was the case with circadian oscillations where fitness effects started to be demonstrated only 40 years after the beginning of the field (Ouyang et al. 1998; Dodd et al. 2005) and as it is currently still happening with oscillations in electrical activity in the brain (Buzsáki 2006). In the pollen tube field, such skepticism is expected since oscillations in growth and other processes have been reported in vitro, with no evidence of their occurrence in vivo (Iwano et al. 2009), although Ca^{2+} spiking behavior is thought to underlie aspects of the communication between the male and female tissue (Iwano et al. 2012; Denninger et al. 2014; Hamamura et al. 2014; Ngo et al. 2014).

Oscillations in Ca^{2+}, including spikes, have interesting characteristics for regulating signal transduction including providing specificity in the activation of specific downstream targets through particular frequencies and efficiency in the usage of intracellular Ca^{2+} concentrations (Schuster et al. 2002; Bodenstein et al. 2010). Oscillations can transiently increase the cytosolic concentration above the activation threshold of certain targets, abrogating the need of maintaining high steady-state levels that would be inefficient and could, in addition, form potentially lethal insoluble salts. Alternatively, oscillations may simply be hard to prevent due to nonlinearities in the underlying regulatory system, potentially having no specific biological function or even reflecting pathological states (Schuster et al. 2002).

Since the pollen tube is a model for polarized growth and guidance, some insight into the role of oscillations can be obtained from analogous systems involved in intracellular polarity and chemotaxis, where some molecular details are better understood. A general scenario emerging in many systems, from uni- to multicellular organisms, consists of oscillations in polarity-associated molecules (often small GTPases as Rac/Rho) that occur while there is no directional cue guiding the cell (Wu and Lew 2013; McClure and Lew 2014). These polarity molecules assemble at random intracellular sites until an external signal acts by stabilizing them at an intracellular polarity site, inhibiting the formation of new sites and abolishing the oscillations. Under this perspective, rather than being functional per se, oscillations could reflect a particular dynamical regime of the system responsible for establishing intracellular polarity. In fact, such a system may not formally be an oscillator but rather what is called an excitable system, able to amplify signaling events and induce waves and other spatial patterns (Xiong et al. 2010). While the oscillatory regime could be reached by such an excitable system alone, coupling with or among oscillators based on actin dynamics has been proposed in different systems. In neutrophils, motion was suggested to stem from the coupling of two oscillatory systems with different frequencies, the signaling pathways (featuring Ras and Rac GTPases), which compose a slower excitable system, and the actin dynamics, which generate faster oscillations (Huang et al. 2013). In mast cells, actin traveling waves were converted to oscillations confined in space (standing waves) upon coupling with Ca^{2+} oscillations (Wu et al. 2013). In *Dictyostelium* excessive coupling between distinct actin oscillators in different membrane sectors produced large amplitude oscillations, considered to be pathological states since they would hinder cellular responses to intra- and extracellular cues (Hoeller et al. 2016). Indeed, Meinhardt (1999) predicted that oscillations could decrease sensitivity in signal detection by creating a phase dependency in the response of chemotactic cells.

Thus, considering examples in the other systems, oscillations could be a by-product, a pathological state, or even a fundamental way to link different intracellular systems to establish polarity, allow motion, and produce guidance behavior. Modular systems capable of oscillating, one related to signaling and polarity closer to the membrane and the other related to actin dynamics spread further into the

cytoplasm, seem to be a recurrent and offer interesting perspectives for explaining pollen tube growth regulation.

15.5 Conclusion and Perspectives

In short, a surge in the tendency to describe many oscillators at the pollen tube tip is expected to happen, although most works will be likely pointing toward the same underlying oscillatory system. However, this does not mean that there is a single pacemaker at the pollen tube tip. Here we propose that pollen tube oscillations could stem from a similar organization as other systems involved in polarity and chemotaxis, namely, two oscillatory modules: a signaling-based oscillator and a motion-based oscillator (similar to Huang et al. 2013).

The signaling-based oscillator would be localized at the tip membrane and directly or indirectly involve ion dynamics and other signaling pathways as ROPs, being responsible for establishing and maintaining polarity, as well as dictate the pace of growth. Calcium ion waves would relay signals from the tip to a motion-based oscillator more localized at the tube shank, essentially composed of actin filaments that would depolymerize with signals from the tip, possibly also synchronizing their polymerization/depolymerization among themselves and with the tip oscillations. Depending on the coupling between signaling and motion oscillators, different synchronization regimes could be predicted. In the fully synchronous regime, both oscillators would adopt a common frequency with a defined phase relationship, while the fully desynchronized regime could include the signaling oscillator running independently of motion. This latter uncoupled regime could be the basis to explain oscillations in Ca^{2+} observed in the absence of growth (Iwano et al. 2009; Damineli et al. 2017), perhaps requiring a resonance frequency with the motion-based oscillator to promote growth, which could resolve the conundrum posed by most models discussed previously.

In conclusion, the signaling-based oscillator currently seems to be the best candidate for a pacemaker and where efforts to understand oscillations would be more prolific. A systems approach to theoretical and experimental work is fundamental since the regulatory characteristics of biological oscillations are emergent properties and cannot be accounted by any of its elements separately. Mathematical modeling with the adequate level of details (Mogilner et al. 2012) will be necessary to investigate the complex and often elusive function of oscillations that, even if absent, allow a glimpse into the relationships between key cellular processes involved in their generation (Feijó et al. 2001).

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